

How Many Bystander Effects Are There?

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The "Bystander Effect" is not new. As early as the 1940s there were reports that the inactivation of biological entities may be brought about equally by ionizations produced within the entity or by the ionization of the surrounding medium (1-4). By 1947, Kotval and Gray (5) had shown that alpha particles which pass close to the chromatid thread, as well as those which pass through it, have a significant probability of producing chromatid and isochromatid breaks or chromatid exchanges.

The term used today to describe such phenomena is the "Bystander Effect," a name borrowed from the gene therapy field, where it usually refers to the killing of several types of tumor cells by targeting only one type of cell within a mixed population (6, for example).

In the radiation field, it has come to be loosely defined as the induction of biological effects in cells that are not directly traversed by a charged particle, but are in close proximity to cells that are.

Data now available concerning the bystander effect fall into two quite separate categories, and it is not certain that the two groups of experiments are addressing the same phenomenon. First, there are experiments involving the transfer of medium from irradiated cells, which results in a biological effect in unirradiated cells. Second, there is the use of sophisticated single particle microbeams, which allow specific cells to be irradiated and biological effects studied in their neighbors; in this case communication is by gap junction.

Medium transfer experiments have shown a bystander effect for cell lethality, chromosomal aberrations and cell cycle delay. The type of cell, epithelial versus fibroblast appears to be important, though data are conflicting. Experiments suggest that the effect is due to a molecule secreted by irradiated cells which is capable of transferring damage to distant cells. Use of a single particle microbeam has allowed the demonstration of a bystander effect for chromosomal aberrations, cell lethality, mutation and oncogenic transformation. When cells are in close contact, allowing gap junction communication, the bystander effect is a much larger magnitude than the phenomenon demonstrated in medium transfer experiments.

Evidence comes from experiments with V79 cells, where the endpoint observed was cell lethality. Lines of hygromycin and neomycin resistant V79 cells were produced. Before exposure the hygromycin resistant cells were stained with a low concentration of a vital nuclear dye. They were then plated in micro wells in the proportion nine neomycin-resistant for every one hygromycin resistant cell. The computer was programmed to irradiate only the 10% of cells stained with a nuclear dye with various numbers of alpha particles from 1 to 16, aimed at the centroid of the nucleus. The cells were then removed and cultured for survival in the

appropriate growth media, which made it possible to obtain survival curves for hit and non-hit cells. The data are shown in Figure 1. There is a considerable degree of cell killing in the non-hit cells, implying a substantial bystander effect. The magnitude of the bystander effect in these studies is much greater than that reported by the Gray Institute for Cancer Research where only 5 to 10% lethality is seen in non-hit cells, using protons or soft-x-rays in a microbeam. The difference is probably accounted for by the cell density. In the Gray Institute studies, only about 200 cells were seeded in an area of 10 x 10 mm. The average distance between cells, therefore, was some hundreds of microns, so it is likely that communication via gap junction did not contribute to the effect observed (Barry Michael, Private Communication). By contrast, in the studies reported here, 1,000 to 1,200 cells were plated, in a mini-well of 6.3 mm diameter so that 50 to 60% were in contact, allowing gap junction communication that has been demonstrated to be of importance in mutation studies with the microbeam. Therefore, these data support the notion that communication via the medium and communication via gap junctions are separate phenomenon because the magnitude of the effect is so different.

A very large bystander effect was observed in studies of oncogenic transformation in C3H 10T1/2 cells, where, in

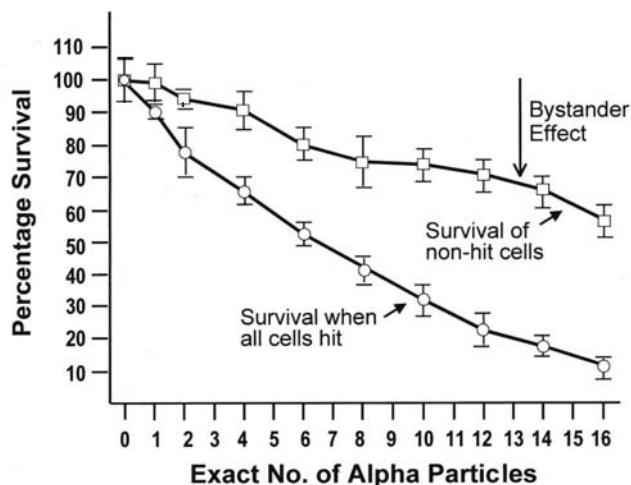


Fig. 1. The bystander effect for cell survival in V79 cells. Each data point (mean \pm SE) on the line with circles refers to the survival of cells when all cell nuclei on each dish were exposed to the same exact numbers of alpha particle traversals using the microbeam system. The squares show survival for various numbers of alpha particles, from 1 to 16, traversing 10% of the cell population. The extent to which this falls below the 100% survival for the non-hit is an indication of the magnitude of the bystander effect. Each data point represents the mean \pm SD of the clonogenic survivals from three culture plates. [Redrawn from Sawant et al. (7)].

order to have sufficient cells for this assay, cells were plated at high density and therefore were in gap-junction communication.

The data are shown in Figure 2 and illustrates that (a) more cells can be inactivated by alpha particles than were actually traversed by an alpha particle. (b) When 10% of the cells on a dish are exposed to two or more alpha particles, resulting frequency of induced oncogenic transformation is indistinguishable from that when all the cells on the dish are exposed to the same number of alpha particles. In these experiments mouse fibroblasts (C3H 10T1/2) cells were plated in a monolayer, and the computer was programmed to irradiate either every cell, or every tenth cell, selected at random with one to eight alpha particles directed at the centroid of the cell nucleus. The cells were subsequently removed by trypsinization, replated at low density, and transformed foci were identified 6 weeks later by their morphologic appearance.

References

1. Dale WM. The effect of x-rays on enzymes. *Biochem J* 34:1367, 1940.
2. Dale WM. The effect of x-rays on the conjugated protein amino-acid oxidase. *Biochem J* 36:80, 1942.
3. Dale WM. Effect of x-rays on aqueous solutions of biologically active compounds. *Brit J Radiol* 16:171, 1943.
4. DE, Smith KM, Holmes B and Markham R. Direct and indirect actions of radiation on viruses and enzymes. *Parasitology* 36:110, 1944.
5. Kotval JP and Gray LH. Structural changes produced in microspores of *Tradesantia* by radiation. *J of Genetics* 48:135-54, 1947.
6. Cheng TL, Wei SL, Chen BM, Chen JW, Wu MF, Liu PW and Roffler SR. Bystander killing of tumour cells by antibody-targeted enzymatic activation of a glucuronide prodrug. *Br J Cancer* 79:1378-85, 1999.
7. Sawant SG, Zheng W, Hopkins KM, Randers-Pehrson G, Lieberman HB and Hall EJ. The radiation-induced by-

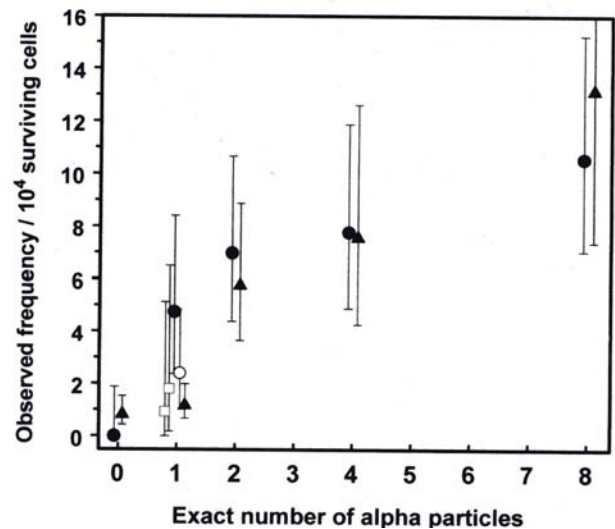


Fig. 2. Yield of oncogenically transformed cells per 10^4 surviving C3H 10T1/2 cells produced by nuclear traversals by 5.3 MeV α -particles. Triangles represent to exposure of all cell nuclei on each dish to exact numbers of α -particles, using the microbeam system. Solid circles represent exposure of 1 in 10 cell nuclei on each dish to exact numbers of α -particles. Open squares represent subsequent repeats of the experiment in which 1 in 10 cell nuclei were exposed to exactly one α -particle. Open circle represents combined data for all the experiments in which 1 in 10 cell nuclei were exposed to one α -particle including these repeat experiments (with caveats described in the text). Standard errors (\pm SD) were estimated assuming an underlying Poisson-distributed number of transformed cells. [Redrawn from the data of Sawant et al. (8).]

stander effect for clonogenic survival. *Radiat Res* 157:361-4, 2002.

8. Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ and Hall EJ. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. *Radiat Res* 155:397-401, 2001. ■

Identification of Signal Transduction Pathway(s) in High LET Radiation Induced Bystander Response by cDNA Microarray Analysis

Adayabalam S. Balajee, Brian Ponnaiya and Charles R. Geard

“Bystander effect” (BE) is the result of the ability of the cells directly affected by an agent to convey the manifestation of the damage to neighboring cells that are not directly targeted thereby eliciting a response similar to that of targeted cells. BE can be triggered either through direct contact

with the damaged cells or through the growth factors released from the targeted cells (1). Although the signaling pathways responsible for bystander response are largely unknown at this moment, this multifaceted phenomenon is expected to have a significant impact on the radio- and che-

motherapy of tumors. A better understanding of molecular steps involved in BE is pivotal for modulation and evaluation of the protocols designed to improve the efficacy of the radio and chemotherapy treatments. In an attempt to understand the molecular basis for BE, we have undertaken a cDNA microarray approach to identify the components of diverse signal transduction pathways that mediate the response.

Primary fibroblast cells (normal human dermal fibroblasts, NHDF) were obtained from Clonetics. The cells were routinely maintained in fibroblast basal medium supplemented with 15% fetal bovine serum, vitamins, essential amino acids, non-essential amino acids and antibiotics (Gibco BRL). The cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. NHDF cells were seeded on double-sided Mylar dishes and the dishes were irradiated only on one side with different doses of track segment alpha particles (1 and 5 Gy). The protocol for preparation and irradiation of cells in double-sided Mylar dishes has been previously described (2). Total RNA was isolated from both irradiated (bottom) and non-irradiated (top) cells. cDNA synthesis was carried out using biotin-16-dUTP and the biotinylated cDNA samples were denatured and hybridized with cDNA signal transduction pathway finder array procured from Super Array, MD, USA. This array contains 96 marker genes associated with 18 different signal transduction pathways (Mitogenic pathway, Wnt pathway, Hedgehog pathway, TGF pathway, Survival pathway, p53 pathway, stress pathway, NFkB pathway, NFAT pathway, CREB pathway, Jak-Stat pathway, Estrogen Pathway, Androgen pathway, calcium and Protein kinase C pathway, phospholipase C pathway, insulin pathway, LDL pathway and Retinoic acid pathway). After hybridization, the signal was detected using streptavidin-alkaline phosphatase as per the instructions of the manufacturers. The membranes were used to expose Kodak BiomaX light films. Digital images were generated using the ScanAlyze2 programme (developed by Michael Eisen at Lawrence Berkeley National Laboratory). The analysis was done using GE array analyzer (Super Array). A representative example of hybridization patterns obtained for control direct hit and bystander cells is shown in Figure 1.

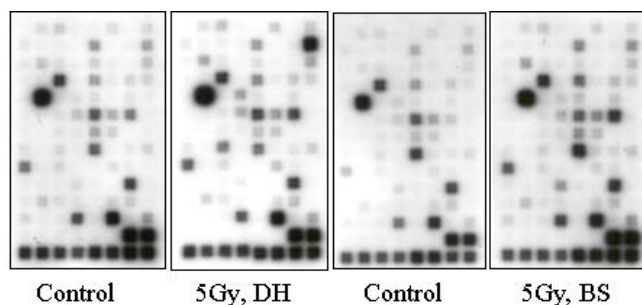


Fig. 1. cDNA expression profiling of genes associated with 18 signal transduction pathways in control, direct hit (DH) and bystander (BS) cells. Exponentially growing NHDF cells were irradiated with 5 Gy of α -particles. Total cellular RNA was isolated 3 hrs after irradiation and 1 μ g of the RNA was used for cDNA synthesis with biotin 16-dUTP for hybridization to super array filters.

The results of cDNA arrays indicated that 15 genes [Bcl-2, Bcl2L1 (Homo sapiens BCL-2 like protein 1), BIRC 1, BIRC2 (Homo sapiens baculovirus IAP repeat containing proteins), BRCA1, CD5 (T-cell surface glycoprotein), CDK2 (Cyclin dependent kinase 2), CDKNIA (p21), CDX1 (Homo sapiens caudal type homeo box transcription factor 1), CEBPB (Homo sapiens CCAAT/enhancer binding protein beta), FLJ12541 (Homo sapiens hypothetical protein FLJ12541 similar to mouse Stra6), GADD45A, HK2 (Homo sapiens hexokinase2, nuclear gene encoding mitochondrial protein), KLK2 (Prostrate kallikrein 2) and MDM2] out of a total of 96 belonging to p53, survival, TGF, androgen and Retinoic acid pathways showed a 2-4 fold increase in induction in direct hit NHDF cells as compared to unirradiated control cells. Of all, p21 showed the highest induction being 7.34 fold more than that of control cells. Distinct differences in the expression patterns were observed between direct hit and bystander cells. In contrast to direct hit cells, genes representing NF-kB, protein kinase C and p53 pathways were selectively induced in bystander cells. Out of 96 genes, 3 genes [IGFBP3 (insulin-like growth factor binding protein 3), NFKBIA (Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha) and PRKCE (protein kinase C epsilon)] in bystander cells showed a 2 fold more induction than control cells. Both IGFBP3 and PRKCE showed a 2.8 fold more induction than the unirradiated cells. The unique expression patterns observed for direct hit and bystander cells indicate that the factors triggering the signal transduction pathways may differ between BE and radiation response.

We are presently doing cDNA experiments using RNA samples isolated at different post-irradiation times (30 min, 3 hrs, 6 hrs, 9 hrs and 24 hrs) to follow the differential expression of genes in both direct hit and bystander cells. Additionally, pathway specific arrays are being carried out to confirm the initial observations. Efforts are also underway to verify the cDNA array results by western blotting and RT-PCR. It would be interesting to know whether the signal transduction pathways that mediate the bystander response are defective in radiosensitive mutants. We plan to use several human mutant cells defective in important DSB repair genes to determine whether DSB repair efficiency contributes to bystander response. This project will be initiated by culture of wild type and AT cells in double-sided Mylar dishes to determine whether or not ATM kinase plays a role in radiation induced bystander response in human cells. This approach will be gradually extended to other double strand break repair defective cell lines with mutations in Nbs1 and DNA-PK gene products.

References

1. Iyer R and Lehnert BE. Effects of ionizing radiation in targeted and nontargeted cells. *Arch Biochem Biophys* **376**:14-25, 2000.
2. Geard CR, Jenkins-Baker G, Marino SA and Ponnaiya B. Novel approaches with track segment alpha particles and cell co-cultures in studies of bystander effects. *Radiat Prot Dosimet* **99**:233-6, 2002. ■

Involvement of Replication Protein A in Ionizing Radiation Induced Bystander Response in Human Cells

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“Bystander effect” (BE) is an interesting biological phenomenon where cells not directly affected by DNA damaging agents elicit a response similar to that of targeted cells. Increased levels of sister chromatid exchange, micronuclei, p21 and p53 proteins in bystander cells indicate that a complex DNA damage response pathway may mediate BE. Identification of the DNA repair and signal transduction proteins involved in BE may help in understanding the molecular cascade of events leading to this complex phenomenon. With this objective, we have studied the expression of replication protein A, which is involved in diverse DNA metabolic activities such as replication, repair and recombination.

RPA is induced in bystander cells

Using γ -rays as a DNA damaging agent, RPA expression was analyzed in both direct hit and bystander primary human fibroblast cells (MRC5 and WI38) by immunofluorescence and western blot techniques. Unirradiated control cells exhibited a punctuated pattern of RPA foci ranging from 30-40 in number per cell. The pattern of RPA distribution was essentially the same in cells treated for 30 min with irradiated-conditioned medium without cells. In contrast, intense numerous RPA foci were observed in cells treated with conditioned medium derived from irradiated MRC5 cells. The fluorescence intensity was 2-fold more than that of control cells. The pattern of RPA foci observed 30 min after treatment in bystander cells was different from cells that were directly irradiated with either 5 Gy or 10 Gy of γ -rays. The bystander cells were characterized by numerous intense RPA foci while the irradiated cells displayed large 30-40 distinct focal sites of RPA. Treatment of MRC5 cells with hydrogen peroxide (which predominantly induces single strand breaks and oxidative base lesions) also triggered numerous intense RPA foci 30 min after treatment similar to that observed in ionizing radiation (IR) induced bystander cells.

Western blot analysis was next carried out to determine whether the induced RPA was found either in the soluble fraction or in the chromatin bound fraction. For this purpose, total cellular proteins extracted using low (soluble) and high salt (insoluble) buffers were size fractionated on 4-20% SDS-PAGE and RPA was detected immunologically. Consistent with immunofluorescence data, RPA induction was observed in both soluble and chromatin bound protein fractions derived from bystander MRC5 cells. Bystander cells showed a 2-3 fold induction of RPA as compared to control cells and RPA induction was more pronounced in the chromatin bound fraction. In directly irradiated WI38 cells, RPA induction was hardly detectable in both soluble and insoluble protein fractions at 30 min after treatment. RPA induction was however observed at later times in WI38 cells yet

the fold of induction was lower in the chromatin bound fractions during the first 2 hrs after irradiation. In bystander cells, RPA induction, which was rapid in the insoluble protein fractions at 30 min after treatment, showed a gradual decline at 4 hr with a subsequent increase at 6 hr after treatment. This biphasic kinetics of RPA induction observed in bystander cells was not detectable in direct hit cells. In addition to RPA, p53 induction was analyzed in both bystander and direct hit cells. Unlike RPA, p53 induction was noticed only in the insoluble protein fraction and the kinetics of induction was grossly similar in both bystander and direct hit cells reaching a peak at 4 hr after treatment. To clarify whether or not the increased level of RPA (observed by immunofluorescence and western blot analyses) is due to post-translational modifications, RT-PCR was carried out using the cDNA synthesized from total cellular RNA. RT-PCR analysis showed a 2-fold more induction of RPA in both direct hit and bystander cells as compared to untreated control cells, illustrating the transcriptional activation of RPA in response to DNA damage.

In order to verify whether or not the differences between the two-fibroblast cell lines (WI38 and MRC5) contribute to bystander effects, RPA induction was analyzed in bystander MRC5 cells treated with the transfer of medium derived from irradiated MRC5 cells. In corroboration with earlier results, RPA induction was observed both in the soluble and insoluble protein fractions of MRC5 bystander cells. RPA induction was detected at 30 min after treatment, which slowly declined to the level of control cells by 24 hr. To determine whether the induction of RPA in bystander cells was mainly due to the release of soluble factor(s) from the irradiated cells, RPA induction was analyzed in cells treated with complete medium that was irradiated without cells. RPA induction was not detectable in either of the protein fractions isolated from cells that were treated with irradiated medium alone (without cells) and the RPA level was approximately the same in both control and treated samples at different treatment times. This observation strongly suggests that the irradiated medium alone did not contribute to RPA induction but the signal elicited by the irradiated cells was chiefly responsible for increased RPA expression in bystander cells.

γ -H2AX, an indicator of DNA double strand breaks, is not elevated in radiation induced bystander cells

In order to determine the nature of DNA lesions leading to RPA induction, the expression of the phosphorylated form of histone H2AX (γ -H2AX) was analyzed in both direct hit and bystander cells. γ -H2AX has been shown to specifically bind to DNA double strand breaks induced by ionizing ra-

diation and radiomimetic chemicals. Western blot analysis indicated a 2.5 fold increase in the induction of γ -H2AX at 30 min after treatment, which gradually declined to the control level with increasing recovery times. On the contrary, bystander cells did not exhibit any increase in γ -H2AX level. The lack of γ -H2AX induction in bystander cells suggests that lesions other than DNA double strand breaks may be responsible for RPA induction in bystander cells.

In this study, we have shown that replication protein A, which is a key player in base excision repair (BER) pathway, is specifically induced in the bystander cells. The rapid induction of RPA and its subsequent decline with increasing recovery times suggest that the DNA lesions, which are substrates for BER pathway, are inflicted in the genomic DNA of bystander cells. ■

Gene Expression as a Window on Bystander Effects

Sally A. Amundson

Exposure of mammalian cells to ionizing radiation (IR) induces damage in multiple cellular compartments, resulting in complex biological responses, many of which are mediated through alterations in gene expression. While direct damage to DNA has long been considered the major initiator of cellular responses to IR, the more recent recognition of “non-targeted” effects of IR, such as radiation-induced bystander effects, is altering our understanding of radiation damage and response. Intra-cellular signaling from neighboring irradiated cells is thought to mediate bystander effects in cells not directly irradiated. Documented bystander effects include sister chromatid exchanges, reduced clonogenic survival, chromosome aberrations, apoptosis, micronucleation, oncogenic transformation, mutation induction, and changes of gene expression. Gene expression changes can represent effector responses, the mobilization of the molecular machinery that will execute the cellular endpoints observed, but they can also provide insight into the signal transduction pathways underlying bystander responses.

A functional genomics approach, such as microarray hybridization analysis (1), can survey expression changes in thousands of genes simultaneously. This will enable the identification of potential mediators of bystander signaling, including soluble factors, such as cytokines. This approach should also prove useful in determining the effects of bystander factors on gene expression in unirradiated cells, and suggesting key signaling pathways that might be engaged or blocked to mimic or prevent bystander responses.

Considering that cytokines, such as IL8 (2) and TGF β (3), are known to be released from irradiated cells and have been implicated in mediation of bystander effects in unirradiated cells, preliminary microarray studies examined whether additional cytokine-related genes showed IR-responsiveness and to determine if a soluble factor(s) from irradiated cells could affect gene expression in unirradiated (bystander) cells. As shown in Figure 1, expression of many cytokine-related and extracellular signaling genes increased at various times after IR in human myeloid ML-1 cells as well as after IR or UV radiation in the breast line MCF-7. We have also seen induction of cytokine mRNAs, including

IL1A, *IL1B*, *IL6*, and *A4* in human peripheral blood lymphocytes (4) irradiated *ex vivo*. In addition, we have treated

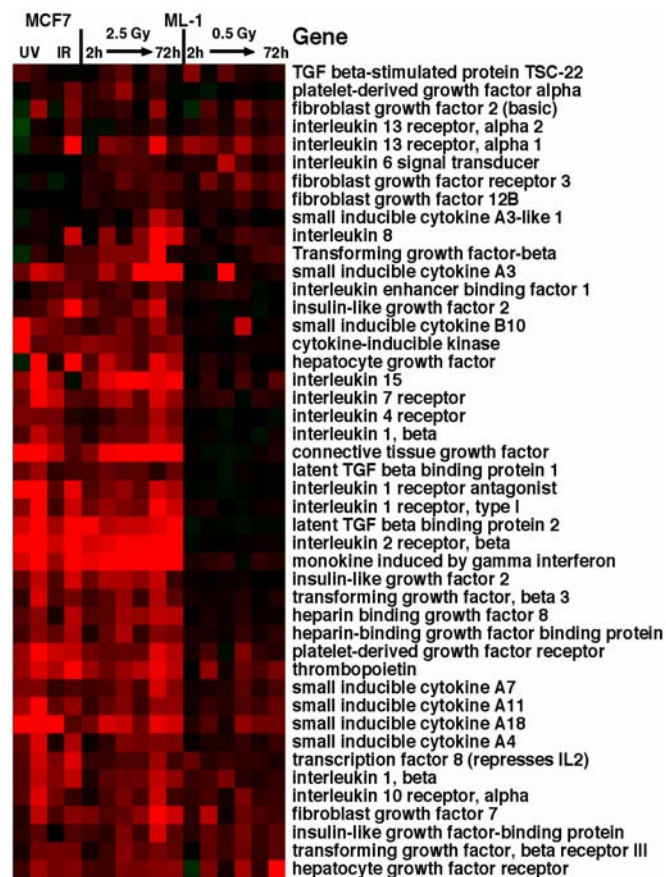


Fig. 1. Hierarchical clustering of radiation induced genes with roles in extracellular signaling (cytokines, interleukins and growth factors from a 6728 member microarray.) The experiments are (from left to right) MCF7 12 hours after 125 J/m² UVB, 6 hours after 4 J/m² UVC, 4 hours after 2.5 Gy α -particles, 24 hours after 2.5 Gy α -particles. These are followed by results for ML-1 2, 4, 8, 24, 48 and 72 hours after 2.5 Gy γ -rays, then at the same times following 0.5 Gy γ -rays.

Table I.
Bystander gene induction in TK6 cells.

HCT116		MCF7	
4 h	24h	4h	Gene
2.9	7.1	4.0	ESTs
2.8	7.4	4.2	SIAT8
2.8	6.4	2.3	CYP1B1
2.7	8.6	4.5	THBS2
2.6	5.9	2.0	LYZ
2.6	6.1	4.4	ESTs
2.5	7.1	2.9	PAI2
2.5	6.0	2.7	CCL11
2.5	6.2	3.4	UNG2
2.5	6.7	2.5	CEACAM1
2.4	6.5	3.6	CTGF
2.4	6.1	2.8	TGM3
2.3	5.9	3.3	TXN
2.3	7.0	3.8	CNGA1
2.3	4.9	2.8	MNDA
2.3	7.2	2.8	ESTs
2.3	9.5	2.4	MGC11271
2.3	5.9	2.9	AMPH
2.2	4.6	2.4	CTSG
2.2	4.9	3.3	ADH4
2.2	4.5	3.1	ESTs
2.2	4.9	3.2	FOXO1A
2.2	5.9	3.3	CDH11
2.2	6.1	3.3	VDR
2.2	6.0	2.8	BRAF
2.1	6.3	2.7	GSTA2
2.1	8.1	3.7	PYGL
2.1	5.8	3.1	CD8A
2.1	5.9	2.6	PPBP
2.1	5.6	2.9	PCYOX1
2.1	5.2	2.8	ESTs
2.1	5.8	3.3	FGA
2.1	4.5	2.2	TNA
2.0	7.2	3.4	LTBP2
2.0	6.9	2.5	ESTs
2.0	5.9	2.7	ESTs
2.0	6.8	2.4	TGFB3
2.0	5.1	2.2	SMPD1

Genes induced in TK6 cells by (4 or 24 hours) exposure to conditioned medium produced by cells irradiated with 5 Gy gamma-rays, as identified from hybridization to 6728 member microarrays.

lymphoid cells, which we previously found to be most sensitive to radiation-induced gene expression changes, with cell-free conditioned media collected 30 minutes after γ -irradiation of HCT116 or MCF-7 cells. Genes showing consistent induction after 4 and 24 hours of exposure to γ -ray conditioned medium are listed in Table I with the magnitude of induction as determined by microarray hybridization. Significant gene induction was also observed in MCF-7 cells similarly treated with MCF-7 conditioned medium, although this response was of lower magnitude, consistent with the response to direct irradiation of this cell line. These results indicate that soluble factors from irradiated cells can have an appreciable effect on gene expression in unirradiated cells of the same and different tissue type. We can conclude from these studies that soluble IR-induced factors do affect gene

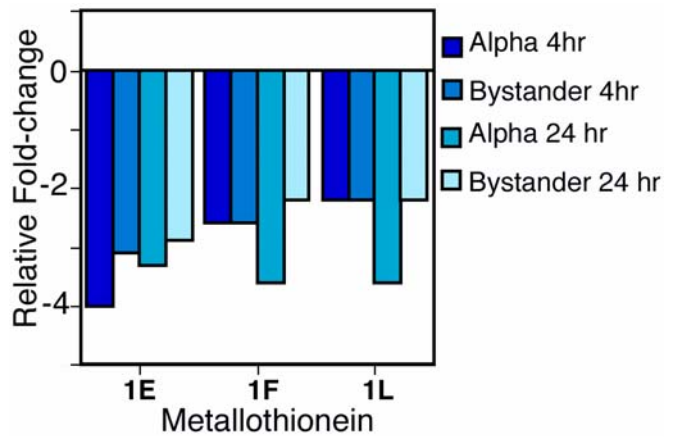


Fig. 2. Relative decrease in expression of metallothionein genes 1E, 1F, and 1L in MCF7 directly and bystander irradiated with 2.5 Gy α -particles, as measured by microarray 4 and 24 hours after irradiation.

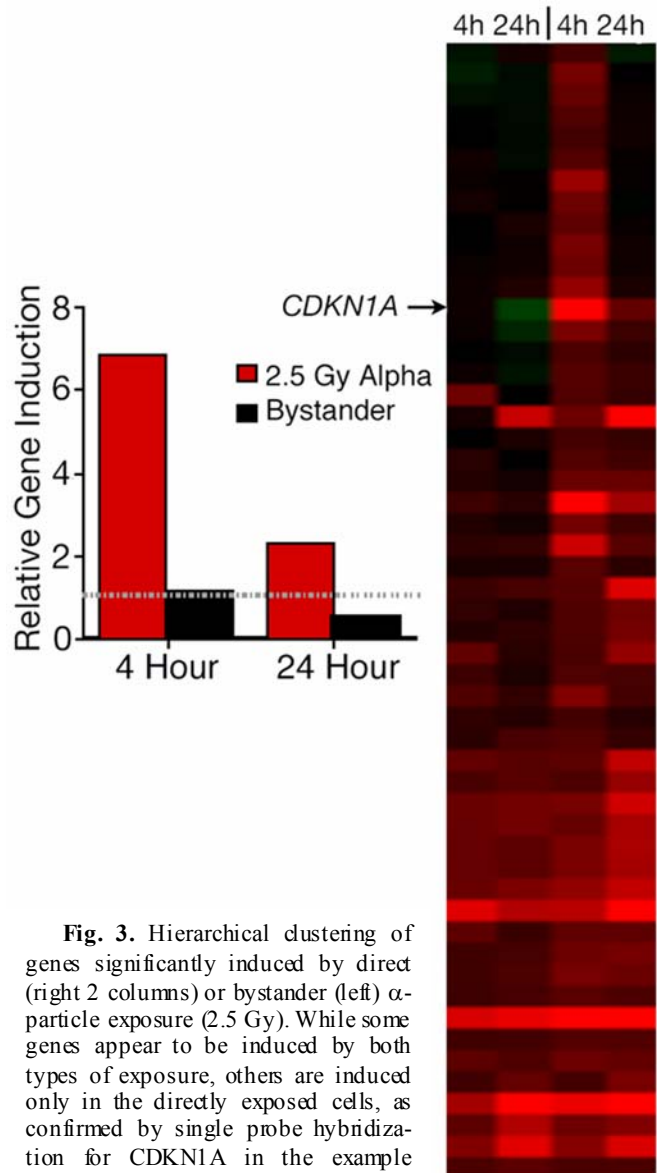


Fig. 3. Hierarchical clustering of genes significantly induced by direct (right 2 columns) or bystander (left) α -particle exposure (2.5 Gy). While some genes appear to be induced by both types of exposure, others are induced only in the directly exposed cells, as confirmed by single probe hybridization for CDKN1A in the example shown above.

expression in bystander cells.

A pilot study using the track segment facility at RARAF has also indicated bystander gene responses following alpha-particle irradiation. In this experiment, MCF7 cells were grown in mylar-bottomed dishes, and half of each dish was irradiated with 2.5 Gy 120 keV/μm α-particles, while the other half was shielded using an aluminum mask. A striking feature of the emerging bystander signature was the apparent coordinate down-regulation of a number of metallothionein genes (Figure 2). Hierarchical clustering of the genes induced in the directly irradiated and bystander halves of the dishes is illustrated in Figure 3. While induction of some genes, such as the illustrated *CDKN1A*, occurred only in the directly irradiated cells, other genes did respond in bystander cells.

The bystander effect is likely to be a natural phenomenon with relevance to IR exposures in humans, and careful gene expression profiling experiments have the potential to dramatically shape our understanding of the signaling and response mechanisms involved. As a naturally occurring physiological response of whole organisms, bystander effects should ideally be studied in an *in vivo*-like multicellular system with preserved 3-D tissue micro-architecture and microenvironment. Future experiments will move toward this goal, building on the results of ongoing experiments in well-characterized cell culture systems and our prior gene expression studies (4, 5, 6, 7).

References

1. Schena M, Shalon D, Davis RW and Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**:467-70, 1995.
2. Narayanan PK, LaRue KE, Goodwin EH and Lehnert BE. Alpha particles induce the production of interleukin-8 by human cells. *Radiat Res* **152**:57-63, 1999.
3. Iyer R and Lehnert BE. Factors underlying the cell growth-related bystander responses to alpha particles. *Cancer Res* **60**:1290-8, 2000.
4. Amundson SA, Shahab S, Bittner M, Meltzer P, Trent J and Fornace AJ Jr. Identification of potential mRNA markers in peripheral blood lymphocytes for human exposure to ionizing radiation. *Radiation Res* **154**:342-6, 2000.
5. Amundson SA, Bittner M, Chen YD, Trent J, Meltzer P and Fornace AJ Jr. cDNA microarray hybridization reveals complexity and heterogeneity of cellular genotoxic stress responses. *Oncogene* **18**:3666-72, 1999.
6. Amundson SA, Do KT and Fornace AJ Jr. Induction of Stress Genes by Low Doses of Gamma Rays. *Radiat Res* **152**:225-31, 1999.
7. Amundson SA, Lee RA, Koch-Paiz CA, Bittner ML, Meltzer P, Trent JM, Fornace AJ Jr. Differential responses of stress genes to low dose-rate gamma irradiation. *Mol Cancer Res* **1**:445-52, 2003. ■

The Bystander Response in C3H 10T½ Cells: The Influence of Cell-to-Cell Contact

Stephen A. Mitchell, Stephen A. Marino, David J. Brenner and Eric J. Hall

It is now widely accepted that radiation-induced heritable effects in mammalian cells are not solely the result of direct DNA damage and there is now evidence for a number of non-targeted effects, including the bystander response, which do not require a direct nuclear exposure (1). The bystander effect is defined as the observation of a biological response in cells which have not been directly traversed by ionizing radiation but which results from signals initiating in cells in which energy has been deposited.

Although reproducible bystander effects have now been demonstrated for a range of biological endpoints, the mechanisms by which the biological insult is transmitted from targeted to non-targeted cells have not been fully elucidated and may be dependent on the experimental protocol employed (reviewed in (2)). One causative agent may be the secretion from irradiated cells of a soluble factor(s) into the media which then elicits a biological response in unirradiated cells, often over some considerable distance (3). Alter-

natively, in densely-irradiated cultures the signal may be transmitted by cell-to-cell communication between adjacent cells via gap junctions (reviewed in (4)).

Previous studies using the Columbia microbeam have shown a significant bystander effect for the endpoints of clonogenic survival and oncogenic transformation in C3H 10T½ cells (5, 6). The aim of the present study was to assess whether the magnitude of this effect observed for both endpoints was dependent upon cell-to-cell proximity at the time of irradiation. To achieve this, cells were plated at both high and low density and targeted with a range of 6 MeV α-particles aimed at the centroid of the nucleus. When approximately 2000 cells were plated on a microbeam dish, the vast majority (>90%) of the cells were in direct contact with neighbors via membranes and intercellular gap junctions when irradiated 18 h later. In contrast, when 200 cells were plated using the same protocol, very little contact between cells (<10%) was seen with the majority of cells appearing

as isolated entities, separated by many tens of microns from their neighbors.

The experimental protocol and results for clonogenic survival have been reported previously (<http://crr-cu.org/reports2002/b4.htm>). Briefly it was observed that at both cell densities, the surviving fractions fell progressively as more α -particles traversed the nucleus but the amount of cell killing was significantly greater at the high cell density compared with low-density cultures ($P < 0.0001$). The study has now been extended to examine the influence of cell density on oncogenic transformation. To assess this parameter, 10% of the cells were exposed to 8 α -particles. Following irradiation, cells were replated into 100 mm culture dishes at a low density of about 300 viable cells per dish. The cells were incubated for 7 weeks with culture medium changed every 12 days, before being fixed and stained with Giemsa to identify morphologically-transformed types II and III foci, as described elsewhere (7).

Results are shown in Table I. In these studies, a total of approximately 3.1×10^5 cells were individually imaged, positioned and irradiated. At high density, a transformation frequency of $9.6/10^4$ viable cells was seen, which is similar to that found previously in high-density cultures (5). Using previously published data (8) it is possible to calculate that when 10% of the cells in a population are irradiated with 8 α -particles, the expected transformation frequency in the absence of a bystander effect would be $2.1/10^4$ viable cells. This is lower than that seen in the present study at both cell densities, although again the difference is only significant in the case of the high-density cultures ($P < 0.0001$ vs. $P = 0.28$ at low density). A statistically significant three-fold decrease in the transformation frequency was observed in the low relative to high cell-density cultures ($P < 0.0004$).

These data indicate that the magnitude of the bystander effect is cell-density dependent in C3H 10T $\frac{1}{2}$ cells, implicating the involvement of gap junction mediated intercellular

communication in transmitting the bystander effect. Several studies have now shown that inhibition of this gap-junction activity in cells irradiated in close contact results in decreased levels of the bystander effects for a variety of biological endpoints (9, 10). An alternative, but unlikely, explanation is that the observed effect is due to some factor released into the media, which, because of a very short half-life can only migrate small distances from the irradiated cell. This is unlikely because it has been estimated that for the irradiation protocol used in the present study, any bystander signal induced could travel over a large distance through the media during irradiation (approximately 600-700 μ m) (11).

The results obtained for low-density cultures did deviate from those expected in the absence of a bystander effect, suggesting that such an effect may still be operative. Considering oncogenic transformation, in the absence of a bystander effect a transformation frequency of $2.1/10^4$ viable cells is expected which is less (although not significantly) than the observed frequency of 3.3 (Table I). A similar result was seen previously for clonogenic survival with a non-significant increase in cell killing. However, any bystander effect evident in the low-density cultures is likely to result from interaction of a secreted cytotoxic factor with unirradiated cells, rather than direct communication due to the very low frequency of cell-to-cell contact. This has been confirmed in a previous study on low-density cells where a random distribution of damaged cells throughout the population was seen, suggestive of an extracellular factor (12).

In conclusion, the present study confirms that when cells are exposed to low doses of α -particles the degree of cell-to-cell contact at the time of irradiation is important in transmission of the bystander signal. When cells are in close contact, gap-junctions play a major role, whereas if the degree of contact is poor, the bystander effect is mediated by the release of factors into the surrounding environment.

References

1. Mothersill C and Seymour C. Low-dose radiation effects: Experimental hematology and the changing paradigm. *Exp Hematol* **31**:437-45, 2003.
2. Morgan WF. Non-targeted and Delayed Effects of Exposure to Ionizing Radiation: I. Radiation-Induced Genomic Instability and Bystander Effects In Vitro. *Radiat Res* **159**:567-80, 2003.
3. Mothersill C and Seymour C. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *Int J Radiat Biol* **71**:421-7, 1997.
4. Ballarini F, Biaggi M, Ottolenghi A and Saporita O. Cellular communication and bystander effects: a critical review for modelling low-dose radiation action. *Mutat Res* **501**:1-12, 2002.
5. Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ and Hall EJ. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T $\frac{1}{2}$ cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. *Radiat Res* **155**:397-401, 2001.
6. Sawant SG, Randers-Pehrson G, Metting NF and Hall

Table I.

Clonogenic survival rates, number of dishes exposed, numbers of viable cells exposed in transformation studies, number of transformed clones produced, and transformation frequencies for microbeam irradiations.

No. of cells plated, No. of α -particles	Clonogenic surviving fraction (plating efficiency) (\pm SEM)	No. of dishes exposed	No. of viable cells exposed/ 10^4	No. of transformants produced	Transformation frequency/ 10^4 surviving cells
200, 0 α	(0.23 \pm 0.02)	71	0.9	1	1.1
2000, 0 α	(0.17 \pm 0.03)	60	2.1	1	0.5
200, 8 α	0.93 \pm 0.02	155	1.8	6	3.3
2000, 8 α	0.81 \pm 0.01	73	1.5	14	9.6

* Estimated, accounting for plating efficiency and clonogenic survival.

- EJ. Adaptive response and the bystander effect induced by radiation in C3H 10T(1/2) cells in culture. *Radiat Res* **156**:177-80, 2001.
7. Reznikoff CA, Bertram JS, Brankow DW and Heidelberger C. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res* **33**:3239-49, 1973.
 8. Miller RC, Randers-Pehrson G, Geard CR, Hall EJ and Brenner DJ. The oncogenic transforming potential of the passage of single alpha particles through mammalian cell nuclei. *Proc Natl Acad Sci USA* **96**:19-22, 1999.
 9. Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ and Hei TK. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc Natl Acad Sci USA* **97**:2099-104, 2000.
 10. Azzam EI, de Toledo SM and Little JB. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha-particle irradiated to nonirradiated cells. *Proc Natl Acad Sci USA* **98**:473-8, 2001.
 11. Nikjoo H and Khvostunov IK. Biophysical model of the radiation-induced bystander effect. *Int J Radiat Biol* **79**:43-52, 2003.
 12. Belyakov OV, Malcolmson AM, Folkard M, Prise KM and Michael BD. Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. *Br J Cancer* **84**:674-679, 2001. ■

The Bystander Effect and Adaptive Response in C3H 10T $\frac{1}{2}$ Cells

Stephen A. Mitchell, Gerhard Randers-Pehrson, David J. Brenner and Eric J. Hall

Evidence has now emerged for a number of biological phenomena which may be important in determining the cellular response to low doses of radiation (1). These include but are not limited to the bystander effect and adaptive response. Although both these phenomena are important at low doses, they have opposite effects on cell survival with the bystander effect transmitting damage from irradiated to non-hit cells while the adaptive response confers resistance to radiation following an initial low priming dose. Therefore they may operate in opposite directions to produce an overall biological effect, but to date there are only limited studies concerning their direct interaction (2, 3).

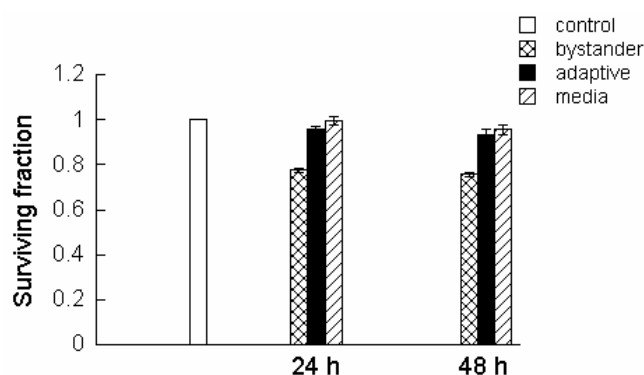


Fig. 1. Surviving fraction of bystander C3H 10T $\frac{1}{2}$ cells co-cultured either with cells ('bystander') or culture media ('media') irradiated with 5 Gy of α -particles. Results are also shown for bystander cells pretreated with a 2 cGy priming dose 5 h prior to co-culture with irradiated cells ('adaptive'). Data were pooled from at least three independent experiments (mean \pm SEM).

We used a novel radiation setup to examine the relationship between these two processes for the endpoints of clonogenic survival and oncogenic transformation. The experimental protocol employed has been described previously (4).

As shown in Figure 1, a significant decrease in surviving fraction from control levels was observed in the non-hit bystander cells following both 24 h and 48 h co-culture with cells irradiated with 5 Gy of α -particles (24 h: SF=0.77 \pm 0.01; p<0.0001). There was no significant difference in survival between the two time points studied. At the density at which the cells were plated, the vast majority of cells (>90%) were in close contact at the time of irradiation. Therefore it is possible that the irradiated cells could transmit the bystander signal to non-hit cells either through the secretion of a soluble extracellular factor into the medium and/or through direct cell-to-cell communication via gap junctions. However co-culture of cells with irradiated medium alone had no effect on survival of the non-hit bystander cells at either time point (24 h: SF=1.00 \pm 0.02). When cells were exposed to a 2 cGy priming dose 5 h prior to being co-cultured with irradiated cells, the majority of the bystander killing was lost and the surviving fraction was not significantly different from control levels at both time points (24 h: SF=0.96 \pm 0.02). This confirms the findings of previous microbeam-based experiments in this laboratory (2, 3).

Table 1 shows the oncogenic transformation frequencies obtained following 24 h of co-culture with irradiated cells. Bystander cells showed a significant increase in transformation frequency over spontaneous control levels (p<0.0001). As was observed for clonogenic survival, cells pretreated with the priming dose showed a 2.7-fold significant decrease in transformation frequency from that observed in bystander

Table I.

Clonogenic survival rates, numbers of viable cells exposed in transformation studies, number of transformed clones produced, and transformation frequencies for bystander C3H 10T½ cells co-cultured for 24h with cells ('bystander') or media ('media') exposed to 5 Gy α -particles. Results are also shown for cells irradiated with a 2 cGy priming dose 5h prior to co-culture with irradiated cells ('adaptive'). Data were pooled from at least three independent experiments (mean \pm SEM).

Irradiation conditions	Clonogenic surviving fraction (plating efficiency)	*No. of viable cells exposed/ 10 ⁴	No. of transformants produced	Transformation frequency/ 10 ⁴ surviving cells
0 Gy	(0.45 \pm 0.004)	11.2	6	0.5
Media	1.0 \pm 0.02	8.5	5	0.6
Bystander	0.77 \pm 0.01	14.7	51	3.5
Adaptive	0.96 \pm 0.02	11.2	14	1.3

* Estimated, accounting for plating efficiency and clonogenic survival.

cells (p<0.0001) to a level that was not significantly different from control levels. Again, no significant increase in transformation frequency was seen following co-culture with irradiated medium only.

Both an adaptive response (5, 6) and bystander effect (7) have been shown to be induced via the transfer of supernatant from irradiated cells onto unirradiated cells. In the present study, we set out to confirm whether such effects could be demonstrated in C3H 10T½ cells.

To examine the adaptive response, confluent, density-inhibited C3H 10T½ cells were sham-irradiated or exposed to a 2 cGy dose of x-rays and 18 h after exposure the supernatants were transferred onto unirradiated cells. Cells treated with the transferred culture medium were then exposed to a 4 Gy dose of x-rays 5 h later and immediately trypsinised for assessment of clonogenic survival and oncogenic transformation as described previously. For comparison, cells were also directly irradiated with 2 cGy and challenged 5 h later with 4 Gy.

The results are shown in Table II. Pretreatment of cells for 5 h with irradiated-conditioned medium prior to the 4 Gy challenge dose had no significant effect on clonogenic survival compared with cells directly irradiated with 4 Gy or treated with sham-irradiated medium. This is in contrast to previous studies (5, 6) and highlights the cell phenotype specific nature of the adaptive and bystander responses. However, an approximate two-fold reduction in the oncogenic transformation frequency was observed in cells treated with irradiated supernatant compared with directly irradiated cells or those treated with sham-irradiated medium, although it did not quite reach statistical significance (p=0.06). This suggests that supernatant from cells exposed to 2 cGy of x-rays may contain a factor(s) which acts on unirradiated, by-

Table II.

Clonogenic survival rates, numbers of viable cells exposed in transformation studies, number of transformed clones produced, and transformation frequencies for C3H 10T½ cells. Cells were either directly exposed to 4Gy X-rays or: (i) supernatant from sham-irradiated cells (sham/4Gy); (ii) supernatant from cells exposed to 2 cGy of X-rays (media/4Gy); or (iii) a 2 cGy priming dose (2 cGy/4Gy). Following a further 5 h incubation at 37°C, these cells were challenged with 4 Gy and processed immediately.

Irradiation conditions	Clonogenic surviving fraction (plating efficiency)	*No. of viable cells exposed/ 10 ⁴	No. of transformants produced	Transformation frequency/ 10 ⁴ surviving cells
0Gy	(0.48 \pm 0.01)	7.2	3	0.4
4Gy	0.34 \pm 0.01	11.7	78	6.7
Sham/4Gy	0.35 \pm 0.01	8.7	54	6.2
Media/4Gy	0.33 \pm 0.02	8.3	23	2.8
2cGy/4Gy	0.34 \pm 0.01	9.0	27	3.0

* Estimated, accounting for plating efficiency and clonogenic survival.

stander cells, reducing their susceptibility to oncogenic transformation, but not cell killing. A similar result was seen for both endpoints when cells were directly irradiated with 2 cGy prior to being exposed to the challenge dose.

It is interesting to note that in the present study, cells directly irradiated with a 2 cGy priming dose followed by a subsequent 4 Gy challenge dose showed no increase in survival (Table II), in contrast to bystander cells in the double-ring experiments which were treated with a priming dose followed by co-culture with irradiated cells and which showed a significant adaptive response for survival (Figure 1). This suggests that following exposure to a priming dose of x-rays and consequent induction of the adaptive mechanism(s), C3H 10T½ cells are less sensitive to the deleterious effects of a bystander signal, but just as susceptible to damage from direct, high-dose exposure to x-rays.

The protocol used to assess the induction of a bystander response via media transfer has been described elsewhere (7). Figure 2 shows the clonogenic survival obtained when unirradiated cells were treated with either irradiated (5 Gy) or unirradiated medium taken from cells 18 h post irradiation. Growth in irradiated medium significantly reduced the clonogenic survival of the cells (p<0.002; SF = 0.90 \pm 0.03). This has been seen in previous studies and is suggestive of the fact that irradiated cells secrete a cytotoxic factor into the medium which is then able to elicit a bystander effect in unirradiated cells (7). Cells treated with medium from unirradiated control flasks had a non-significant increase in survival (SF=1.08 \pm 0.04). This may be due to the medium becoming conditioned from the high-density cultures during the 18 h incubation period and then conferring a survival

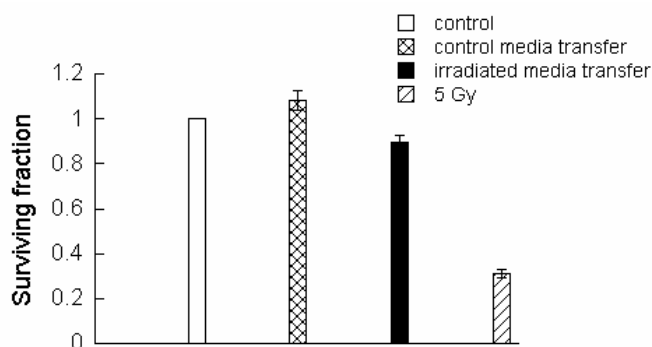


Fig. 2. Surviving fraction of unirradiated C3H 10T½ cells cultured in media from either unirradiated donor cells or cells irradiated with 5 Gy of X-rays 18 h prior to donation. Survival for cells directly irradiated with 5 Gy are also shown. Data were pooled from at least three independent experiments (mean \pm SEM).

advantage on the cells to which it is transferred.

Although there are several differences in the protocols used making a direct comparison difficult, the amount of bystander cell killing seen in the medium transfer experiments was two-fold less than that seen when using the double-ring protocol (Figure 2 vs. Figure 1). This may be a result of the bystander signal being transmitted between cells via gap junctions in addition to the secretion of a cytotoxic factor into the medium in the high-cell density double-ring protocol. This may lead to a subsequent increase in cell killing confirming the importance of cell-to-cell contact at the time of irradiation in transmitting the bystander response (8, 9).

In conclusion, these results indicate that following radiation exposure, the resulting biological effect is dependent upon the interaction between the adaptive response and the bystander effect.

1. Upton AC. The state of the art in the 1990's: NCRP Report No. 136 on the scientific bases for linearity in the dose-response relationship for ionizing radiation. *Health Physics* **85**:15-22, 2003.
2. Sawant SG, Randers-Pehrson G, Metting NF and Hall EJ. Adaptive response and the bystander effect induced by radiation in C3H 10T(1/2) cells in culture. *Radiation Research* **156**:177-80, 2001.
3. Zhou H, Randers-Pehrson G, Geard CR, Brenner D, Hall EJ and Hei TK. Interaction between radiation induced adaptive response and bystander mutagenesis in mammalian cells. *Rad Research* **160**:512-6, 2003.
4. Stephen A. Mitchell, Stephen A. Marino, David J. Brenner and Eric J. Hall. The Bystander Effect in Radiation Oncogenesis. *Center for Rad. Research Annual Report* p. 20-1, 2002 [http://cr-cu.org/reports2002/b5.htm].
5. Iyer R and Lehnert BE. Alpha-particle-induced increases in the radioresistance of normal human bystander cells. *Radiation Research* **157**:3-7, 2002.
6. Iyer R and Lehnert BE. Low dose, low-LET ionizing radiation-induced radioadaptation and associated early responses in unirradiated cells. *Mutation Research* **503**:1-9, 2002.
7. Mothersill C and Seymour C. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology* **71**:421-7, 1997.
8. Azzam EI, De Toledo SM and Little JB. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha-particle irradiated to nonirradiated cells. *Proceedings of the National Academy of Sciences* **98**:473-8, 2001.
9. Zhou H, Randers-Pehrson G, Suzuki M, Waldren CA and Hei TK. Genotoxic damage in non-irradiated cells: contribution from the bystander effect. *Radiation Protection Dosimetry* **99**:227-32, 2002. ■

Stimulation of Clonogenic Survival in Radiation Induced Bystander Cells

Rajamanickam Baskar, Adayabalam S. Balajee and Charles R. Geard

“Bystander effect” is an interesting phenomenon where cells directly targeted by radiation transmit the damaging signal to the non-targeted cells. Bystander effect can be mediated either through gap-junctions or through soluble factors released from irradiated cells. There has been a considerable amount of data obtained on “bystander effects” from various cell types in culture following low and high LET

radiation exposure. Available reports on bystander effects show alterations in growth potential, cell killing, gene mutation and modifications in gene expression (1). Radiation-induced bystander effects are multifaceted and often appear to be cell type and genotype dependent, suggesting a need for more studies to understand the molecular mechanism(s) for radiation induced bystander effects. In the present study,

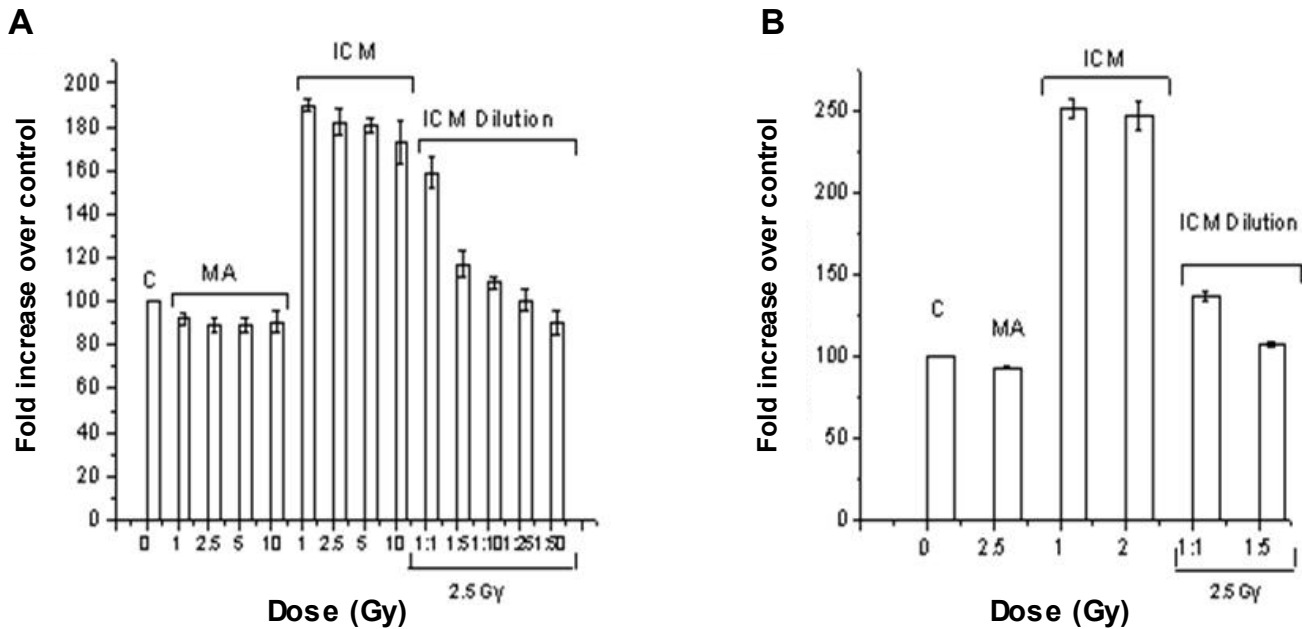


Fig. 1 Stimulation of clonogenic survival in bystander cells. **A.** Survival of the GM637H cells treated with ICM derived from irradiated MRC-5 cells. **B.** Survival of the GM637H cells treated with ICM derived from irradiated GM637H cells. C - Control; MA - Medium alone without cells, irradiated and transferred; ICM - Irradiated conditioned medium with cells; ICM Dilution - ICM (2.5 Gy) was diluted to different concentrations. Each data point represents the mean \pm SE of three independent determinations.

using gamma rays as a DNA damaging agent, we have evaluated the role of bystander effect on the clonogenic potential of human fibroblasts.

Primary and SV40 transformed fibroblasts derived from normal (MRC-5 and GM637H) cells were procured from the Coriell Cell Repository, in Camden, New Jersey. Cells were maintained in 2X Eagle's minimal essential medium (EMEM) supplemented with 15% fetal bovine serum, vitamins, essential amino acids, non-essential amino acids and antibiotics (Gibco BRL). The cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. MRC-5 cells, synchronized at G1 phase by growing them to confluence, were irradiated with different doses (1, 2.5, 5 and 10 Gy) of gamma rays using a ¹³⁷Cs source at a dose rate of 0.98 Gy/min (Gammacell 40, Atomic Energy of Canada, Canada). The irradiated MRC-5 cells were incubated for 1 hr at 37°C, and the medium collected from the irradiated cells (designated as irradiated conditioned medium, ICM) was used to treat the unirradiated cells for determining of bystander effects. A clonogenic survival assay was used for assessing the growth potential in bystander cells. For this purpose, GM637H cells were seeded at a density of 750 cells/6 cm dish 24 hrs prior to treatment. As a negative control, complete medium alone was irradiated with different doses of gamma rays and incubated for 1 hr at 37°C.

Colonies were fixed in 70% ethanol after two weeks and stained with coomassie blue solution (0.5%). The number of colonies obtained for untreated control cells was considered

as 100% and the colonies observed in the treated samples were normalized to the control cells for determining the effect on clonogenic survival. Bystander cells treated with ICM of different radiation doses showed a 1.7 to 1.8-fold enhancement in clonogenic survival (Figure 1A). Cells treated with medium alone did not show any increase in cell survival indicating that the signal released from the irradiated cells is chiefly responsible for an elevated clonogenic potential. If the soluble factors released from the irradiated cells are responsible for clonogenic stimulation in bystander cells, dilution of the factors with unirradiated medium is expected to diminish the bystander effects. To test this possibility, ICM (2.5 Gy irradiated) was serially diluted with complete medium. The findings presented in Fig-1A showed a gradual decline with increasing ICM dilution. Similar results were obtained when ICM derived from 1 Gy and 2.5 Gy gamma rays treated GM637H cells was used (Figure 1B).

Studies using different repair proficient and deficient (primary vs. transformed) human cells are in progress to characterize the biochemical nature of the factor(s) responsible for the clonogenic stimulation in bystander cells.

References

1. Mothersill C and Seymour CB. Radiation-induced bystander effects: Past history and future directions. *Radiat Res* **15**:759-67, 2001. ■

Mechanisms of the Bystander Effect: Assessment of Low LET Radiation-Induced Bystander Effect in a Three-Dimensional Culture Model

Rudranath Persaud, Hongning Zhou, Tom K. Hei and Eric J. Hall

The radiation-induced Bystander effect has been demonstrated for a variety of endpoints, using a range of rodent and human cell culture models, mainly with high LET alpha particles. However, there is a need to ascertain whether a similar response can be observed with low LET radiation at doses relevant to environmental exposure. It is equally desirable to determine if such a response can be demonstrated in a three-dimensional culture system, modeling a normal tissue microenvironment. In the present study, a three-dimensional cell culture model comprised of human-hamster hybrid (A_L) and Chinese hamster ovary (CHO) cells as multi-cellular clusters was used to investigate low LET radiation-induced bystander genotoxicity. Separation of A_L and CHO cells was achieved with ~99% efficiency using a magnetic cell separation technique (MACS). Briefly, CHO cells were mixed with A_L cells in various proportions ranging from 10 to 50% and centrifuged briefly to produce a spheroid of 4×10^6 cells. Clusters were incubated overnight, resuspended into single cell suspensions, passed twice through MACS separation columns and the efficiency of separation determined using Fluorescence-Activated Cell Sorter (FACS) analysis.

To establish if low LET radiation induces bystander toxicity, CHO cells were labeled with tritiated thymidine ($^3\text{HdTTP}$, 30 μCi) for 12 hrs and subsequently incubated with A_L cells in multi-cellular spheroids for 24 hrs at 11°C. Labeled CHO cells showed a 50% survival, while the non-

labeled, bystander A_L cells showed a surviving fraction of 80% compared to similarly treated controls (Figure 1), thus demonstrating a significant bystander effect induced by irradiation of neighboring cells with low LET electrons.

To determine whether low LET radiation can induce bystander mutation using this spheroid model, CHO cells were labeled with 12 μCi $^3\text{HdTTP}$ for 12 hrs and subsequently incubated with A_L cells for 24 hrs at 11°C. Subsequent to magnetic separation, the bystander A_L cells were subjected to a 7-day expression period and mutants were scored utilizing the CD59 Antibody-Complement Cell Lysis Assay. Since the separation of A_L and CHO cells within the cluster may not be entirely efficient, mutant colonies were detected by implementing a centromeric probe toward the human chromosome 11 present in the hybrid A_L cells. Preliminary results have indicated a dose of 12 μCi $^3\text{HdTTP}$ induces 169 mutants/ 10^5 survivors (Figure 2). Additional experiments are underway to establish whether similar mutations can occur at the low dose range of 0.5 to 1.0 μCi $^3\text{HdTTP}$.

Results of the present study should provide important information on the relevance of the bystander effect under *in vivo* conditions. Furthermore, mechanism(s) underlying the bystander effect are likely to be complex and may involve both primary and secondary signaling events. A better understanding of the biochemical and molecular changes governing the bystander process will help in radiation risk assessment and management. ■

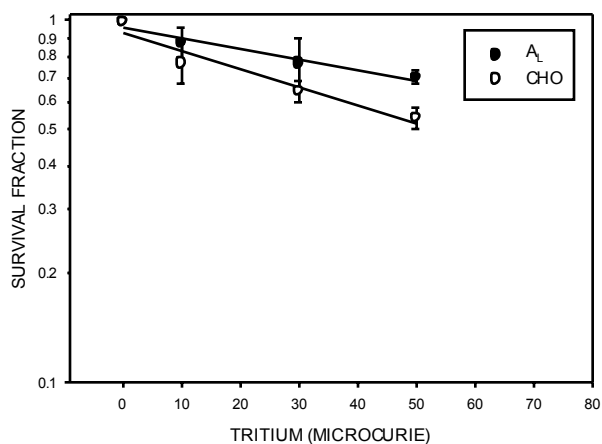


Fig. 1. Survival fraction of A_L and CHO cells treated with Tritium (0-50 microcurie).

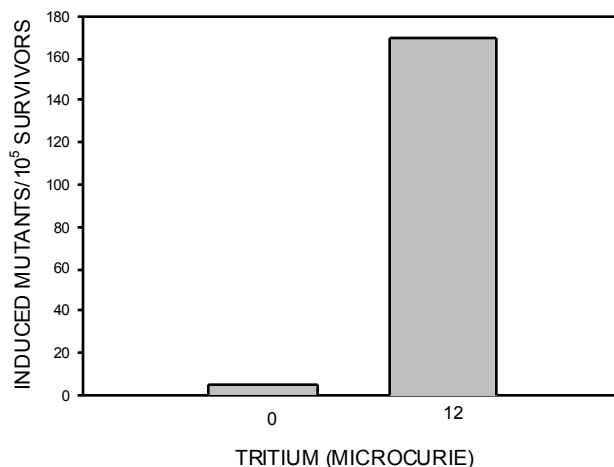


Fig. 2. Mutation induction by Tritium in bystander A_L cells.

Radiation Induced Bystander Effects in Normal Human Fibroblasts

Hongning Zhou, Rudranath Persaud and Tom K. Hei

Based principally on the cancer incidence found in survivors of the atomic bombs in Japan, the International Commission on Radiation Protection (ICRP) and the United States National Council on Radiation Protection and Measurements (NCRP) have recommended that estimates of cancer risk for low dose exposure be extrapolated from higher doses where data are available using a linear, no-threshold model (1, 2). This recommendation is based on the dogma that the DNA of the nucleus is the main target for radiation-induced genotoxicity and, as fewer cells are directly damaged, the deleterious effects of radiation proportionally decline. However, evidence is now emerging that extra-nuclear or extra-cellular targets may also be important in mediating the genotoxic effect of irradiation. Early evidence for this bystander effect came from studies in which the frequency of SCE in populations of cells exposed to low fluences of alpha particles was significantly higher than expected from target theory calculations of the number of cells that had actually received an alpha particle (3, 4). Furthermore, such biological effects as induction of micronuclei (5), gene mutation (6-8), expression of stress-related genes (9-11), and malignant transformation *in vitro* (12) can occur in a significantly higher proportion of cells than in those traversed by an alpha particle. In addition, medium from cultures of cells irradiated with gamma rays can kill unirradiated cells. Cells in contact with cells internally irradiated by short-range ^3H - β particles also have a reduced clonal survival (13). However, the mechanism and nature of these bystander signaling processes remain unclear.

The newly designed strip mylar dishes will be used in this experiment. Briefly, the bottom of the well-fit outer and inner stainless rings is covered with 6 μm and 38 μm thick mylar sheets, respectively. The mylar of the inner rings is cut as strips with a specifically designed tool. Exponentially growing normal human lung fibroblasts are seeded in the specially constructed dishes and allowed to grow to confluence. Cells are irradiated with graded doses of alpha particles from the bottom using the track segment mode as described (14-16). Since the fibroblasts seeded on the 38 μm thick mylar strips will not be irradiated due to the short penetrating distance of the alpha particles, these cells will effectively be the bystander cells seeded right next to cells plated on the 6 μm mylar dishes that are directly irradiated. We found that alpha particle irradiation would induce a bystander response in non-irradiated bystander cells using the strip dishes (Figure 1). This unique method provides a sufficient number of bystander cells as well as irradiated cells for further investigation.

Using cDNA signal transduction pathway finder array Analysis (Super Array, MD), preliminary experiments have shown that the COX-2 gene was up-regulated in the bystander cells, while the IGFBP-3 gene was significantly down-regulated in the bystander cells (Figure 2A). Using technique based on RT-PCR, we confirm the finding of down-regulation of the IGFBP-3 gene (Figure 2B). The IGFBP-3 gene is 8.9kb in length and is expressed in a large number of tissues. In addition to its demonstrated growth-promoting roles, IGFBP-3 is also a well-documented in-

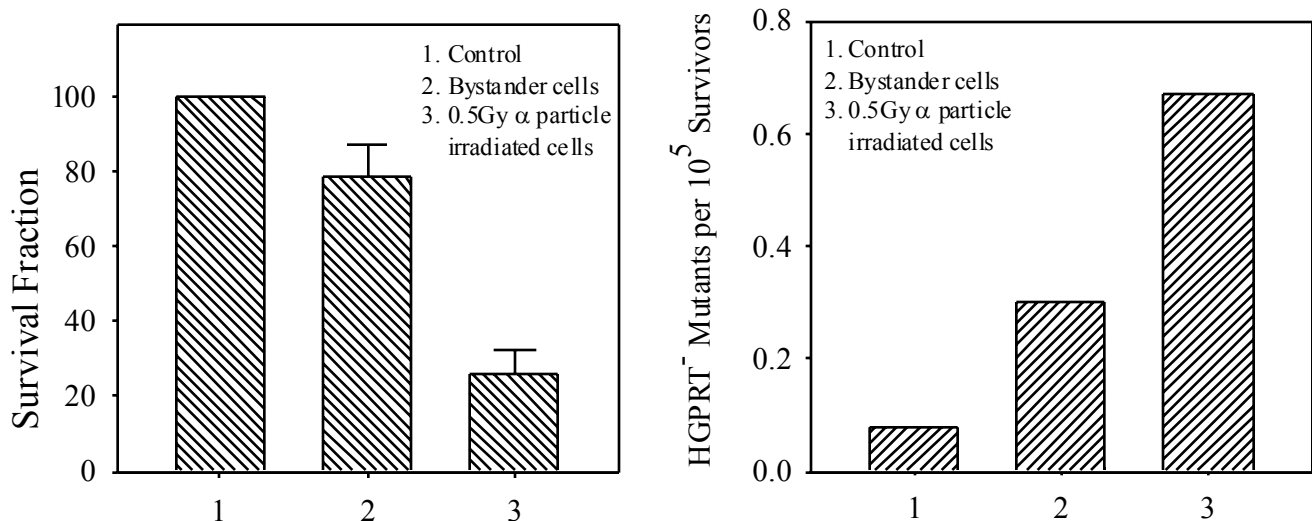


Fig. 1. Survival fraction and HGPRT mutation of bystander and directly irradiated cells (0.5 Gy alpha particle radiation) in strip dishes. Data are pooled from 2-3 independent experiments. Bars represent \pm SD.

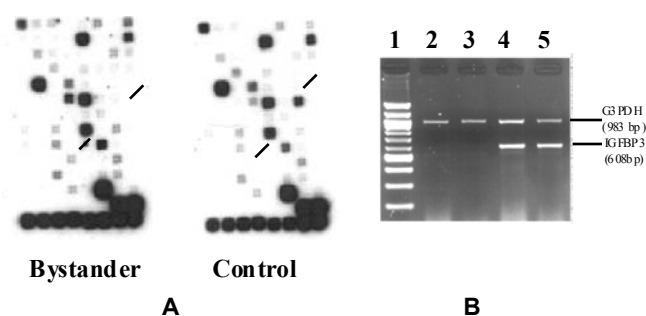


Fig. 2. **A.** Preliminary data showing differentially expressed signaling genes between control and bystander normal human lung fibroblasts using Super Array cDNA signal transduction pathway finder array. **B.** Confirmation of IGFBP3 down regulation in bystander cells by RT-PCR. Lane 1: 100bp marker; Lanes 2 and 3: bystander cells from different experiments; Lanes 4 and 5: control cells from different experiments.

hibitor of cellular proliferation. It has been reported that over-expression of a transfected IGFBP-3 gene strongly inhibits cell proliferation, with or without added IGF (17). Blocking IGFBP-3 expression with antisense IGFBP3 oligodeoxynucleotides can also attenuate the potent anti-proliferative action induced by transforming growth factor- β (TGF- β). More recently, Levitt et al found that IGF-1 inhibited the anti-proliferative effects of celecoxib, COX2 inhibitor, on pancreatic cancer cells, and IGFBP-3 enhanced celecoxib-induced growth inhibition, implying the possible interaction between IGFBP-3 and COX2 (18). In contrast to the expression of COX1, COX2 is not detected in most normal tissues. However, it is induced by mitogenic and inflammatory stimuli, which results in enhanced synthesis of prostaglandins in neoplastic and inflamed tissues. There is considerable evidence that links COX2 to the development of cancer (19). Since the COX2 gene plays an important role in arachidonic acid metabolism, the finding of COX2 over-expression implied that small soluble molecules associated with the arachidonic cascade are essential in mediating the bystander signaling process.

References

1. International Commission on Radiological Protection, Recommendations, Report no. 60 (New York: Pergamon Press), 1991.
2. National Council on Radiation Protection and Measurements, Report 116 (Bethesda, Md, NCRP), 1993.
3. Nagasawa H and Little J. Induction of sister chromatid exchanges by extremely low doses of α -particles. *Cancer Res* **52**:6394-6, 1992.
4. Deshpande A, Goodwin EH, Bailey SM, Marrone BL and Lehnert BE. Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. *Radiat Res* **145**:260-7, 1996.
5. Prise KM, Belyakov OV, Folkard M and Michael BD. Studies of bystander effects in human fibroblasts using a charged particle microbeam. *Int J Radiat Biol* **74**:793-8, 1998.
6. Nagasawa H and Little J. Unexpected sensitivity to the

- induction of mutations by very low doses of alpha-particle radiation: evidence for a bystander effect. *Radiat Res* **152**:552-7, 1999.
7. Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ and Hei TK. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc Natl Acad Sci USA* **97**:2099-104, 2000.
8. Zhou H, Randers-Pehrson G, Suzuki M, Waldren CA, Vannais D, Chen G, Trosko JE and Hei TK. Radiation risk to low fluences of α -particles may be great than we thought. *Proceeding of National Academy of Science USA* **98**:14410-5, 2001.
9. Hickman AW, Jaramillo RJ, Lechner JF and Johnson NF. α -Particle-induced p53 protein expression in a rat lung epithelial cell strain. *Cancer Res* **54**:5797-800, 1994.
10. Azzam EI, de Toledo SM, Gooding T and Little JB. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluencies of alpha particles. *Radiat Res* **150**:497-504, 1998.
11. Azzam EI, de Toledo SM, Spitz DR and Little JB. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from alpha-particle-irradiated normal human fibroblast cultures. *Cancer Res* **62**:5436-42, 2002.
12. Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ and Hall EJ. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T 1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. *Radiat Res* **155**:397-401, 2001.
13. Bishayee A, Rao DV and Howell RW. Evidence for pronounced bystander effects caused by nonuniform distributions of radioactivity using a novel three-dimensional tissue culture model. *Radiat Res* **152**:88-97, 1999.
14. Zhou H, Zhu LX, Li K and Hei TK. Radon, Tobacco Specific Nitrosamine and Mutagenesis in Mammalian Cells. *Mutation Research* **430**:145-53, 1999.
15. Hei TK, Piao CQ, Willey JC, Thomas S and Hall EJ. Malignant transformation of human bronchial epithelial cells by radon-simulated alpha-particles. *Carcinogenesis* **15**:431-7, 1994.
16. Zhu LX, Waldren CA, Vannais D and Hei TK. Cellular and molecular analysis of mutagenesis induced by charged particles of defined linear energy transfer. *Radiat Res* **145**:251-9, 1996.
17. Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM and Rosenfeld RG. Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. *Int J Biochem Cell Biol* **28**:619-37, 1996.
18. Levitt RJ and Pollak M. Insulin-like growth factor-I antagonizes the antiproliferative effects of cyclooxygenase-2 inhibitors on BxPC-3 pancreatic cancer cells. *Cancer Res* **62**:7372-6, 2002.
19. Subbaramaiah K and Dannenberg AJ. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends in Pharmacol Sci* **24**:96-102, 2003. ■

Analysis of Radiation-Induced Bystander Effects in Mouse Embryonic Stem Cells Differing in the Status of *Mrad9* Reveals Complexities in the Process

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It has recently been found that multiple cellular responses to ionizing radiation are not limited to those cells directly exposed, but can often be demonstrated in neighboring “bystander” cells (for review see ref. 1, 2). This bystander effect involves the production of a biological response in cells neighboring those that are actually “hit” by radiation. This implies that cells directly exposed to radiation can transmit a signal to other cells nearby, thus in a sense amplifying the initial damage signal. In essence, this suggests that models taking into account only direct hits in mediating a biological response underestimate the true deleterious effects of radiation exposure, including potential health risks.

The bystander effect was first suggested by Nagasawa and Little (3, 4) when they reported that the calculated, expected nuclear traversal of 1% of cells in a population by a flux of alpha particles caused 30% of the cells to undergo sister chromatid exchanges. Subsequently, bystander effects have been demonstrated for cell survival, mutation and oncogenic transformation (for review see 1). The use of a microbeam to target alpha particles to individual cells also provided more evidence that a cell does not have to be hit directly by an alpha particle to demonstrate mutation or changes in survival. These experiments have been performed by using either of two strategies. In the first, a lethal 20-hit dose of alpha particles could be delivered to 5% of the cells in a population, and mutations would arise in frequency similar to that observed when 100% of cells are hit with a single particle (5). Alternatively, Zhou et al. (6) showed that when 10% of a population is exposed to a single alpha particle, which is sublethal, many more cells in the population demonstrated chromosome aberrations and mutation than just the small percentage exposed. Interestingly, the mutation spectrum for bystander cells differed from that obtained spontaneously or after cytoplasmic irradiation, suggesting that different mutagenic mechanisms are involved (5).

Genetic makeup is also important in terms of understanding how a cell or individual will respond to irradiation. Hundreds of genes participating in multiple DNA repair and cell cycle checkpoint control pathways have been identified and, when mutated, reduce radioresistance and increase the mutagenic or oncogenic potential of radiation exposure (7). Genes involved in signal transduction pathways often have multiple roles in promoting cell survival after radiation exposure, and in maintaining genomic stability in treated cells or even those not exposed to an exogenous DNA damaging agent. Such gene alterations can have dire consequences, including the generation of a high frequency of chromosome

aberrations, mutation and cancer. Interestingly, Nagasawa and Little (5) demonstrated that an *xrs-5* mutation in CHO cells significantly enhances chromosome aberration yields due to bystander effects induced by low fluences of alpha particles. They interpreted their results by stating that the *xrs-5* mutation reduced repair of double strand DNA breaks caused by the alpha particles, and this prolonged a signal that mediates the bystander effect. These results suggest that there is a genetic control component to the bystander response, perhaps at the level of damage processing or at another stage. However, regardless of the mechanism responsible, the array of genes involved and their precise roles have not been determined.

The *RAD9* gene, first identified in the fission yeast *Schizosaccharomyces pombe* (8, 9), then subsequently found as orthologues in human *HRAD9* (10) and mouse *Mrad9* (11), is an important genetic element that regulates multiple radioresponses as part of signal transduction pathways. The human and mouse genes partially complement the sensitivity of *S. pombe rad9::ura4+* cells to ionizing radiation, UV and the DNA synthesis inhibitor hydroxyurea, as well as the associated cell cycle checkpoint defects. We created homozygous *Mrad9* knockout mouse ES cells and demonstrated that they are highly sensitive to ionizing radiation, UV and HU (data not shown), and also show defects in the maintenance of ionizing radiation-induced G2/M checkpoint and UV-induced delays in DNA replication, similar to several specific point mutants of the fission yeast *rad9* gene (12). Furthermore, WT human *HRAD9* or mouse *Mrad9* complements the sensitivity defects of the *Mrad9*^{-/-} mouse cells.

The protein encoded by the human *HRAD9* gene is a nucleoprotein, and a nuclear localization signal (NLS) has been identified within (13). We also found in collaboration with Dr. Eva Lee’s group at the University of Texas, San Antonio, that ATM can phosphorylate HRAD9 on Ser-272 and the event is important for G1 checkpoint control (14). In collaboration with Dr. Hong-Gang Wang’s group at the Moffitt Cancer Center in Tampa, FL, we demonstrated that HRAD9 protein (and surprisingly *S. pombe rad9* as well) contains a BH3-like domain at its N-terminal region that can bind the anti-apoptotic proteins BCL-2 and BCL-xL, and can cause apoptosis when aberrantly expressed in human cells (15, 16). Therefore, RAD9 regulates two fundamental responses to DNA damage, cell cycle checkpoint control and apoptosis.

The goal of this project is to assess whether mouse embryonic stem (ES) cells express a bystander effect, and to define the role of the cell cycle checkpoint control gene

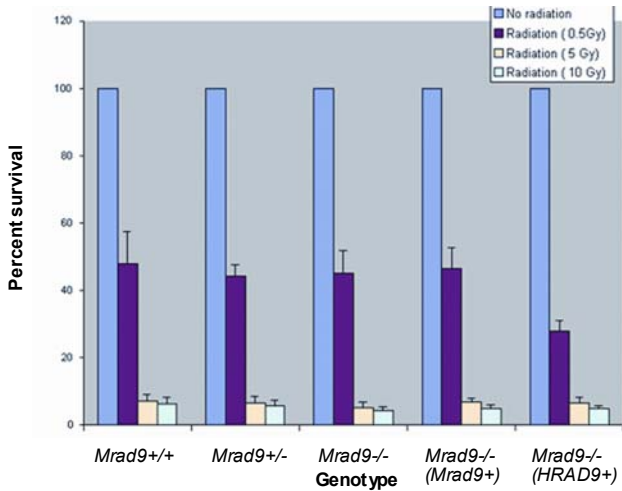


Fig. 1. Radiation-induced cell killing. Radiosensitivity was assessed by measuring colony formation in irradiated versus unirradiated populations. Points represent the average of three trials, +/- S.D.

Mrad9 in the process. In this report, mouse ES cells differing in the status of *Mrad9* were examined for bystander survival, micronuclei formation and apoptosis in response to broad beam 120 keV alpha particle treatment, using specially designed mylar strip dishes.

When cells differing in the status of *Mrad9* were directly exposed to alpha particles, all the populations demonstrated essentially equivalent killing curves (Figure 1). Interestingly, since *Mrad9*+/- cells are more sensitive than *Mrad9*+/+ cells to gamma rays, and the homozygous deletion mutant shows even greater sensitivity (data not shown), these results indicate that the role of *Mrad9* in mediating the cellular response to ionizing radiation is LET dependent. In addition, we found that all cells, regardless of *Mrad9* status, demonstrated an equivalent bystander reduction in survival after alpha particle exposure (Figure 2).

We also examined these cells for apoptosis, induced both by direct exposure to alpha particles and as a bystander effect. And as indicated in Figure 3, all cell populations examined demonstrated an increased frequency of apoptosis after

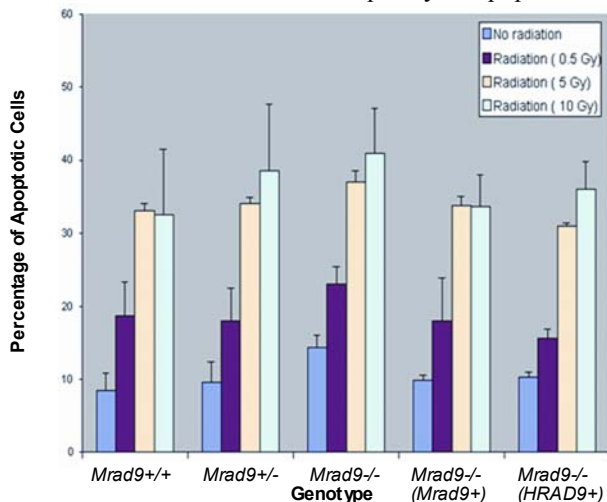


Fig. 3. Radiation-induced apoptosis. Cells either mock treated or irradiated were processed using the Annexin V-FITC Apoptosis Detection Kit from Oncogene. Flow cytometry was used to assess apoptosis in a minimum of 10,000 cells from each population, and points represent the average of three trials, +/- S.D.

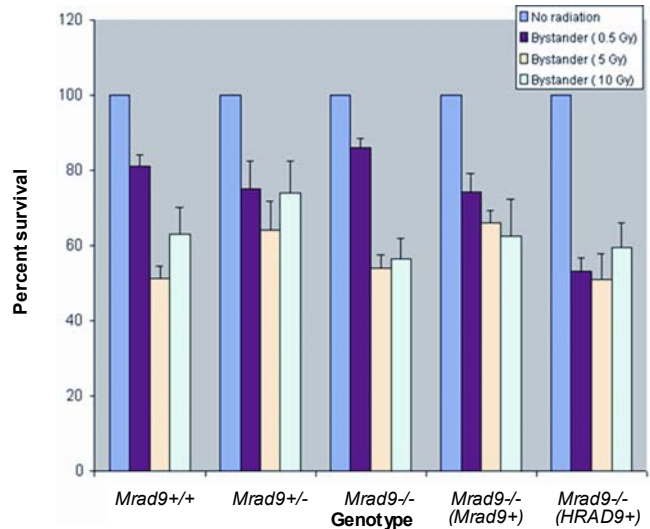


Fig. 2. Radiation-induced bystander cell killing. Radiosensitivity was assessed by measuring colony formation in indirectly irradiated versus unirradiated populations. Points represent the average of three trials, +/- S.D.

direct irradiation. For most exposures, induction was dose dependent. Interestingly also is the fact that even *Mrad9*-/- cells showed an induction of apoptosis, contrary to what would be expected by the deletion of the mouse orthologue of the human gene *hRAD9*, shown previously to be a pro-apoptotic element. Likewise, all cells demonstrated an alpha particle-induced bystander apoptotic effect (Figure 4). These results for apoptosis are consistent with the gene not influencing cell survival after exposure to alpha particles (Figure 1, 2).

Micronuclei formation was also examined in the cells differing in *Mrad9* status. As indicated in Figures 5 and 6, background levels of micronuclei were relatively high in the *Mrad9*-/- cells. However, direct exposure to alpha particles induced micronuclei approximately equally above background in all the cells examined. Interestingly, there was little or no bystander effect with regard to micronuclei formation in all the populations examined except those contain-

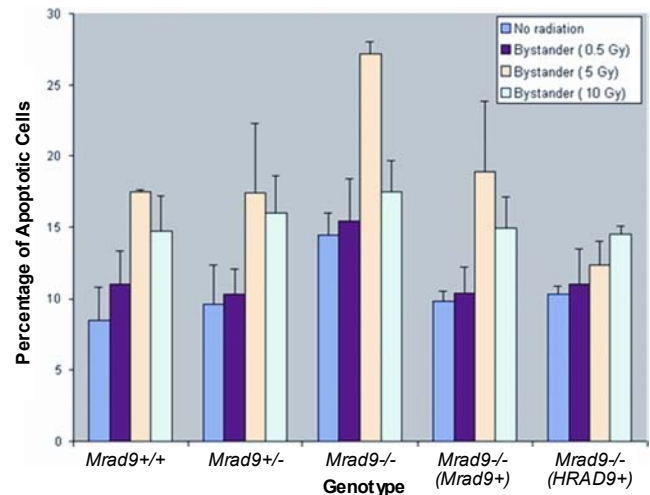


Fig. 4. Radiation-induced bystander apoptosis. Cells either mock treated or irradiated indirectly were processed using the Annexin V-FITC Apoptosis Detection Kit from Oncogene. Flow cytometry was used to assess apoptosis in a minimum of 10,000 cells from each population, and points represent the average of three trials, +/- S.D.

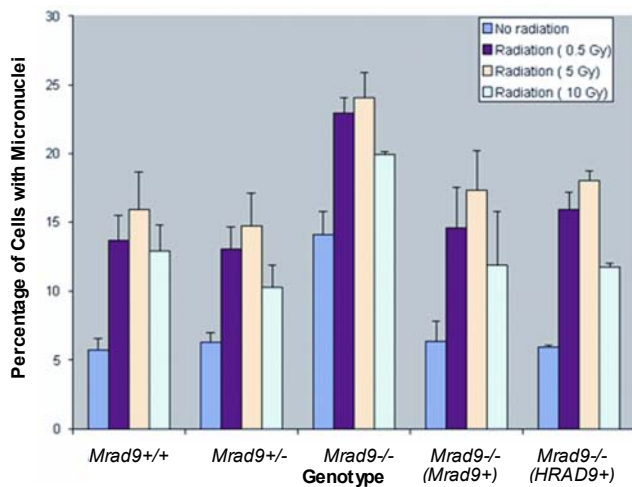


Fig. 5. Radiation-induced micronuclei formation. Irradiated or mock-treated cells were fixed, stained with DAPI, and then scored for micronuclei. Between 500 and 2000 cells were scored per population per experiment. Points represent the average of three trials, +/- S.D.

ing the *Mrad9*^{-/-} mutation (Figure 6).

In summary, we found that wild-type mouse ES cells are capable of expressing an alpha particle induced bystander effect for cell survival and apoptosis, but not for micronuclei formation. Interestingly, although *Mrad9*^{-/-} cells demonstrated high background levels of apoptosis, the mutation did not effect the bystander induction of programmed cell death or cell killing. In contrast, wild-type ES cells did not exhibit a bystander effect with regard to micronuclei formation, but the *Mrad9*^{-/-} mutation allowed such an effect to be observed. These results suggest that the mechanism of bystander induction is complex and may differ with regard to different endpoints examined.

References

- Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, Clastogenic factors and transgenerational effects. *Radiat Res* **159**:581-596, 2003a.
- Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. *Radiat Res* **159**:567-80, 2003b.
- Nagasawa H and Little JB. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Res* **52**:6394-6, 1992.
- Nagasawa H and Little JB. Bystander effect for chromosomal aberrations induced in wild-type and repair deficient CHO cells by low fluences of alpha particles. *Mutat Res* **508**:121-9, 2002.
- Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ and Hei TK. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc Natl Acad Sci USA* **97**:2099-104, 2000.
- Zhou H, Suzuki M, Randers-Pehrson G, Vannais D, Chen G, Trosko JE, Waldren CA and Hei TK. Radiation risk to low fluences of alpha particles may be greater than we thought. *Proc Natl Acad Sci USA* **98**:14410-5, 2001.
- Friedberg EC, Walker GC and Siede W. *DNA Repair*

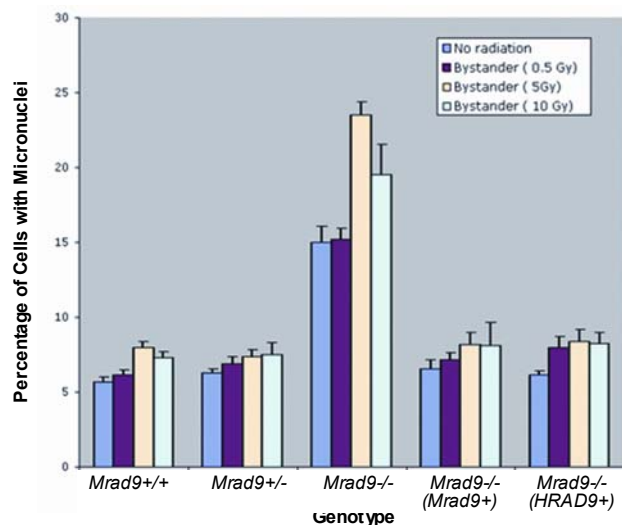


Fig. 6. Radiation-induced bystander micronuclei formation. Indirectly irradiated or mock-treated cells were fixed, stained with DAPI, and then scored for micronuclei. Between 500 and 2000 cells were scored per population per experiment. Points represent the average of three trials, +/- S.D.

and Mutagenesis. ASM Press, Washington, D.C., 1997.

- Murray JM, Carr AM, Lehmann AR, Watts FZ. Cloning and characterisation of the rad9 DNA repair gene from *Schizosaccharomyces pombe*. *Nucl Acids Res* **19**:3525-31, 1991.
- Lieberman HB, Hopkins KM, Laverty M and Chu HM. Molecular cloning and analysis of *Schizosaccharomyces pombe rad9*, a gene involved in DNA repair and mutagenesis. *Mol Gen Genet* **232**:367-76, 1992.
- Lieberman HB, Hopkins KM, Nass M, Demetrick D and Davey S. A human homologue of the *Schizosaccharomyces pombe rad9+* checkpoint control gene. *Proc Natl Acad Sci USA* **93**:13890-5, 1996.
- Hang H, Rauth SJ, Hopkins KM, Davey SK and Lieberman HB. Molecular cloning and tissue-specific expression of *Mrad9*, a murine orthologue of the *Schizosaccharomyces pombe rad9+* checkpoint control gene. *J Cell Physiol* **177**:241-7, 1998.
- Hang H, Rauth SJ, Hopkins KM and Lieberman HB. Mutant alleles of *Schizosaccharomyces pombe rad9(+)* alter hydroxyurea resistance, radioresistance and checkpoint control. *Nucleic Acids Res* **28**:4340-9, 2000.
- Hirai I and Wang HG. A role of the C-terminal region of human Rad9 (hRad9) in nuclear transport of the hRad9 checkpoint complex. *J Biol* **277**:25722-7, 2002.
- Chen M-J, Lin Y-T, Lieberman HB, Chen G and Lee EY-HP. ATM-dependent phosphorylation of human Rad9 is required for ionizing radiation-induced checkpoint activation. *J Biol Chem* **276**:16580-6, 2001.
- Komatsu K, Miyashita T, Hang H, Hopkins KM, Zheng W, Cuddeback S, Yamada M, Lieberman HB and Wang H-G. Human homologue of *S. pombe Rad9* interacts with Bcl-2/Bcl-xL and promotes apoptosis. *Nature Cell Biol* **2**:1-6, 2000a.
- Komatsu K, Hopkins KM, Lieberman HB and Wang H-G. *Schizosaccharomyces pombe Rad9* contains a BH3-like region and interacts with the anti-apoptotic protein Bcl-2. *FEBS Letters* **481**:122-6, 2000b. ■