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Quantifying a bystander response following microbeam irradiation using single-cell RT-PCR analyses

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Objective. **There is growing recognition that the effects of ionizing radiation may extend to more than those cells that directly suffer damage to DNA in the cell nucleus. Data from several investigators have indicated that cells neighboring those that are irradiated also demonstrate several responses seen in hit cells—the so-called bystander effect. The microbeam facility at the Center for Radiological Research is particularly well suited for the study of this bystander effect, since it has the ability to place known numbers of charged particles (protons or α-particles at LETs from 20 to 180 KeV/μm) at defined positions relative to individual cells. That is, some known fraction of cells in a population can be irradiated through the nucleus, or the cytoplasm or even adjacent to cells through the media. Therefore, using the microbeam it is possible to examine individual cell responses in both hit and nonhit cells in the same population.**

Method and Results. **Alterations in the cyclin-dependent kinase inhibitor CDKN1a (p21/Cip1/WAF1) were quantified at the mRNA level in single normal human fibroblasts following precise delivery of 0 or 10 α-particles per cell at 90 KeV/μm to 50% of cells in a population. Semi-quantitative RT-PCR of individual hit cells demonstrated increases in the levels of CDKN1A message that followed the kinetics previously described for irradiated populations. Furthermore, nonhit bystander cells also showed increased (though lesser) levels of CDKN1a message.**

Conclusion. **Data presented here demonstrate the power of this approach, which combines the ability of the microbeam to irradiate specific cells in a population and the ability to quantify the response to the irradiation in individual targeted and bystander cells.** **© 2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

While it is readily accepted that ionizing radiation can induce tumors, very little is known about the mechanisms underlying this process. Ionizing radiation has long been known to be capable of inducing a broad spectrum of genetic lesions and it is generally thought that the carcinogenic effects of ionizing radiation are related to its clastogenic and/or mutagenic properties. The underlying assumption in this concept is that the cell nuclear DNA is the target. In recent years this basic concept, and the risk-oriented extrapolations based on the concept, have been brought into question. An increasing amount of data from radiation studies have led to the proposal that the frequency at which a particular endpoint is observed in an irradiated population is contributed to, in part, by cells that are not directly hit by the initial irradiation. The nonirradiated cells that respond have been termed "bystanders," a term borrowed from a similar phenomenon previously reported from viral transfection experiments [1–4].

Early studies of radiation-induced bystander responses 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 [5,6]. These nonhit, 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 specific genes, including CDKN1a, in more cells than were estimated to have been hit by α-particles [7]. Subsequent studies were confirmatory and pointed to extracellular factors as being responsible for these effects, with reactive oxygen species implicated [8–11].

Other investigators have transferred media from irradiated cells onto nonhit cells and have observed enhanced cell death and specific gene induction in the nonirradiated populations [12,13]. These results have been interpreted as indicating that the irradiated cells release factors into

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the media that result in the observed changes in the recipient cells.

More definitive studies of the bystander effect have used a charged-particle microbeam. A microbeam has the ability of delivering a defined number of α-particles (including one) at very precise locations in a cell population. Thus it is possible to target the nuclei of single cells with a defined number of particles. This is in contrast to whole-population irradiations, where only a fraction of the particles actually produce damage in the nucleus. Furthermore, the dose delivered to a population typically follows a Poisson distribution and therefore any analysis of the population is by definition an average of that population. The microbeam, however, allows the precise analysis of each cell in the population, in which all hit cells are homogeneous with respect to the dose received, as are the zero-dose nonhit cells in the population. 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 [14,15]. Importantly, both mutation induction [16] and oncogenic transformation [17] have been shown to be enhanced in bystander cells following microbeam irradiation of known proportions of cells in a population.

It must be noted that in most of the above-mentioned studies, the presence of a bystander effect has been inferred rather than directly demonstrated. Since it was not possible to distinguish the hit cells from the bystanders, the presence of a bystander effect relied on calculations indicating that biological responses were observed in more cells than were irradiated. Recently, we developed a modified staining technique that allows the direct microscopic identification of hit and bystander cells [18]. This approach takes full advantage of the ability of the microbeam to target specific cells in a population, and was used in the first studies that directly visualized known hit and bystander cells in the same population. Using this protocol we have demonstrated the induction of micronuclei as well as cell cycle delays in both irradiated and bystander normal human fibroblasts. In addition, studies in this laboratory as well as other reports indicated that the p53/p21WAF1 pathway may be involved in the bystander effect.

Here we report on the use of a single-cell assay to quantify alterations in gene expression in individual irradiated and bystander cells. This approach combines the ability of the microbeam to target specific cells in a population with single-cell analyses to study the bystander effect.

Materials and methods

Cell culture and microbeam irradiation

Normal human fibroblasts (Clonetics, Walkersville, MD, USA) were grown to plateau phase in T-25 flasks for 3 to 7 days prior to experiments. Cells were then split 1:2 and reseeded onto T-25 flasks. Once the cells had attached, one flask was stained with 100 nM Cell Tracker Orange (Molecular Probes, Eugene, OR, USA) for 30 minutes. At this concentration the dye does not cause any cell perturbations and gives a clear cytoplasmic signal through 3 to 4 cell divisions. The other flask was stained with 50 nM Hoechst 33342 for 30 minutes, as done routinely for microbeam studies [16,17]. Flasks were then rinsed and incubated with fresh media for 30 minutes. Both sets of cells were then trypsinized, counted, mixed in a 1:1 ratio, and seeded in a 2-μL drop onto Cell-Tak coated microbeam dishes, at a density of 500 cells per dish (250 cells of each type). This resulted in a mean distance of 300 μm between cells. Construction of the microbeam dishes and the microbeam irradiation protocol used have been described elsewhere [19]. Only the Hoechst-stained cells were irradiated with the indicated number of α-particles, while the CTO dye–stained cells were the bystander cells. Cells were irradiated within 5 hours of plating to minimize progression through the cell cycle and movement relative to one another.

Analyses of CDKN1a expression by single-cell RT-PCR

Following irradiation, 2 mL media was added to the cells and incubated for 1 hour, then rinsed in HBSS and incubated with 20 μL trypsin for 30 seconds. The trypsin was then carefully removed and 20 μL HBSS was added. Individual Hoechst (irradiated)- or CTO dye (nonhit)–stained cells were isolated using a combination of phase-contrast and fluorescent microscopy, with a micromanipulator (Narashige, Long Island, NY, USA) fitted with a microcapillary attached to a microsyringe (Narashige). Single cells were put into 0.2-mL PCR tubes kept on ice. Typically about 40 appropriately stained individual cells can be picked and placed in individual tubes in 30 minutes. The challenging nature of this procedure restricts extensive sample collections on a time-dependent basis. RT-PCR for β-actin and CDKN1a messages were conducted in a one-step RT-PCR reaction (Qiagen, Valencia, CA, USA) with primers previously published [20] according to the manufacturer's protocol. Following reverse transcription and amplification (~70% success rate), samples were electrophoresed on 2% agarose gels (Nuseive 3:1, FMC Bioproducts, Rockland, ME, USA), stained with ethidium bromide, and photographed (Kodak EDAS 120, Kodak, New Haven, CN, USA). Quantification of amplified products was done using Kodak 1D analysis software. Induction of CDKN1a in irradiated and bystander cells was expressed as relative increases over control cells after normalizing with β-actin signal.

Results

Previous immunofluorescence studies in this laboratory have demonstrated strong nuclear signals for p53 and CDKN1a in both irradiated and bystander human fibroblasts. A semiquantitative, single-cell RT-PCR protocol was used to determine whether there were quantitative differences in the induction of CDKN1a between individual irradiated and bystander cells.

Initial studies were confined to examining the CDKN1a response in the irradiated cells. Normal human fibroblasts were microbeam irradiated with 10 α-particles and individual Hoechst-stained cells were isolated at 30 minutes and 1, 2, and 3 hours postirradiation. Control cells were isolated at

30 minutes following sham irradiation, i.e., cells were stained with Hoechst or CTO and imaged but not irradiated. Following RT-PCR, a 631-base-pair β-actin fragment and a 383-base-pair CDKN1a fragment was amplified from single cells (Fig. 1A). Expression of CDKN1a in single irradiated cell was normalized using the β-actin signal and is expressed as fold increase over control cells (Fig. 1B). As can be seen, there was an induction of CDKN1a in the irradiated cells that was elevated at the earliest time examined—30 minutes. At this time the irradiated cells had between an eightfold and a ninefold increase in CDKN1a expression over controls. This expression seemed to peak at 1 hour and was reduced at 2 and 3 hours postirradiation.

Having established that it was possible to analyze CDKN1a expression in single human fibroblasts as a function of time postirradiation, alterations in CDKN1a expression were next examined in bystanders and compared to irradiated cells. Hoechst-stained cells were microbeam irradiated with 10 α-particles. One hour postirradiation, both Hoechst-and CTO-stained cells were individually isolated and analyzed for CDKN1a expression (Fig. 2A). This particular time was selected since maximum induction was observed at this point. There were no significant differences in CDKN1a mRNA levels between control Hoechst-stained and control CTO-stained cells (data not shown). Quantification of the CDKN1a signal following normalization with the β-actin signal indicates that CDKN1a expression in all irradiated and some bystander cells was elevated. In irradiated cells, these levels ranged between sixfold and 10-fold higher than controls. This is similar to increases detected previously (Fig. 1B). Known nonhit, bystander cells also demonstrated between twofold and sixfold increases in 21/WAF1 expression, clearly showing a dramatic bystander response.

One interesting observation in the previous experiments was the degree of variability in expression levels in both irradiated and bystander cells. To further examine this finding, more cells were analyzed under the same conditions as previously used, i.e., irradiated with 10 α-particles and isolated at 30 minutes and 1, 2, and 3 hours postirradiation. Expression of CDKN1a levels in single irradiated or bystander cells was compared to the mean of 20 control cells, following normalization with the β-actin fragment. As can be seen in Figure 3A, there was a similar pattern of induction of CDKN1a in irradiated cells. There was an increase in expression levels at 30 minutes postirradiation, peaking at 1 hour and reduced at 2 and 3 hours postirradiation. The mean of all irradiated cells at 30 minutes was a fourfold increase over controls, rising to sevenfold at 1 hour before decreasing to 2.5- to 3.5-fold increases at the final time points. However, there were a few cells that did not have elevated levels of CDKN1a at 2 and 3 hours postirradiation, while other cells at the same time points showed much higher peak levels that were similar to those seen at 30 minutes and 1 hour postirradiation. Certainly there is

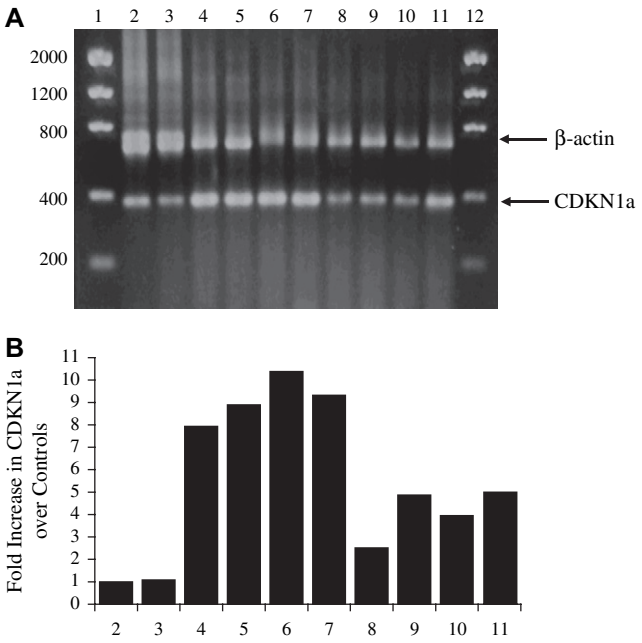

Figure 1. **(A)** Semiquantitative RT-PCR of β-actin and CDKN1a mRNA from individual control (lane 2,3) and microbeam-irradiated (lanes 4–11) cells. Cells were isolated 30 minutes (lanes 2–5), 1 hour (lanes 6 and 7), 2 hours (lanes 8 and 9), and 3 hours (lanes 10 and 11) after irradiation with 10 α-particles. Lanes 1 and 12 are molecular weight markers. **(B)** Quantification of CDKN1a expression in control and microbeam-irradiated cells in (A). Numbers correspond to lane numbers in (A).

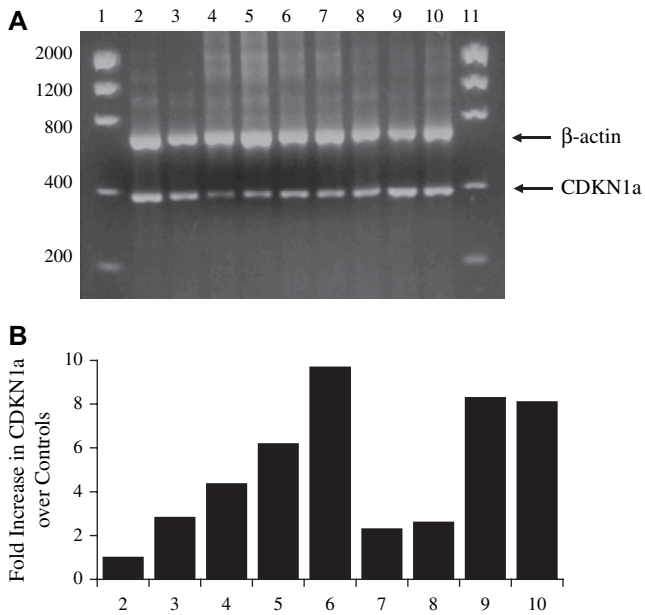

Figure 2. **(A)** Semiquantitative RT-PCR of β-actin and CDKN1a mRNA from individual control (lane 2), bystander (lanes 3, 4, 7, and 8), and irradiated cells (lanes 5, 6, 9, and 10) one hour after microbeam irradiation with 10 α-particles. Lanes 1 and 11 are molecular weight markers. **(B)** Quantification of CDKN1a expression in control and microbeam-irradiated cells as seen in (A). Numbers correspond to lane numbers in (A).

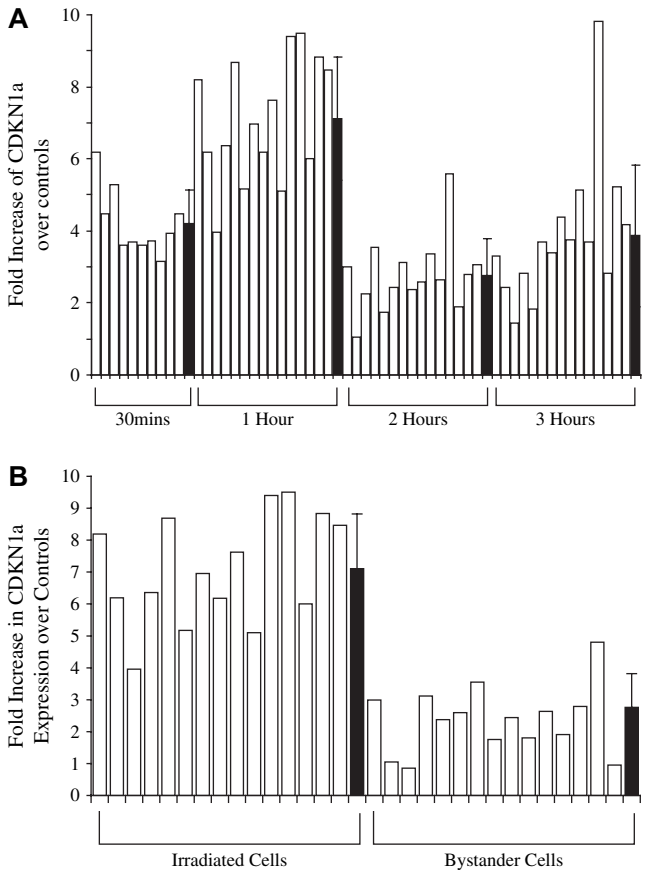

Figure 3. **(A)** Relative induction of CDKN1a in single irradiated cells over controls, at 30 minutes and 1, 2, and 3 hours following exposure to 10 α-particles. Clear bars represent single cells, black bars represent the mean of 10 irradiated cells (error bars are SD). **(B)** Relative induction of CDKN1a in single irradiated or bystander cells over controls, one hour following exposure to 10 α-particles. Clear bars represent single cells, black bars represent the mean of 10 irradiated or bystander cells (error bars are SD).

substantial intercellular variability in response kinetics following insult for both precisely hit and bystander cells.

In addition, expression levels in bystander cells were compared to irradiated cells at 1 hour postirradiation (Fig. 3B). Again, expression levels in bystander cells were variable. In some bystander cells (3/15) CDKN1a expression levels were similar to that of controls, while in others it ranged up to fivefold higher than background. The mean of all bystander cells examined was a 2.8-fold increase, intermediate between that of irradiated cells and controls.

Discussion

The bystander effect has now been proposed to be capable of leading to cell death, induction of sister chromatid exchanges, mutations, and transformation of cells neighboring those that have been irradiated. The data presented here are the first that attempt to quantify a molecular aspect of the bystander effect at the level of individual hit and nonhit cells. From the data it is clear that given an equal number of irradiated and bystander cells, there are increases in CDKN1a levels in all irradiated and most of the bystander cells. Almost 80% of the bystander cells showed elevated levels of CDKN1a. This frequency is one of the highest observed in bystander studies and implicates a highly efficient initiating pathway emanating from irradiated cells.

The variation observed between individual irradiated cells might point to differences between cells that are inherent in the population. In other words, there may be genetic heterogeneity in the population that results in differences in the way cells respond to damage following irradiation. Alternatively, these variations may be due to the delays in response of a fraction of the population (such as that seen in the cell with an almost 10-fold increase at 3 hours postirradiation). Variations in bystander cells may also reflect such differences. It is apparent, however, that the kinetics of these molecular responses are not markedly different between hit and nonhit cells, indicating a rapid induction in hit cells, release, and then activation in bystander cells.

An alternative possibility for variations in the bystander cells is that not all bystanders are affected to the same degree. It has been suggested that irradiated cells release as-yet-unidentified factors into the media that are responsible for the effect observed in the bystander cells [13]. If this is indeed the case, it is possible that the factors released do not migrate to all bystander cells, and the amount of factor/s that reach a bystander cell is dependent on concentration gradients and media movement following irradiation. Thus is it possible that not all bystander cells are exposed to the same level of released factor/s, and in turn do not respond to the same degree. This is in keeping with other reports of micronuclei induction in bystander cells following microbeam irradiation [14,21]. It was observed that following irradiation of 4 cells with 5 α-particles, ∼2.5% of about 3000 cells showed micronuclei, compared to 1% of the control cells showing micronuclei. Importantly, it was observed that the responding bystander cells were randomly distributed in the dish and there was no correlation between distance from the irradiated cell and bystander response.

A possible role of the p53/CDKN1a pathway in the propagation of the bystander effect has previously been proposed [7,11]. When confluent populations of human fibroblasts were irradiated with low fluences of α-particles such that only a small fraction of the cells were estimated to have been traversed by an α-particle, clusters of cells showed increased levels of CDKN1a as determined by immunofluorescence. This might reflect one (or more) hit cells surrounded by bystander cells. Western blotting of these populations indicated that induction of CDKN1a was higher than that of only hit cells. However, when treated with lindane (an inhibitor of gap junctions), the response in the bystander cells was reduced. This was interpreted

as the requirement of functional gap junctions to induce CDKN1a in bystander cells. Interestingly, another study [22] reports that transferring media from α-irradiated fibroblasts to unirradiated cells resulted in the suppression of p53 and CDKN1a levels. This was accompanied by increases in PCNA and CDC2 levels, as well as increased cellular proliferation in the unirradiated cells. Since the media was filtered prior to transfer, there was no contact between irradiated and bystander cells, and thus in this case the transfer of the bystander effect cannot be due to gap junction communications. The experiments presented in this report were set up such that there was no contact between cells, and therefore the data indicate that the increases in CDKN1a levels in bystander cells does not require gap junction communications. It must be kept in mind that these contradictory results come from different types of experiments.

In conclusion, the data presented here supports earlier studies demonstrating that the effects of ionizing radiation are not confined to the directly hit cells, but also affect neighboring nonhit cells. The presence of this bystander effect impacts both basic mechanisms and risk extrapolations. It is also clear that the approach to utilized microbeam irradiation of specific cells in a population combined with single-cell analyses provides a powerful tool to study this bystander effect.

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