Connexin-43 regulates p38-mediated cell migration and invasion induced selectively in tumour cells by low doses of γ-radiation in an ERK-1/2-independent manner

Soma Ghosh, Ashish Kumar, Rajendra Prashad Tripathi and Sudhir Chandra

Natural Radiation Response Mechanisms Group, Division of Radiation Biosciences with 1Institute of Nuclear Medicine & Allied Sciences, Brig. Mazumdar Road, Delhi-110054, India

*To whom correspondence should be addressed. Tel: +91-011-23977027; Fax: +91-011-23919509; Email: sudhirchandra@yahoo.com

Radiotherapy exposes certain regions of solid tumours to low sublethal doses of γ-radiation that may cause secondary malignancies. Therefore, evaluating low-dose-γ-radiation-induced alterations in tumorogenic potential and understanding their mechanisms could help in improving radiotherapy outcome. Limited studies have indicated connexin (Cx) up-regulation by low doses, whereas Cxs are independently shown to alter cell migration in unirradiated cells. We investigated low-dose-γ-radiation-induced alterations in Cx43 expression and cell proliferation/migration/invasion in various tumour cell lines, along with the putative molecular pathways such as p38 and extracellular signal-regulated kinase-1/2 (ERK-1/2)–mitogen-activated protein kinases (MAPKs). Interestingly, a narrow range of low doses (10–20 cGy) enhanced Cx43 expression and also selectively induced glioma cell migration without altering cell proliferation, accompanied by sustained activation of p38 and up-regulation of p21γ activation, whereas the lowest (5 cGy) dose induced cell proliferation coupled with enhanced p-ERK1/2, proliferating cell nuclear antigen and p-H3 levels without inducing cell migration. Most importantly, low-dose-γ-radiation-induced cell migration and p38 activation was strongly inhibited by knocking down Cx43 expression, thereby demonstrating latter’s upstream role, whereas the knock-down had no effect on ERK-1/2 or cell proliferation. Silencing Cx43 caused near-complete inhibition of radiation-induced cell migration/invasion in all tumour cell lines (U87, BMG-1, A549 and HeLa), whereas no cell migration/invasiveness was induced in the γ-irradiated primary VH10 or transformed A549 fibroblasts. Our study demonstrates for the first time that low-dose-γ-radiation induces p38-MAPK mediated cell migration selectively in tumour cells. Further, this effect is regulated by Cx43, which could thus be an important mediator in radiation-induced secondary malignancies and/or metastasis.

Introduction

Conventional radiotherapy as well as the more recent advanced modalities, such as intensity-modulated radiotherapy and image-guided radiotherapy, may involve sublethal low-dose-γ-radiation exposure of cells/tissue located within the peripheral regions of solid tumours. Although this unwanted low-dose exposure to tumour tissue is grossly encountered during the conventional modes of radiotherapy (1), the other modalities may also pose this problem to some extent (2), thereby making low-dose-γ-irradiation of tumour cells a common consequence of radiotherapy apart from the undesirable exposure of surrounding normal tissues (3,4). While the biological effects of low-dose-γ-radiation exposure to solid tumours need more investigation, it could very well contribute to post-radiotherapy secondary malignancies that are reported to occur quite frequently (5–7). Therefore, evaluating the potential of low-dose-γ-radiation for enhancing tumorigenic potential, and understanding the mechanisms thereof, seems imperative for identifying new potential drug targets for controlling/preventing such incidences.

Biological effects of ionizing radiation exposure include induction of cellular DNA damage followed by stimulation of DNA repair mechanisms and cell cycle checkpoints, which may lead to radiation-induced cellular lethality or survival based on the extent of damage and proficiency of cellular repair mechanisms (8–10). Low doses (<50 cGy) of low-linear energy transfer ionizing radiation (X-rays; γ-rays) are also known to induce certain intriguing cellular responses including hyper-radiosensitivity (11) and bystander effect (12), which could have important implications for biological manifestations including mutagenesis (13,14). Besides, a number of other cellular signalling responses known to be elicited by low-dose γ-radiation include extracellular signal-regulated kinase-1/2 (ERK-1/2)–mitogen-activated protein kinase (MAPK) and p38-MAPK pathways, which may mediate or regulate cellular responses such as proliferation, migration and even transformation/carcinogenesis (15–18). X-ray doses <50 cGy have also been shown to significantly increase the connexin-43 (Cx43) expression that seems to be associated with corresponding increase in gap junctional intercellular communication (GJIC) (19). Independent of radiation response, Cx43 has received much attention for its role as tumour suppressor, and several reports have additionally indicated its role in cell migration (20–24). In human tumour cells with low or absent endogenous expression of Cx43, its over-expression resulted in increased motility and/or invasion, whereas the down-regulation of Cx43 decreased cell motility and invasion (25–28). Although these studies indicate that Cx43 may regulate tumour cell migration/motility, it is not yet known whether radiation-induced alterations in tumour cell migration and Cx43 expression is inter-related, especially, because the latter effect is reported only occasionally.

In the present study, we investigated the effect of low-dose-γ-radiation on Cx43 expression, GJIC as well as on cell migration and invasion activity in tumour cells of varying origins and alterations in MAPK pathways. Interestingly, our study demonstrates that low doses of γ-radiation induce significant migration activity in all tumorigenic cells and this effect was indeed dependent on post-irradiation increase in the expression of Cx43, thus demonstrating a possible regulatory role of Cx43 in the low-dose-γ-radiation-induced increase in tumour cell migration/invasion.

Materials and methods

Cell lines and treatments

Human malignant glioma cell lines U87 and BMG-1, cervical cancer cell line HeLa, adenoacarcinoma cell line A549 and non-malignant cell lines AA8 (transformed Chinese hamster fibroblasts) and VH10 (primary human fibroblasts) were maintained as monolayer at 37°C in Dulbecco’s modified Eagle’s medium supplemented with fetal calf serum. BMG-1 and U87 cell lines were obtained from the National Institute of Mental Health and Neurosciences (Bangalore, India) (29). A549 cell line was obtained from National Centre for Cell Sciences (Pune, India) and HeLa cell line was obtained from North-Eastern Hill University (Shillong, India). Non-malignant cell line AA8 (Chinese hamster ovaries) and VH10 (human primary fibroblast) were provided by Prof. Mats Harms-Rangdahl, Stockholm University, Sweden.

Exponentially growing cells were irradiated using a 60Co γ-radiation source (Teletherapy Unit, BBharton-II, Panacea Medical Technology Pvt Ltd, Bangalore) at 1.03 Gy/min dose rate. For inhibition of ERK-1/2, cells were incubated with a specific inhibitor of ERK-1/2 activation (PD98059; Calbiochem) for 1 h prior to irradiation. For transient inhibition of cell proliferation, 1 μg/ml of aphidicolin (Sigma), an inhibitor of DNA
polymerase-α, was added into growth medium 6 h prior to irradiation, and cell cycle analysis was performed using flow cytometry. Axiovert-200 Zeiss inverted DIC microscope (Carl Zeiss) was used for routine morphological observations.

**Quantitative real-time PCR analysis**

Cx43 expression was studied using real-time PCR. Primers were designed using Beacon Designer (Stratagene) for Cx43 (accession no. NM_000165.3). Five micrograms of total RNA isolated using RNaseasy Mini Kit (Qiagen) from cells was used for synthesis of complementary DNA using M-MuLV reverse transcriptase (Fermentas). An equal volume of complementary DNA was used for real-time PCR using QuantiTect SYBR green PCR Kit (Qiagen) in Stratagene Mx3005P system as per the manufacturer’s instructions. The sequences of primers for Cx43 were 5’GAGCCGACCTATTGCGACACG3’ (forward) and 5’ACCCAGGGAGATGGCGAGAG3’ (reverse) and annealing temperature was set at 63°C. Glyceraldehyde 3-phosphate dehydrogenase was used as loading control housekeeping gene.

**Western blotting**

For protein expression analysis, western blotting was performed as described earlier (30). Anti-Cx43, anti-phospho-p38, anti-p38, anti-phospho-ERK, anti-ERK, anti-phospho-Histone H3 and anti-proliferating cell nuclear antigen (PCNA) (all purchased from Santa Cruz) were used. Non-fat dry milk (Santa Cruz) was used as blocking agent. Anti-β-actin antibody (Santa Cruz) was used as loading control. Appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and chemiluminescence detection kit (Pierce) were used for detecting the protein bands. Quantification of protein bands was performed by normalizing with loading control using Image Quant software (GE Healthcare).

**Cell cycle analysis**

Cell cycle distribution was analyzed by relative DNA content measurement using flow cytometry of ethanol-fixed cells stained with 50 µg/ml of propidium iodide. Cells were washed twice with phosphate-buffered saline (PBS without Ca²⁺, Mg²⁺, pH 7.4) by centrifugation at 600g for 5 min, fixed in 70% ethanol and stored at 4°C overnight. Fixed cells were washed twice in PBS and treated with 200 µg/ml RNase-A at 37°C for 30 min before staining with propidium iodide for 30 min. Relative DNA content and cell cycle distribution were analyzed with the help of FACSCalibur flow cytometer (Becton Dickinson) using the CellQuest software (v.3.0.1; Becton Dickinson) for acquisition and ModFit LT software (v.2.0; Verity Software House) for cell cycle analysis.

**Scrape-loading assay**

Lucifer yellow-based scrape-loading assay was used for analyzing GJIC. Because lucifer yellow can only migrate through functional gap junctions, the distance travelled by the dye from scrape-wounded region through the neighbouring cells indicates GJIC activity. Briefly, the confluent cultures grown in 60 mm Petri dishes were washed with PBS containing Ca²⁺ and Mg²⁺ and scrape wounds were created in cell monolayer with a sterile scalpel. Subsequently, cells were incubated with 0.05% lucifer yellow for 4 min in dark at 37°C. Dye was then removed and cells were washed with PBS thrice before observing under the Axiovert-200 inverted fluorescence microscope (Carl Zeiss).

**Small interfering RNA transfection**

For knocking down Cx43 expression, pre-validated small interfering RNA (siRNA) from Santa Cruz Biotechnology as well as a custom-made siRNA (5’ GGTTGCGCGTCGACTTT 3’) from Eurofins MWG Operon (Bangalore, India) was used. For transfection with siRNA, RNAiFect reagent (Qiagen) was used as per the manufacturer’s instruction. The knock-down level was assessed 12 and 24 h after transfection by western blot and real-time PCR.

**MTT assay**

Metabolic viability of U87 cells was estimated using MTT assay. Briefly, cells grown in 96-well plate were irradiated at doses 5 cGy–2 Gy and MTT (1 mg/ml) was added in growth medium 24 h post-irradiation. Following incubation of cells with MTT at 37°C for 2 h, dimethyl sulfoxide was added and absorbance was recorded at 570 nm in ELISA plate reader (ECIL, India). The fluorescence intensities were normalized relative to unirradiated control.

**Migration (wound-healing) assay**

For assessing radiation-induced alterations in cell migration, wound-healing assay was used. Confluent cultures grown in Petri dishes were scraped using sterile scalpel, rinsed twice with PBS and supplemented with fresh growth medium. Cells were then irradiated and observed using DIC mode under Axiovert-200 Zeiss inverted microscope (Carl Zeiss; ×20 magnification) immediately or 9 h following doses 5 cGy–2 Gy. The number of cells migrated per unit distance (µm) from the edges of scraped region towards the centre were counted using the Axiovision (Carl Zeiss) software.

**Fig. 1.** Low-dose γ-radiation selectively activates Cx43 expression and gap junctional activity in U87 glioma cells. (A) Western blot analysis of Cx43 following different radiation doses. Cells were harvested 2 h post-irradiation. Representative blots from three independent experiments are shown. Densitometry of Cx43 protein bands relative to β-actin bands is shown (**P ≤ 0.01). (B) Alterations in Cx43 mRNA expression using real-time quantitative PCR analysis 2 h following γ-irradiation at different doses. Glyceraldehyde 3-phosphate dehydrogenase was taken as the standard loading control. Data (mean ± SD) are presented after normalizing with respect to untreated control from three independent experiments (**P ≤ 0.01; ***P ≤ 0.001). (C) Gap junctional activity visualized with lucifer yellow dye transfer assay following γ-irradiation at 20 cGy. Representative images are shown from three independent experiments that yielded similar results (magnification = ×20).
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Cell invasion assay
To study the low-dose-γ-radiation-induced alterations in invasion, QCM™ 96-Well Cell invasion assay kit was used (Millipore). Briefly, 50,000 cells were loaded on inserts pre-coated with ECMatrix™. The ECMatrix™ layer occludes membrane pores and blocks non-invasive cells from migrating. Cells invading the lower chamber after 24 h were lysed in lysis buffer containing CyQuant GR Dye. The fluorescence of CyQuant GR Dye was analysed (480/520 nm) using fluorescence plate reader (Spectra Max M2; Molecular Devices).

Statistical analysis
Differences between the mean values were analysed for significance using the unpaired two-tailed Student’s test for independent samples using Microsoft Excel software.

Results
A narrow low-dose window of γ-radiation selectively enhanced Cx43 expression and gap junctional activity in U87 glioma cells
Because low doses have been occasionally shown to up-regulate expression of Cx43 (31), we studied alterations in the Cx43 expression in U87 cells following γ-irradiation at low doses (5–20 cGy) and compared the effect elicited by clinically relevant dose of 2 Gy. Although no significant change in Cx43 expression level could be observed at 5 cGy, a remarkable increase was observed within few hours (2 h) following doses 10 and 20 cGy, and maximum (~3-fold) increase was detected following 20 cGy dose (Figure 1A). The rate of Cx43 messenger RNA (mRNA) expression was also found increased by ~3- to 4-fold following doses 10–20 cGy in comparison to untreated control, as observed using quantitative real time PCR (Figure 1B).

Further, we also checked whether this increase in the expression of Cx43 was associated with enhanced GJIC by using scrape-loading assay as detailed in Materials and methods. Increased expression of Cx43 following 10cGy-20cGy γ-irradiation was indeed found to be associated with significantly higher level of GJIC (intercellular dye transfer) as compared to the untreated control cells (Figure 1C), whereas no significant increase could be observed at other doses (data not shown).

Low doses inducing Cx43 over-expression also enhanced migration activity in tumour cells of varying origin without affecting cell proliferation
Low doses inducing increased expression of Cx43 might also affect cellular adhesion-linked phenomena such as cell growth and mobility. Therefore, we further tested the effect of these doses on cellular migration and proliferation. Corresponding with increase in Cx43 expression and GJIC activity, migration of U87 cells was indeed found to be significantly higher following low doses (10–20 cGy), whereas no effect could be observed at 5 cGy dose (Figure 2A) or immediately at higher doses (50 cGy) (data not shown). Up-regulated expression of Cx43 as well as enhanced cell migration was also evident in other tumour cell lines of varying origin following 20 cGy dose of γ-radiation (Figure 2B). Cell migration
increased highly significantly (~2-fold higher than unirradiated cells) in BMG-1, U87 and HeLa cell lines ($P < 0.002$), whereas this increase was comparatively less in the A549 lung adenocarcinoma cell line (~1.5-fold; $P < 0.01$). Similar pattern in the radiation-induced invasiveness among these cell lines was obtained as shown later. Quite interesting, no significant alteration could be observed in the Cx43 expression as well as cell migration in the non-tumorigenic cell strains, viz., AA8 Chinese hamster fibroblasts (transformed) and VH10 primary untransformed fibroblasts (Figure 2B and C).

In order to further rule out the possibility that enhanced cell migration could be contributed by increased cell proliferation, U87 cells were transiently treated with aphidicolin (an inhibitor of DNA Pol-α that effectively blocks cell proliferation) for 6 h before γ-irradiation. Even in the aphidicolin-treated cells, cell migration was significantly and selectively induced at 10–20 cGy (Figure 2D), thus confirming that the cellular migration induced by these low doses was independent of cell proliferation activity.

Cell proliferation was selectively enhanced following γ-irradiation at 5 cGy without any effect at 10–20 cGy

Cell proliferation was enhanced significantly at 5 cGy as evident from cell counting ($P < 0.02$) as well as from MTT assay ($P < 0.05$), whereas no change could be observed in cell growth following
γ-irradiation at 10–20 cGy (Figure 3A). In these experiments, exponentially growing cells were harvested for counting or MTT analysis at 24 h post-irradiation. This selective alteration in cell proliferation observed at 5 cGy was associated with significantly increased levels of phospho-Histone H3 (P < 0.02) and PCNA, whereas both these indices remained unchanged following 10–20 cGy doses (Figure 3B and C). Quite intriguing, the p21 expression remained unaltered at 5 cGy but was increased with very high significance (P ≤ 0.002) following γ-irradiation at 10–20 cGy, the extent of expression being comparable to increase at the clinically relevant dose of 2 Gy (Figure 3D).

Differential induction of cell proliferation and migration was associated with distinct patterns of activation of ERK-1/2 and p38-MAPK pathways

There is growing evidence that MAPK pathways are involved in the regulation of cell proliferation and migration in which ERK-1/2 and p38 play very important role (32–34). Therefore, exponentially growing U87 cells were analysed for activation of ERK-1/2 as well as p38 following γ-irradiation at doses 5, 10 and 20 cGy and compared with 2 Gy dose at different time points post-irradiation. Following 5 cGy dose, a transient increase was observed in the ERK-1/2 phosphorylation at 1 h, which returned to basal level by 4 h, whereas a sustained effect was observed up

Fig. 2. Low doses (10–20 cGy) induce cell migration without affecting cell proliferation. (A) Wound-healing analysis was done in U87 cells following γ-irradiation at doses 5 cGy, 10 cGy, 20 cGy and 2 Gy, at 0 and 9 h after scraping. Cell migration was calculated by measuring average distance (µm) travelled by cells from edges of scraped region towards the centre. Data (mean ± SD) are presented after normalizing with respect to untreated control from three independent experiments (***P ≤ 0.01; ****P ≤ 0.001). (B) Western blot analysis of Cx43 expression in U87, BMG-1, HeLa, A549 and VH10 cells at 2 h following γ-irradiation at 20 cGy (**P ≤ 0.01). (C) Cell migration was observed using wound-healing assay at 0 and 9 h post-irradiation at 20 cGy in U87, BMG-1, HeLa, A549, AA8 and VH10 cell lines. Cell monolayers were wound scraped immediately following γ-irradiation. Representative images are shown, and data represent mean ± SD from three independent experiments (*P < 0.05; **P < 0.02). (D) Confluent cultures were treated with aphidicolin for 6 h and U87 cells were exposed to doses 5 cGy, 10 cGy, 20 cGy and 2 Gy, and cell migration was assessed at 24 h post-irradiation as shown (magnification ×10; bar = 200 µm). Cell cycle distribution observed at 6 h after release of aphidicolin is shown with significant enrichment of G1/S phase cells. Illustration shows the design of experiment. Dotted lines represent the scraped region.
to 24 h post-irradiation at all the other doses (10 cGy, 20 cGy and 2 Gy) (Figure 4A). Similarly, a transient increase was observed in the phosphorylation of p38 following 5 cGy dose, which peaked at 8 h post-irradiation followed by reversal to basal levels by 24 h post-irradiation (Figure 4B). Although a similar transient pattern of p38 activation was observed at 2 Gy, highly significant activation could be observed following 10–20 cGy doses that sustained up to 24 h, with the most prominent effect observed following 20 cGy dose ($P \leq 0.02$). These results indicate that sustained activation of p38, observed maximally following γ-irradiation at 20 Gy, might be responsible for the enhanced cell migration pattern observed selectively at 10–20 cGy (Figure 2A), whereas transient activation of ERK-1/2 observed selectively at 5 cGy could be associated with the increased cell proliferation (Figure 3A), as discussed later.

Knock-down of Cx43 expression prevented low-dose-γ-radiation-induced cell migration/invasion through selective inhibition of p38 pathway

Because the narrow low-dose range that significantly increased Cx43 expression and GJIC activity also caused activation of ERK-1/2 and p38 proteins, we further investigated whether the effects on these MAPKs were associated with the radiation-induced up-regulation of Cx43 expression. Cells were transfected with Cx43-specific siRNA that successfully
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inhibited Cx43 protein expression up to ~60% within 24 h compared with the untransfected control (Figure 5A), with mRNA levels significantly reduced by 12–24 h (Figure 5B). In cells with Cx43 knock-down, the GJIC activity was also significantly inhibited (Figure 5C). Importantly, the radiation-induced migration activity could not be observed following 10–20 cGy doses in cells with reduced expression of Cx43 (Figure 5D), thus confirming that the low-dose-γ-radiation-induced increase in cellular migration is mediated by Cx43 over-expression.

The study was extended to other cell lines including BMG-1, HeLa and A549 tumour cells, as well as AA8 and VH10 non-tumorigenic cell lines. Similar to U87 cells, the radiation-induced migration following 20 cGy dose was almost completely inhibited in all other tumour cell lines by Cx43 knock-down (Figure 5E). Two different siRNAs were used in all these experiments, whose sequence is given in Materials and methods. Importantly, both siRNAs could inhibit the radiation-induced cell migration in all the cell lines used. The Cx43 knock-down significantly decreased constitutive levels of cell migration in the two non-tumorigenic cell lines, viz., AA8 and VH10, whereas low-dose-γ-radiation-induced cell migration was marginal in AA8 cells and absent in VH10 primary cells (Figure 5E).

Because Cx43 has been implicated as an important factor in malignant glioma cell migration and invasion into the surrounding normal tissues (35), we further studied the role of Cx43 in cell invasion induced by low-dose γ-radiation. Enhanced cell invasion was observed in all tumour cell lines following 20 cGy dose, with maximum increase observed (nearly 2.5-fold) in the two glioma cell lines (Figure 5F), whereas relative percent invasion was much less in the A549 cells. Cell invasion was completely absent in non-tumour cells following γ-irradiation at this dose.

A pronounced inhibition of p38 activation (phosphorylation) was also evident in cells that were irradiated at 20 cGy following treatment with Cx43–siRNA (Figure 5G). On the other hand, radiation-induced ERK activation remained unaffected by Cx43 knock-down (Figure 5H), thereby strongly suggesting that unlike p38, the low-dose-γ-radiation-induced activation of ERK-1/2 is independent of Cx43 up-regulation.
In order to further rule out any possible role of ERK-1/2 activation in the cell migration of U87 cells, we analyzed the effect of PD98059 (a specific inhibitor of ERK1/2 phosphorylation) on radiation-induced increase in migration activity. Inhibition of ERK activation by PD98059 (Figure 6A) could not alter the radiation-induced cell migration (Figure 6B), although it successfully inhibited the cell proliferation induced by 5 cGy (Figure 6C), thereby confirming that although the transient activation of ERK-1/2 was sufficient to increase cell proliferation at this low dose, it did not have any role in the radiation-induced cell migration.

Discussion

Our study presents intriguing evidence wherein closely spaced low doses of γ-radiation significantly induced either cell proliferation or cell migration/invasion activity in various tumour cell lines. All these responses favour tumour progression and importantly indicate the potential of low γ-radiation doses to unscrupulously cause post-radiotherapy secondary malignancies and even metastasis. Most important, this study further shows that Cx43 up-regulation is essentially required for low-dose-γ-radiation-induced cell migration/invasion mediated through activation of p38-MAPK activation, thereby demonstrating a potentially pivotal role of Cxs in regulating radiation-induced secondary malignancies. These findings may well facilitate the development of novel intervention strategies for reducing post-radiotherapy tumour incidence.

Both the cellular responses, viz., cell proliferation or increased migration, were induced in a mutually exclusive manner at two closely spaced low doses that varied by a surprisingly small margin, viz., 5 versus 10/20 cGy (Figures 2A and 3A). That such small variation in low doses could indeed induce these distinct cellular responses was further confirmed by assessing markers of cell proliferation and cell cycle inhibition. The lowest dose tested (5 cGy) could induce significant increase in cell proliferation that was associated with enhanced phospho-Histone H3 (indicating increased cell division) as well as PCNA expression, whereas the neighbouring low doses (10–20 cGy) enhanced cell migration without increasing cell proliferation (Figure 3B and C). This shift in the cellular response observed within a small dose window was additionally associated with differential patterns in the induction of ERK1/2- and p38-MAPK pathways at these neighbouring low doses. Although all these low doses (5–20 cGy) stimulated activation of both ERK-1/2 and p38, both the responses were induced transiently at 5 cGy dose, whereas 10–20 cGy doses induced both pathways in a sustained manner (Figure 4A and B). Inhibiting ERK-1/2 with specific ERK inhibitor PD98059 could successfully suppress cell proliferation induced by 5 cGy dose (Figure 6C), whereas it had no effect on the cell migration induced at 10–20 cGy doses. Activation of ERK is known to be usually associated with low-dose-γ-radiation-induced cell proliferation in tumorigenic (32,36,37) as well as non-tumorigenic cells (38,39). Doses <10 cGy have been earlier shown to selectively induce cell proliferation, with a limited dose range (<50 cGy) causing ERK-1/2 activation without involving p53 accumulation that apparently results in enhanced cell proliferation (32). However, sustained activity of ERK1/2 has been shown to cause accumulation and activation of p53, which acts as transcription factor for a group of genes including p21<sub>waf1/cip1</sub>, resulting in the induction of cell cycle checkpoint (32,40). The present study indeed shows up-regulated expression of p21<sub>waf1/cip1</sub> in U87 glioma cells at doses 10–20 cGy that might be responsible for the lack of cell proliferation (Figure 3D).

Induction of cell migration in the absence of cell proliferation effect might be a common consequence of low-dose exposure in various cell types. Previous studies have reported that low doses <80 cGy induce endothelial cell migration without affecting cell proliferation (18). In various studies on different cell types and conditions, cell migration has been found to be dependent upon p38 activation. For example, activation of p38 through MKK6 transfection was observed to be sufficient for inducing migration and increasing invasiveness of MCF10A
breast carcinoma cells (16). Further, radiation could enhance the invasiveness of glioma cells through activation of p38 and FAK, therefore, further studies were necessary for establishing the dose–response and universality of this effect (41,42). In this study as well, we observed p38 activation following γ-irradiation at doses as low as 5–20 cGy, although the lowest dose (5 cGy) induced transient activation only for few hours post-irradiation (Figure 4B). Interestingly, cell migration was induced selectively by the 10–20 cGy dose window, whereas the 5 cGy dose failed to show the same effect. Therefore, it is evident that sustained p38 activation was associated with increased cell migration induced by this narrow dose range.

A significant up-regulation of Cx43 in U87 glioma cells was evident within few hours following γ-irradiation at doses as low as 10–20 cGy (Figure 1A and B). The effect could not be observed at the clinically relevant dose of 2 Gy and indicates a possibly selective activation of Cx43-mediated signalling pathways at these low doses that
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### F.

**Fold migration**

- U87: **, **
- BMG-1: **, **
- HeLa: *, **
- A549: **, **
- AA8: *, *
- VH10: **, **

**% Relative invasion**

- U87: **, **
- BMG1: **, **
- HeLa: **, **
- A549: **, **
- AA8: *, *
- VH10: **, **
Various cell lines were transfected with Cx43–siRNA and irradiated as described earlier. Cells were trypsinized and counted, and U87 cells pre-treated with Cx43–siRNA were analysed.

Figures 2A–2B

Figure 2A shows Cx43 expression at 12 and 24 h following siRNA transfection in U87 cells. (D) U87 cells were irradiated at different doses with or without siRNA pre-treatment and analyzed for cell migration. Data (mean ± SD) are presented after normalizing with respect to untreated control from three independent experiments. Dotted lines represent the scraped region. (E) Few other tumorigenic cell lines (BMG-1, HeLa and A549) as well as non-tumorigenic cells (AA8 and VH10) were transfected with Cx43-specific siRNA 24 h prior to γ-irradiation at 20 cGy and 2 Gy, and cell migration was observed 9 h post-irradiation (\(^{45}\)). Data are representative (mean ± SD) of three independent experiments. (F) Various cell lines were transfected with Cx43–siRNA and irradiated as described earlier. Cells were trypsinized and counted, and 5 x 10^4 cells from each treatment condition were allowed to invade transwell inserts containing 8 µm pore polycarbonate membranes pre-coated with extracellular matrix for 24 h at 37°C. Cells that had invaded to the lower side of the membrane were quantified using lysis buffer containing CyQuant GR Dye. Samples were read using 480/520 nm filter set. Data are mean ± SD of three experiments. Significance of difference between values is depicted either with respect to untreated cells (\(^{45}\)) or with 20 cGy irradiated cells (\(^{45}\)). Figure 5G illustrates Cx43 expression following low-dose γ-irradiation in tumour cells, which was in complete contrast with the normal primary fibroblasts in which Cx43 expression remained unaltered (\(^{45}\)). Cx43 over-expression following low-dose γ-irradiation in tumour cells was associated with sustained activation of p38-MAPK and enhanced cell migration activity (Figures 2A and 4B). Most important, knocking down the Cx43 expression resulted in inhibition of p38 activation (Figure 5G) and successfully reversed the low-dose-γ-irradiation-induced increase in cell migration (Figure 5D) without affecting the ERK-1/2 activation. Earlier, Cx43 has been shown to increase invasiveness of unirradiated glial cells though several studies have also found otherwise, suggesting a cell-type-specific role for Cx43 (\(^{45,46}\)). For example, using transwell migration assay in the MD-831 breast carcinoma cells, increased motility upon Cx43 knock-down by short hairpin RNA was observed (\(^{44}\)). The same group also showed decreased migration of MDA-MB-231 breast cancer cells upon transfection with Cx43 or Cx26 (\(^{45}\)). In the present study, we found that knock-down of Cx43 could inhibit cell invasion that was selectively induced in tumour cells by low-dose γ-irradiation (Figure 5F). Not many reports are available that indicate any regulatory role of Cx43 upstream to p38-mediated cell migration. Recently, inducing controlled expression of Cx43 was reported to enhance the HeLa cell migration in a p38-mediated manner, although the effect needs to be investigated in greater detail (\(^{46}\)).

The present study demonstrates for the first time that low-dose-γ-irradiation-induced p38-MAPK-mediated cell migration/invasion is regulated by Cx43 in the human tumorigenic cell lines and, thus, presents Cx43 as an important mediator of potential effects such as radiation-induced secondary malignancies and/or metastasis. Importantly, this study also indicates that low doses in the range of few cGy might induce metastasis, which should be further investigated in detail. The selective induction of this effect in tumour cells further warrants in-depth studies on molecular mechanisms that might help understand and exploit the low-dose-γ-irradiation-induced responses in tumour versus normal cells. These studies might prove quite valuable in designing more effective combined therapeutic strategies for improving tumour control and treatment.
Fig. 6. ERK inhibition had no effect on low-dose-γ-radiation-induced cell migration. (A) U87 cells were pre-treated with PD98059 (a specific inhibitor of ERK activation) for 1 h prior to γ-irradiation at 20 cGy. Phosphorylation of ERK1/2 was determined by western blot analysis. (B) U87 cell migration was observed following γ-radiation at doses 5, 10 and 20 cGy following pre-treatment with PD98059. The fold migration data are representative (mean ± SD) of three independent experiments (**P ≤ 0.01). Dotted lines represent the scraped region. (C) Exponentially growing cells were pre-treated with 5 μM of PD98059 for 1 h and irradiated at 5 cGy before incubating for an additional 24 h. Cell counting and MTT assay for cell viability was performed 24 h post-irradiation. Results are presented as mean ± SD of three independent experiments (*P ≤ 0.05; **P ≤ 0.02).
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Funding
Defence Research and Development Organisation, Ministry of Defence, India (INM-311.1.5).

Acknowledgement
Senior research fellowship was received by S.G. and A.K. from the University Grants Commission, India during the course of this study.

Conflict of Interest Statement: None declared.

References

Received June 3, 2013; revised August 13, 2013; accepted August 24, 2013

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