



Review

Detection of chromosomal instability in α -irradiated and bystander human fibroblasts

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Abstract

There is increasing evidence biological responses to ionizing radiation are not confined to those cells that are directly hit, but may be seen in the progeny at subsequent generations (genomic instability) and in non-irradiated neighbors of irradiated cells (bystander effects). These so called non-targeted phenomena would have significant contributions to radiation-induced carcinogenesis, especially at low doses where only a limited number of cells in a population are directly hit. Here we present data using a co-culturing protocol examining chromosomal instability in α -irradiated and bystander human fibroblasts BJ1-htert. At the first cell division following exposure to 0.1 and 1 Gy α -particles, irradiated populations demonstrated a dose dependent increase in chromosome-type aberrations. At this time bystander BJ1-htert populations demonstrated elevated chromatid-type aberrations when compared to controls. Irradiated and bystander populations were also analyzed for chromosomal aberrations as a function of time post-irradiation. When considered over 25 doublings, all irradiated and bystander populations had significantly higher frequencies of chromatid aberrations when compared to controls (2–3-fold over controls) and were not dependent on dose. The results presented here support the link between the radiation-induced phenomena of genomic instability and the bystander effect.

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Keywords: α -Irradiation; Chromosomal instability; Bystander effects; Chromosomal aberrations and normal human fibroblasts

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1. Introduction

There is now a large amount of data of the delayed appearance of mutations [1–4] and chromosomal aberrations [5–7]. In these studies, mutations or chromosomal aberrations have been observed several generations removed from the irradiation and could not have been directly induced by the irradiation. The latent expression of mutations and aberrations in irradiated progeny have been interpreted to be a manifestation of genomic instability [reviewed in 8,9]. It has been hypothesized that radiation-induced genomic instability is the earliest cellular event in the process of radiation-induced tumorigenesis [10,11]. This has been supported by the observation that strain specific tumor induction correlated with induction of chromosomal instability in mammary epithelial cells following irradiation *in vitro* [12] and *in vivo* [13]. Similar correlations between tumor sensitive strains and induction of chromosomal instability have also been demonstrated for hematopoietic cells [14]. However, there have been several reports that have failed to observe chromosomal instability following both *in vitro* and *in vivo* exposure to ionizing radiation [15–17].

The other non-targeted phenomenon that appears to be of significance in regard to radiation-induced carcinogenesis is that of bystander effects [reviewed in 18,19]. It was reported that exposure to very low doses of α -particles initiated sister chromatid exchanges in more cells than it was estimated could have been hit by an α -particle [20,21]. These non-hit, responding cells were then ‘bystanders’ of either directly hit cells or of energy depositions in extracellular medium. Similar types of experiments have also demonstrated the induction of mutations [22] and specific genes in more cells than were estimated to have been hit by α -particles [23]. Subsequent studies were confirmatory and pointed to extracellular factors as being responsible for these effects, with reactive oxygen species implicated [24–27].

Other investigators have transferred media from irradiated cells onto non-hit cells and have observed enhanced cell death [28,29], chromosome damage [30] and increased cell proliferation [31] in the non-irradiated populations. These results have been interpreted as indicating that the irradiated cells release factors into the media that result in the observed changes in the recipient cells. More definitive recent studies of the bystander effect have used a charged particle microbeam. A microbeam was used to irradiate a few cells in a population, with levels of micronuclei and of apoptosis being much higher than expected i.e. demonstration of a bystander effect [32,33]. Importantly, both mutation induction [34] and oncogenic transformation [35] have been shown to be enhanced in bystander cells following microbeam irradiation of known proportions of cells in a population. The ability of bystander cells to express many, if not all, of the biological end-points associated with exposure to ionizing radiation suggests that the bystander effects may contribute to radiation-induced tumorigenesis as well.

There are a few recent studies which link the two phenomena, in which genomic instability has been thought to come from the bystander cells. Re-examination of initial data of chromosomal instability in hematopoietic stem cells [5] has suggested that the instability could be derived from non-irradiated cells. Experiments using a shielding grid to alter the ratio of irradiated to non-irradiated cells demonstrated that changing the ratio of hit to non-hit cells altered the cell survival but not the number of clones expressing instability [36], which indicates that a bystander effect may be responsible, in part at least, for the expression of instability. Subsequent data have shown that chromosomal instability can be induced in bystander cells *in vivo* [37]. More recently, microbeam experiments of lymphocytes have demonstrated a significant bystander

component to instability following α -irradiation [38].

We have previously reported on a novel co-culturing protocol to study bystander effects in which irradiated and bystander cells are cultivated in the same system [39]. This technique takes advantage of the limited penetration of charged particles, and allows for the culture of both irradiated and bystander cells in the same media. Since the two populations are not in physical contact with each other, this protocol can be used to study the bystander effect in which irradiated cells secrete molecules into the media that affect the bystander population. Here we present data from experiments designed to investigate the ability of bystander cells to express chromosomal instability using these co-culturing techniques.

2. Materials and methods

2.1. Cell culture and α -irradiation

Life-span extended human fibroblasts (BJ1-htert) were obtained from Clontech and maintained in culture as recommended in media containing 10% fetal bovine serum. For the irradiation, cells were co-cultured on double sided mylar dishes as discussed previously [39]. These dishes are stainless steel rings with ports on two opposite sides of the ring and have mylar (6 μm) on both surfaces (the distance between the two mylar surfaces is 9 mm). Cells are seeded onto one surface through one of the ports at a concentration of 2×10^5 cells per dish, and allowed to attach and incubated for 48 h. The media is then aspirated through the port and the dish turned over, and cells seeded onto the second surface (at a concentration of 5×10^5 cells per dish) and allowed to attach (1 h). One of the ports is then plugged, the dishes completely filled with media and then the other port is plugged and the dishes returned to the incubator. Populations on both mylar surfaces are confluent in 48 h.

Double sided mylar dishes with confluent populations of BJ1-htert cells on both surfaces were irradiated with 0, 0.1 (~ 1 particle per nucleus), 1 and 10 Gy of 6.1 MeV α -particles using the track segment facility at RARAF. The charged particle beam was generated using a 4 MV Van de Graaff accelerator and passed through a 3 μm thick Havar (cobalt/chromium/nickel

alloy, Hamilton Precision Metals) foil, approximately 8 mm of atmosphere, the mylar dish bottom, and irradiated the sample attached to the mylar. The track segment α -particles release 120 keV/ μm through the cells and penetrate less than 100 μm through the 9000 μm of media. A slot-shaped aperture approximately 6 mm \times 38 mm defined the beam irradiating the samples. The uniformity of the particle flux was $\pm 5\%$ or better over the 38 mm length of the beam and the beam has negligible gamma and X-ray components.

Following irradiation the dishes were returned to the incubator for 1 h. Dishes were incubated with the irradiated surface down to prevent the possibility of irradiated cells detaching from the mylar surface and attaching to the lower surface thereby contaminating the bystander population. After the incubation, the two populations were separated, cells trypsinized and reseeded onto 60 mm dishes.

2.2. Cytogenetic analyses

Control, bystander and irradiated populations were assayed for chromosomal aberrations at the first cell division post-irradiation, i.e. at 24–27 and 27–30 h post-irradiation and at every 5 population doublings thereafter up to 25 population doublings post-irradiation. At the indicated times, cells in 60 mm dishes were treated with colcemid (0.005 mM) for 3 h to disrupt the mitotic spindle and accumulate cells in metaphase. Cells were then removed from each dish by light trypsinization, agitating as necessary since rounded mitoses detach first. The cell suspension was then centrifuged and the pellet resuspended in a small amount of media (200 μl). Cells were then treated with 0.075 M KCl for 11 min at 37 °C. 1 ml of fixative (methanol:acetic acid 3:1) was added to the suspension before the cells were pelleted and resuspended in fixative. Cells were dropped onto clean slides and examined under a phase contrast microscope for optimum separation of the chromosomes. Slides containing metaphase chromosomes were stained for 15 min with 5% Giemsa in Sorensen's buffer (10 mM phosphate, 10 mM sodium citrate).

Cytogenetic aberrations detected in chromosomes stained with Giemsa were scored under light microscopy and classified as detailed earlier [7]. Achromatic discontinuities on one chromatid of a metaphase chromosome that were broader than the width of the adjacent sister chromatid were scored as breaks, as were

clearly displaced fragments; other achromatic discontinuities were scored as gaps. Data of chromatid aberration frequencies in irradiated and bystander cells were statistically analyzed using the χ^2 -test.

3. Results

3.1. Frequencies of chromosomal aberrations at the first division post-irradiation

To determine whether bystander cells express chromosomal damage immediately following irradiation, cytogenetic analyses were conducted on co-cultured bystander and irradiated BJ1-htert cells at the first cell division following exposure to 0.1 and 1 Gy α -particles. Metaphases of the population exposed to 10 Gy were not scored due to the exceeding low frequency of mitotic cells and the extremely high frequencies of aberrations at this dose. Only cells that were bystanders to this dose were scored.

As would be expected, at the first division, frequencies of chromosomal aberrations in the irradiated populations were increased in a dose dependent manner (Fig. 1). The populations that received 0.1 and 1 Gy had means of 0.3 and 1.3 aberrations per cell, respectively. Controls populations had a mean of 0.1 aberrations per cell. The aberrations observed in the irradiated populations were almost all chromosome-type aberrations, including dicentrics, excess acentric fragments

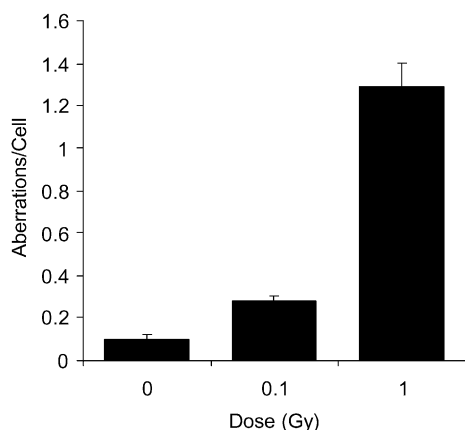


Fig. 1. Frequencies of chromosomal aberrations observed in human fibroblasts at the first cell division following α -irradiation (data are presented as mean \pm S.D. of $n = 4$).

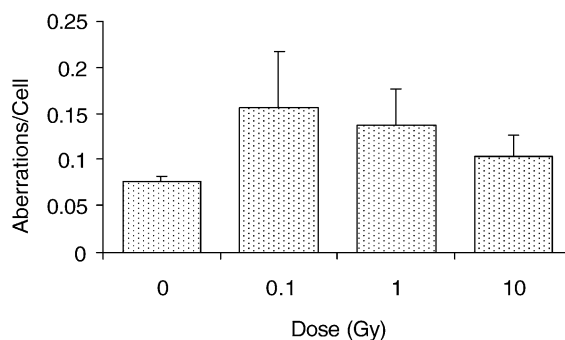


Fig. 2. Frequencies of chromatid-type aberrations in fibroblasts bystanders that were bystanders to α -irradiated fibroblasts at the first cell division post-irradiation (data are presented as mean \pm S.D. of $n = 4$).

and acentric rings. The frequencies of chromatid-type aberrations in all irradiated populations were comparable to that seen in controls (data not shown), which would be expected given that these populations were in the G0/G1 phase of the cell cycle at the time of irradiation.

Increased frequencies of chromosomal aberrations were also detected in the bystander populations. However, unlike the irradiated populations, bystander populations had increased yields of chromatid-type aberrations (Fig. 2). Bystander populations had between 0.1 and 0.16 chromatid aberrations per cell. These were 1.3–2-fold increases in frequencies of aberrations over control bystander cells. Furthermore, all the chromatid-type aberrations observed were of the simple type, namely breaks and gaps. Importantly the elevated yields of chromatid-type aberrations detected in the bystander populations at the first division post-irradiation did not appear to dose dependent.

3.2. Frequencies of chromosomal aberrations at delayed times

To assay for the appearance of delayed chromosomal aberrations, irradiated and bystander populations of BJ1-htert cells were examined for chromosomal aberrations at every 5 population doublings up to 25 population doublings post-irradiation (Fig. 3).

Chromatid aberration frequencies in sham irradiated and bystander control populations ranged between 0.02 and 0.06 per cell over the time points examined. By 10 population doublings all aberrations induced by the

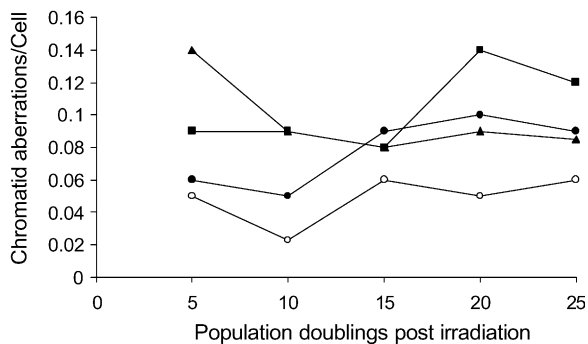


Fig. 3. Delayed chromatid-type aberrations observed in human fibroblasts exposed to 0 Gy (○), 0.1 Gy (●), 1 Gy (▲) and 10 Gy (■) α-particles as a function of time post-irradiation (results presented here are from a single experiment. A repeat of the experiment produced similar trends in the data).

initial irradiation had been removed from the irradiated populations, and the frequencies of chromosome-type aberrations in these cells were comparable to those observed in the controls (data not shown). Over the first 10 doublings the population exposed to 0.1 Gy had between 0.05 and 0.06 aberrations per cell that rose to 0.09–0.1 per cell between 15 and 25 doublings post-irradiation. Similar trends were observed in the other irradiated populations as well. BJ1-htert cells exposed to 1 and 10 Gy α-particles had between 0.08 and 0.14 aberrations per cell, although the patterns of expression of the aberrations as a function of time post-irradiation were not identical in both populations.

Similar increases in chromatid aberration frequencies at delayed times were observed in the bystander

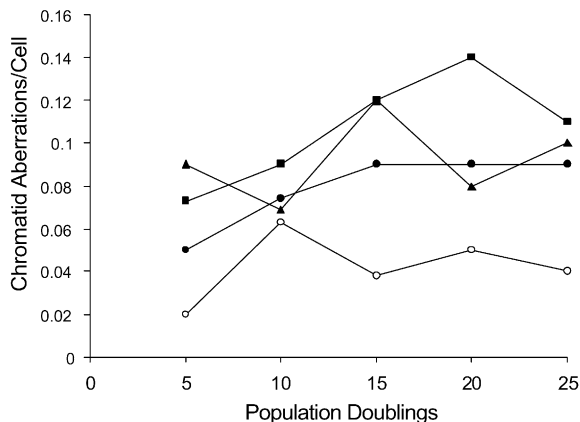


Fig. 4. Delayed chromatid-type aberrations observed in human fibroblasts that were bystander to fibroblasts exposed to 0 Gy (○), 0.1 Gy (●), 1 Gy (▲) and 10 Gy (■) α-particles as a function of time post-irradiation (results presented here are from a single experiment. A repeat of the experiment produced similar trends in the data).

cells (Fig. 4). Control bystander cells had between 0.02 and 0.06 aberrations per cell over 25 population doublings, Aberration frequencies in the populations that were bystander to irradiated cells were between 0.08 and 0.14 per cell at the time points assayed. Interestingly, there did not appear to be a difference in the frequencies of delayed chromatid aberrations between irradiated and bystander populations.

Chromatid-aberration frequencies observed in each sample between 5 and 25 population doublings post-irradiation were pooled and are presented in Table 1. All of the chromatid aberrations observed were of the simple type – breaks and gaps. Additionally, only a few

Table 1

Frequencies and distribution of chromatid-type aberrations observed in control, α-irradiated and bystander cells between 5 and 25 population doublings post-irradiation

Dose	No. of cells scored	Breaks	Gaps	Cells with chromatid-type aberrations (%)	Fold increase over controls
Irradiated					
0	442	6	13	4.3	
0.1	500	10	29	7.4*	1.7
1	482	15	32	9.3*	2.1
10	466	11	38	10.3*	2.4
Bystander to					
0	498	5	14	3.6	
0.1	429	12	29	9.1*	2.6
1	477	9	28	7.8*	2.2
10	550	12	45	10.0*	2.8

* Indicate the values are significantly different from controls by χ^2 -analyses ($P < 0.005$).

of these cells contained more than one aberration, and none had more than two aberrations. More complex types of chromatid aberrations were only very rarely seen and have not been included in the analyses. As can be seen, the ratio of breaks to gaps ranged between 1:2 and 1:3.5 over 25 doublings. All irradiated and bystander populations had significantly higher frequencies of cells containing chromatid aberrations when compared to appropriate controls. Irradiated populations had between 1.7- and 2.4-fold higher yields of cells with chromatid aberrations over the control populations. Similarly, bystander populations had between 2.2- and 2.8-fold higher frequencies of aberration containing cells. Importantly, the yields of delayed aberrations in both irradiated and bystander populations did not appear to be dose dependent.

4. Discussion

The data presented here indicate that α -particle irradiation of human fibroblasts can induce bystander effects in unirradiated human fibroblasts as seen by increased yields of chromatid-type aberrations at the first division post-irradiation. Further, both irradiated and bystander populations demonstrated similar levels of chromosomal instability in later cell generations as seen by the increased chromatid aberration frequencies being 2–3 times in excess of control levels. It must be stressed that in these experiments irradiated and bystander cells were not in contact with each other. Rather, the populations were separated by a considerable thickness of media, suggesting that bystander cells were responding to signaling molecules from the stressed cells that were disseminated through the media volume. At the first cell division following irradiation, bystander cells but not those irradiated demonstrated elevated frequencies of chromatid aberrations. This would suggest that the irradiated cells were not capable of responding to the factor(s) that are responsible for the elevated frequencies of replication/post-replication type aberrations in bystander cells.

Further, there was no detectable increase in chromatid aberrations frequencies in those cells sampled after 0.1 Gy, in which about one third of the cells would have not been traversed by a single particle and therefore would be bystander cells. One might expect that this fraction of cells would behave similarly to

the other bystander populations and display chromatid aberrations. However, given the modest yields of such aberrations in the bystander populations, aberration frequencies in the population exposed to 0.1 Gy might reasonably be indistinguishable from that seen in controls.

It remains possible that the irradiated and bystander cells that demonstrate chromatid aberrations at delayed times also contain chromosome-type aberrations. This has been previously demonstrated for Chinese hamster ovary cells following exposure to X-rays [40]. However, unlike the cells used in those studies, human cells do not tolerate loss of chromosomal material that would occur in subsequent divisions as a result of dicentric and other acentric fragments, and would fail to reach mitosis. Thus, only cells containing transmissible, non-lethal chromosome-type aberrations would reach mitosis and be present at the time of analyses. This could possibly account for the fact that no increase in acentric fragments or other chromosome-type aberrations were detected by Giemsa staining of irradiated or bystander cells at delayed times. However, it remains possible that there is some as yet undetermined explanation for the lack of chromosome-type aberrations in these populations.

The lack of a dose dependent response in the delayed aberrations observed in the irradiated populations is similar to previous findings [7]. MCF-10A cells irradiated with 0.2 or 0.4 Gy neutrons demonstrated similar frequencies of chromatid aberrations at delayed times. This is in contrast to reported dose dependent responses of chromosomal instability observed following X-irradiation or exposure to Fe ions [45,46]. The reason for this difference is not clear.

There appears to be at least two different mechanisms by which bystander effects are propagated. Data from low fluence studies using confluent cultures suggest the bystander effect is transferred from irradiated to non-irradiated cells via gap junctions [27,34,41,42]. The effect can be abolished or diminished to a great extent when the formation of these junctions is prevented either by inhibitors or by use of mutant cells [41,43]. However, this is clearly not possible in media transfer studies where the two sets of cells are never in contact with each other [28–31,44]. In this case it has been postulated that deposition of energy in the media, or in cells, generate factors in the media which can then produce responses in bystander cells that are similar to those observed in the irradiated cells. The

bystander and delayed effects demonstrated by data presented here clearly could not have arisen via gap junctions since the two populations are not in contact with each other but are separated by media. Therefore, the bystander responses and chromosomal instability observed were more likely induced by the second mechanism where factors are released by irradiated cells that diffuse through the media and influence bystander cells.

In conclusion, the data discussed here support the emerging link between the non-targeted effects of radiation-induced genomic instability and bystander effects. While it still remains to be determined whether common mechanisms are involved in their induction, the phenomena may have significant contributions to radiation-induced carcinogenesis especially at low doses of ionizing radiation.

Acknowledgments

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