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Radiation-Induced Bystander Effect: Activation of Signaling Molecules in K562 Erythroleukemia Cells

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Abstract Gap junction independent signaling mechanism was investigated using K562 human erythroleukemia cells. They were exposed to 2, 5, or 10 Gy of ^{60}Co γ irradiation, the medium isolated 20 min post-irradiation and added to fresh cells. Evidence of radiation-induced bystander effect was observed wherein there was activation of p21, nuclear factor-κB (NF-κB), Bax, Bcl-2 and cleavage of poly(ADP-ribose) polymerase in bystander cells. The study implicates the involvement of signaling molecules released into the medium and factors like stable free radicals that are generated in the surrounding medium. The response elicited appears to be primarily via NF-κB and p21 activation. J. Cell. Biochem. 100: 991–997, 2007. © 2006 Wiley-Liss, Inc.

Key words: radiation; bystander; signaling; nuclear factor-κB and p21

The response of cells to impinging ionizing radiation is known to be due to the activation existing signaling pathways of the cell. However, in recent years there has been increasing evidence indicating that the surrounding bystander cells also contribute to the overall signaling response of the tissue [Lyng et al., 2000, 2002; Hall and Hei, 2003; Mothersill and Seymour, 2003, 2005; Prise et al., 2003; Maguire et al., 2005]. Such radiation-induced bystander signals, appear to coordinate a higher order homeostatic regulation as a result of a generalized stress response in tissues or cells. The signals may be produced by all exposed cells but the response may require a minimum threshold for it to be elicited. The major response involving low LET radiation exposure discussed in the existing literature is a death response, which has many characteristics of apoptosis but may be detected in cell lines without p53 expression [Lyng et al., 2000, 2002; Mothersill and Seymour, 2003, 2005; Maguire et al., 2005]. Such bystander responses may be induced through gap junction-mediated communication with the irradiated cell or through signaling molecules released into the surrounding medium by "hit" cells (those that have actually been traversed by ionizing radiation).

The assessment of radiation-induced effects like damage, initiation of carcinogenesis or the development of radioresistance following radiotherapy must, therefore, take into account the direct as well as the bystander effects. In the present study, we have investigated medium-mediated bystander effect in K562 cells with a focus on factors involved in apoptosis and cell cycle.

MATERIALS AND METHODS

Human erythroleukemia K562 cells were grown in RPMI 1640 medium (Sigma, USA), supplemented with 10% fetal calf serum (Sigma). Cells were kept at 37°C in humidified atmosphere with 5% CO_2.

K562 cells where they were exposed to 2, 5, or 10 Gy of ^{60}Co γ irradiation, using Gamma Cell 220 (Atomic Energy of Canada Ltd.), at a dose rate of 6.2 Gy/min, the medium isolated and added to fresh cells. Before designing the experiment there were two aspects that had to be taken care of, one was the likelihood that the replacement of the medium itself caused transient expression of certain signaling factors

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and the second that the components of the medium, which include proteins like growth factors, etc. and are also unavoidably exposed to radiation, contribute to the changes in the bystander cell. The experimental design, therefore, consisted of the following sets: (a) control unirradiated cells (Fig. 1, Lane 1), (b) unirradiated cells receiving medium from unirradiated cells (Fig. 1, Lane 2), (c) unirradiated cells receiving fresh medium (Fig. 1, Lane 3), (d) ^{60}Co γ irradiated cells lysed at ½, 2, and 4 h post-irradiation, (e) unirradiated cells receiving medium from ^{60}Co γ irradiated cells and lysed at ½, 2, and 4 h post-transfer of medium, and (f) unirradiated cells receiving medium irradiated with ^{60}Co γ rays in the absence of cells and lysed at ½, 2, and 4 h post-transfer of medium.

Cells were passaged 24 h before the experiment and were irradiated at various doses in the presence of medium. Irradiated cells were separated from the medium, 20 min post-irradiation, by centrifugation at 100*g* for 10 min and the medium transferred to unirradiated cells which in turn had been harvested from their medium previously.

Nuclei were isolated with nucleiPURE prep nuclei isolation kit (Sigma). Cells/nuclei were harvested at various time periods (½, 2, and 4 h) and were lysed in boiling SDS–PAGE loading buffer (50 mM Tris-Cl, pH 6.8; 100 mM dithiothreitol; 2% SDS and 20% glycerol without Bromophenol blue), proteins were separated by 8% SDS–PAGE (150 μg protein loaded per well), transferred to nitrocellulose membrane, probed with anti-p21, anti-Bax (both Sigma) and bands were detected using secondary antibody and reagents from Lumi Light plus Western blotting kit (Roche, Germany). Subsequently, membranes were stripped with stripping buffer (50 mM Tris pH 7.5, 150 mM NaCl, 100 mM 2-mercaptoethanol, 2% SDS), and reprobed with anti-Bcl-2 (Sigma), anti-NF-κB (p65) (Transduction Laboratories, USA), anti-PARP (Calbiochem, USA) antibodies. Actin (Sigma) was used as loading control.

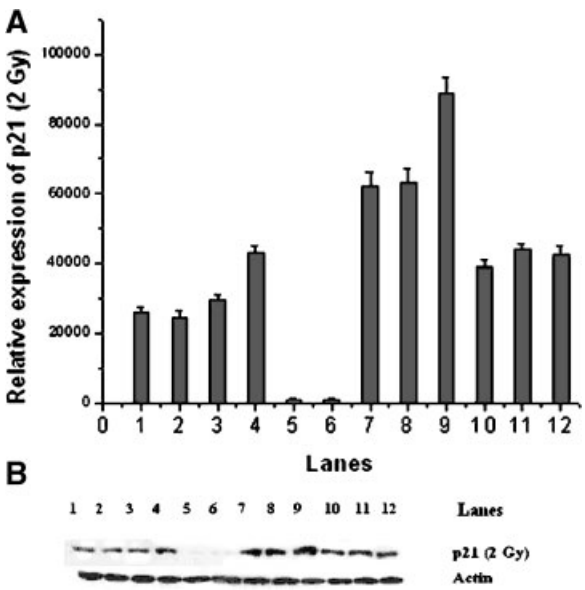

Fig. 1. Expression of p21 in 2 Gy γ-irradiated and bystander K562 cells. The cells were irradiated at 2 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of p21. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

RESULTS

The activation of nuclear factor-κB (NF-κB), a redox regulated signaling factor and expression of p21, which is involved in cell cycle arrest and can be activated by NF-κB, were investigated at 2, 5, and 10 Gy. Since both were found to be altered at 5 Gy, Bax, Bcl-2, and PARP which are factors involved in apoptosis were done at 5 Gy.

The expression of p21, following 2 Gy dose of γ irradiation increased at ½ h (Fig. 1, Lane 4) which was followed by a decrease below control levels at 2 and 4 h (Fig. 1, Lanes 5 and 6, respectively). In the bystander cells there was a strong increase in the p21 levels at all time points with a peak at 4 h (Fig. 1, Lanes 7, 8, and 9). At 5 Gy dose p21 levels increased manifold in the irradiated cells (Fig. 2, Lanes 4, 5, and 6) with a maximum at 2 h (Lane 5) and also in the bystander cells (Fig. 2, Lanes 7, 8, and 9) as compared to controls. At 10 Gy, however, although there was an increase of p21 at early time points in both irradiated (Fig. 3, Lanes 4 and 5) as well as bystander cells (Lane 7), it came back to control levels by 4 h (Fig. 3, Lanes 6 and 9). Surprisingly, in cells where medium had been replaced with medium irradiated without cells, there was an initial increase, coming back to normal at 4 h (Figs. 2 and 3, Lanes 10, 11, and 12). It was interesting to note from the above

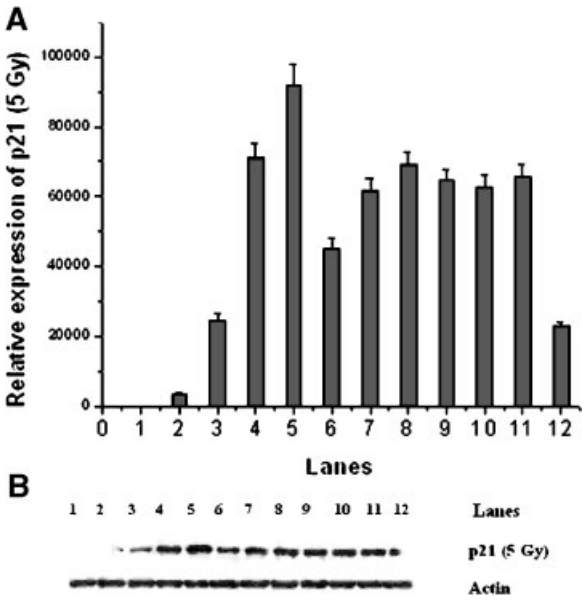

Fig. 2. Expression of p21 in 5 Gy γ-irradiated and bystander K562 cells. The cells were irradiated at 5 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of p21. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

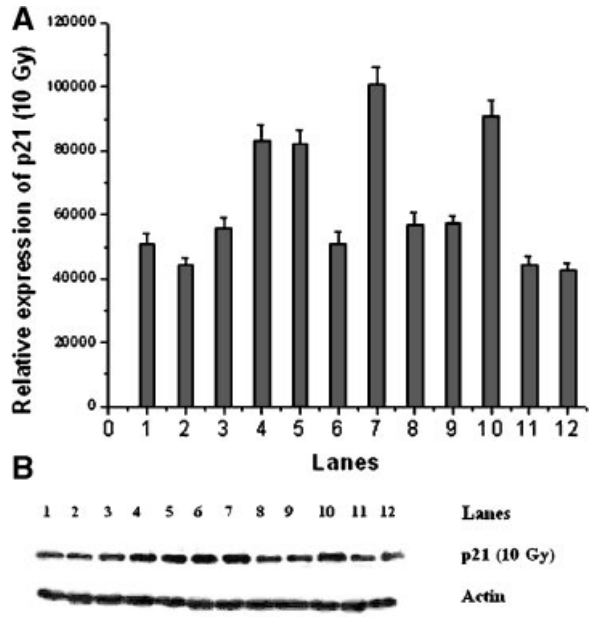

Fig. 3. Expression of p21 in 10 Gy γ-irradiated and bystander K562 cells. The cells were irradiated at 10 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of p21. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

experiments that there was a definite difference in the p21 levels in the bystander cells as compared to the controls and the response differed with the radiation dose that was delivered to the irradiated cells.

The nuclear levels of NF-κB (p65) were found to increase in the 2 Gy irradiated cells at ½ h post-irradiation (Fig. 4, Lane 4), thereafter, decreasing to normal levels (Lanes 5 and 6). However, bystander cells displayed no effect at this dose (Lanes 7–9), while cells receiving medium irradiated without cells showed an increase at 2 h (Lane 11). At 5 Gy NF-κB peaked at 4 h in irradiated cells (Fig. 5, Lane 6) while there was a general increase in bystander cells at all time points (Lanes 7–9). At 10 Gy the changes in NF-κB are not significant in both irradiated and bystander cells (Fig. 6).

The expression of Bax, a proapoptotic protein, increased significantly in both irradiated (Fig. 7, Lanes 4–6) and bystander cells (Lanes 7–9). However, cells exposed to medium irradiated without cells showed maximum levels of Bax (Lanes 10–12). The anti-apoptotic Bcl-2 expression also increased both in 5 Gy irradiated (Fig. 7, Lanes 4–6) and bystander cells (Lanes 7–9) and also in cells exposed to medium irradiated without cells (Lanes 10–12). Interestingly, in the case of irradiated cells, it came back to normal levels at 2 h post-irradiation (Lane 5) while in bystander cells, it peaked at 2 h (Lane 8). The same peak was also observed in cells exposed to irradiated medium (without cells) (Lane 11).

PARP cleavage, a marker of apoptosis, increased significantly in both irradiated (Fig. 8, Lanes 4–6) and bystander cells (Lanes 7–9) at all time points where an increase in the 85 kDa fragment was observed with a concomitant decrease in the 116 kDa fragment. The cleavage of PARP, however, was around control

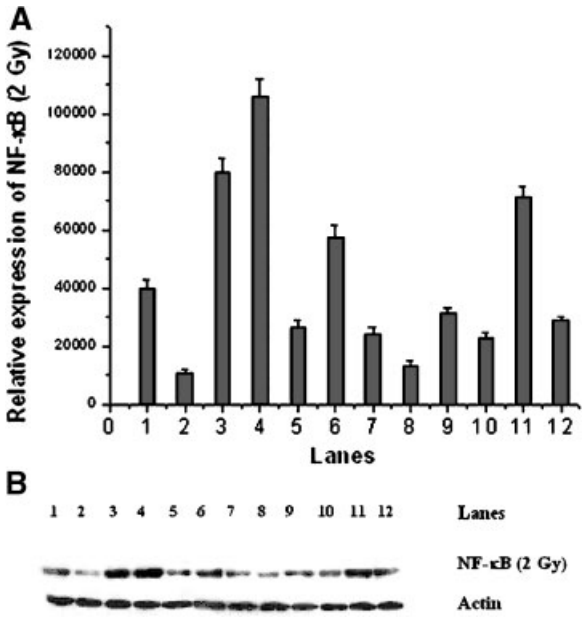

Fig. 4. Nuclear NF-κB in 2 Gy γ-irradiated and bystander K562 cells. The cells were irradiated at 2 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of NF-κB. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

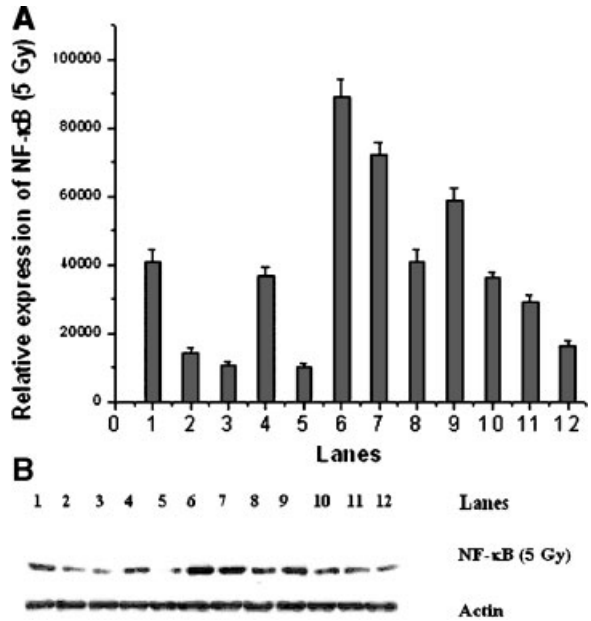

Fig. 5. Nuclear NF-κB in 5 Gy γ-irradiated and bystander K562 cells. The cells were irradiated at 5 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of NF-κB. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

levels for cells exposed to medium irradiated in absence of cells (Lanes 10–12).

DISCUSSION

Essentially two models have surfaced to explain the radiation-induced bystander effect, depending upon the experiments conducted and the cell types used. One hypothesizes the release of transmissible factors into the medium by the irradiated cells, which are then transported through the medium to unirradiated cells, eliciting a bystander response [Mothersill and Seymour, 2003, 2005]. The other model proposes communication from the irradiated to bystander cells via gap junctions [Azzam et al., 2003; Hall and Hei, 2003; Prise et al., 2003]. In the present study, K562 cells which are an erythroleukemia line, that is, of hematopoietic origin were used. They grow in suspension culture and hence most cell-to-cell communication would occur through molecules released in the medium. Such a cell line was very appropriate for the experimental design used wherein medium from irradiated cells was transferred to unirradiated cells. The assumption was that the signal required by the bystander cells would already have been released into the medium by the irradiated cells. This in turn, would elicit a response in the bystander cells.

Reactive oxygen species (ROS) may have a role to play in both the systems [Kadhim et al., 2004]. In the present study also, it is observed that NF-κB, which is known to be involved in redox signaling [Shishodia and Aggarwal, 2004], gets strongly activated in the bystander cells following irradiation of the target cells at 5 Gy. A noteworthy finding of this study is the fact that the cells that are exposed to medium irradiated without cells also show activation of

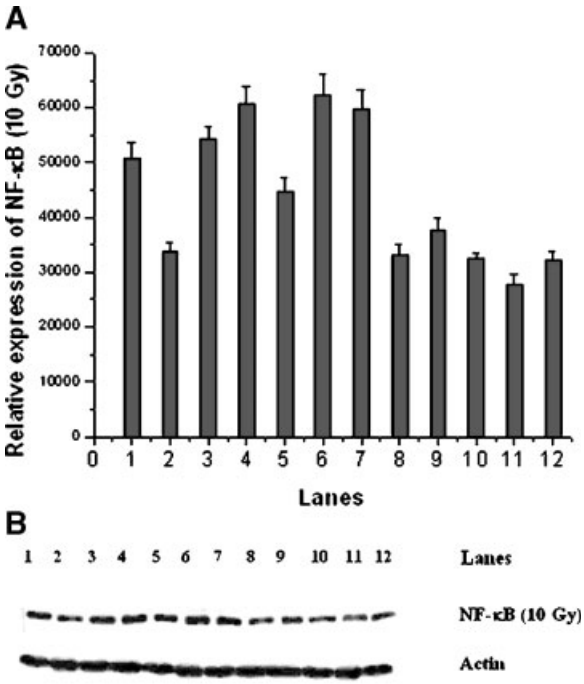

Fig. 6. Nuclear NF-κB in 10 Gy γ-irradiated and bystander K562 cells. The cells were irradiated at 10 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of NF-κB. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

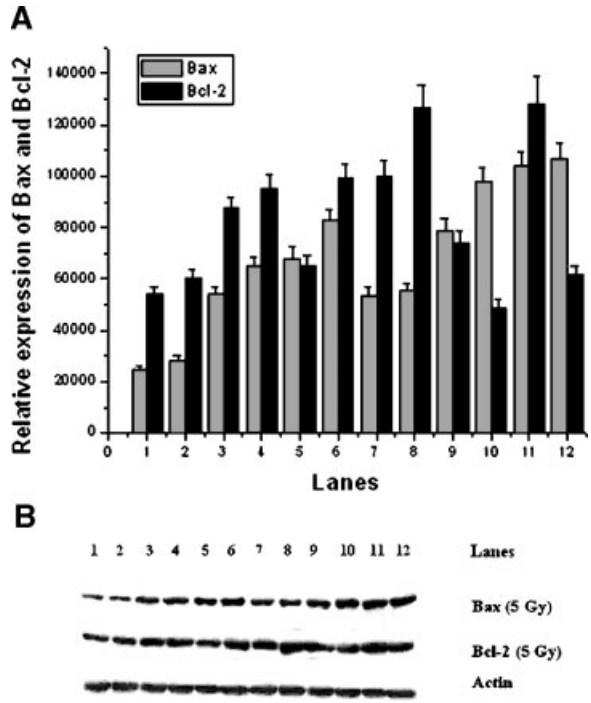

Fig. 7. Expression of Bax and Bcl-2 in γ-irradiated and bystander K562 cells. The cells were irradiated at 5 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of Bax and Bcl-2. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

NF-κB at 2 Gy dose. Moreover, these cells showed a response similar to the bystander cells but lesser in magnitude for p21 and Bcl-2. Interestingly, PARP cleavage—which is a marker of apoptosis—was much less in these cells as compared to bystander cells. This suggested a lesser apoptotic response in them. In case of Bax, however, the response was even more than that of the bystander cells. Therefore, the effect seen in the bystander cells is a combination of effects produced by ionizing radiation in the medium and signals released by the "hit" cells. The effective stimulus generated in the medium irradiated without cells may involve stable ROS produced by ionizing radiation. In the case of the bystander cells, the effect observed may be sum total of the effect of a number of factors, including ROS generated in the medium and signaling molecules released by the "hit" cells.

Literature implicates very small (less than 1,000 kDa) peptide molecule, biogenic amines or possibly that long-lived radicals in transduction of the signal to the bystander cells [Emerit et al., 1997; Azzam et al., 2002; Balcer-Kubiczek et al., 2002; Davies, 2003]. These could be acting individually or in combination lead to the formation of relatively more stable molecular species that act as the true effectors [Mothersill and Seymour, 2004]. Cytokines like interleukins may also be good candidates for such functions by being secreted by the irradiated cells and elicit a response from the bystanders. In the light of the above, it would not be unreasonable to speculate that recipient cells

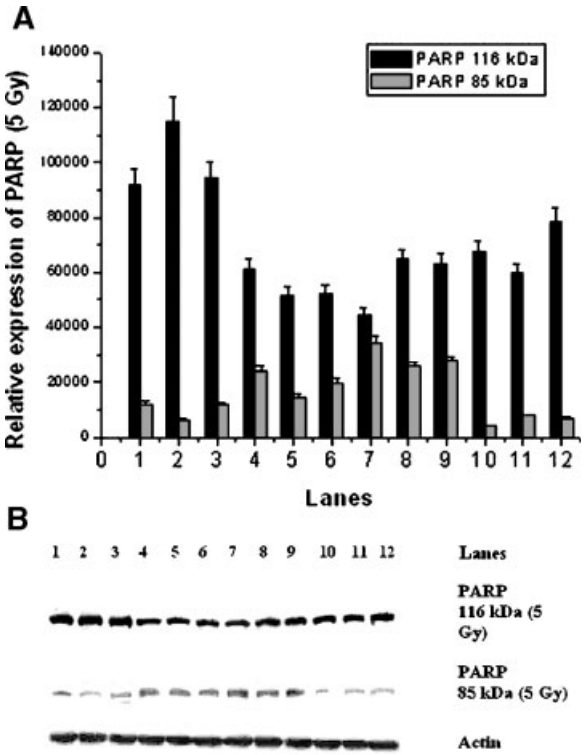

Fig. 8. PARP cleavage products in γ-irradiated and bystander K562 cells. The cells were irradiated at 5 Gy, lysed after treatment as described in Materials and Methods Section. Lysates were resolved on 8% SDS–PAGE and probed with specific antibody. **A**: Plot and (**B**) Western blots showing protein levels of PARP cleavage products. Data represents mean ± SE of three independent experiments. Key: **Lane 1**, unirradiated control; **Lane 2**, receiving medium from unirradiated cells; **Lane 3**, unirradiated control receiving fresh medium; **Lane 4**, irradiated cells at ½ h post-irradiation; **Lane 5**, irradiated cells at 2 h post-irradiation; **Lane 6**, irradiated cells at 4 h post-irradiation; **Lane 7**, bystander cells at ½ h; **Lane 8**, bystander cells at 2 h; **Lane 9**, bystander cells at 4 h; **Lane 10**, cells with irradiated medium at ½ h; **Lane 11**, cells with irradiated medium at 2 h; **Lane 12**, cells with irradiated medium at 4 h.

have receptors for bystander signals. Whether these are external or internal is unknown.

Belyakov et al. [2005], have shown that unirradiated human cells in normal three dimensional human tissue systems can respond to radiation-induced cellular damage that occurs in cells as much as 1 mm away. This is in support of the present work where we speculate that the signal is transduced from the target cells to the bystander cells through the release of signaling molecules into the surrounding medium.

The current study is a clear evidence of radiation-induced bystander effect being involved in adaptive responses in the bystander cells. Both the proapoptotic and anti-apoptotic pathways seem to be activated as is clear from the Bax and Bcl-2 induction. However, since the Bax induction appears to be sustained while that of Bcl-2 transient, the predominant response may be apoptotic. This is further supported by the fact that PARP cleavage is enhanced in the bystander cells. At the same time enhanced induction of p21 at both 2 and 5 Gy in the bystander cells indicate the initiation of cell cycle arrest (G2-M) in the bystander cells. A similar induction of p21 was also observed by Yang et al. [2005] in human fibroblasts in a study involving medium-mediated bystander communication after X-ray irradiation. This induction of p21 might be taking place through the activation of NF-κB that is observed in the bystander cells. Previously, Wuerzberger-Davis et al. [2005] have demonstrated enhanced G2-M arrest in leukemic cells by NF-κB dependent p21 induction.

Just as in the cells that are irradiated directly, the response of the signaling factors in the bystander cells varied with the dose given to the target cells. The probable explanation for this is that the dose given determines the response elicited and hence the signal transduced by the target cells.

The observed bystander responses clearly indicate that there are signals transmitted from the target cells in a paracrine manner which in this case probably lead to a proapoptotic response as indicated by Bax and PARP cleavage or a cell cycle arrest through p21 or both. The response of the bystander cell is a cumulative response of the effect of the signaling molecules produced by the irradiated cells which get transported via the medium to the unirradiated cells and also certain factors like ROS that are generated by the irradiation of the surrounding medium. This is evident through the effects observed in the bystander cells and the cells exposed to medium irradiated without cells. This is particularly relevant in vivo where the cell is surrounded by other cells and bathed in extra cellular fluids. Here a component of the bystander effect may be due to the signals from the "hit" cell and a part may be due to the irradiated surrounding fluids.

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