

## Human cell responses to ionizing radiation are differentially affected by the expressed connexins

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In multicellular organisms, intercellular communication is essential for homeostatic functions and has a major role in tissue responses to stress. Here, we describe the effects of expression of different connexins, which form gap junction channels with different permeabilities, on the responses of human cells to ionizing radiation. Exposure of confluent HeLa cell cultures to <sup>137</sup>Cs  $\gamma$  rays, 3.7 MeV  $\alpha$  particles, 1000 MeV protons or 1000 MeV/u iron ions resulted in distinct effects when the cells expressed gap junction channels composed of either connexin26 (Cx26) or connexin32 (Cx32). Irradiated HeLa cells expressing Cx26 generally showed decreased clonogenic survival and reduced metabolic activity relative to parental cells lacking gap junction communication. In contrast, irradiated HeLa cells expressing Cx32 generally showed enhanced survival and greater metabolic activity relative to the control cells. The effects on clonogenic survival correlated more strongly with effects on metabolic activity than with DNA damage as assessed by micronucleus formation. The data also showed that the ability of a connexin to affect clonogenic survival following ionizing radiation can depend on the specific type of radiation. Together, these findings show that specific types of connexin channels are targets that may be exploited to enhance radiotherapeutic efficacy and to formulate countermeasures to the harmful effects of specific types of ionizing radiation.

**Keywords:** gap junction permeability; cohort effects; radiotherapy; stress response; linear energy transfer/radiation quality

### INTRODUCTION

In multicellular organisms, different modes of intercellular communication have evolved to coordinate the activities required for normal tissue homeostasis and proper response to injury. Cells communicate with each other via direct contact and by paracrine and endocrine pathways with extensive crosstalk between the pathways. In our studies of the cellular responses to oxidative stress induced by ionizing radiation, we have focused on the role of direct intercellular contact via gap junctions in modulating the levels of DNA damage, stress-responsive proteins and survival in irradiated human cells [1, 2].

A large body of evidence indicates that gap junction intercellular communication is a critical mediator of human cell

responses to various forms of stress, including ionizing radiation, chemotherapeutic agents and hyperthermia [1–12]. Upon exposure to densely ionizing radiations, junctional communication in confluent normal human cell cultures enhanced induction of DNA damage, stress-responsive proteins, lipid peroxidation, protein oxidation and lethal effects in both targeted and non-targeted (i.e. bystander) cells [13–19] (reviewed in [20–22]). In contrast, other studies with human cells exposed to low doses of sparsely ionizing radiations suggested that junctional communication enhances the expression of protective effects against clastogenic and lethal damages [23, 24]. Together, the studies support the concept that the nature of the effects of junctional communication in response to irradiation or other toxic agents greatly depend on the type and dose of the

stressful agent, the biochemical change produced in the targeted cells and the genotype and/or phenotype of the targeted and bystander cells [5, 25, 26]. Depending on connexin, gap junction channels mediate the propagation of biochemical signals that either enhance or mitigate stressful effects in targeted cells, and in some cases result in important biological changes in non-targeted cells in the vicinity that can persist over multiple cellular divisions (reviewed in [27, 28]). These findings make it clear that gap junctional communication can have positive and negative effects in response to stress. However, delineation of the effects mediated by different connexins has remained unexplored.

Gap junctions are dynamic transmembrane channels that connect the cytoplasm of contiguous cells [29]. They are central to normal tissue development, and their dysregulation contributes to numerous pathologies and to cancer progression [29]. The aqueous pores of gap junction channels mediate the intercellular movement of a wide variety of small cytoplasmic molecules [30]. By allowing free passage of metabolites between coupled cells, they provide a powerful pathway for direct molecular signaling that facilitates the formation of networks determining tissue functions (reviewed in [29, 30]). Gap junction channels are composed of connexin proteins, of which there are at least 20 members [29]. Each of the connexins forms channels with distinct permeability properties [31]. The junctional channel selectivity among cytoplasmic permeants is not simply on the basis of size or charge, but appears to be tuned for selectivity among biological molecules [32–34]. Depending on their composition, connexin channels can discriminate between highly similar second messengers (e.g. cAMP, cGMP, inositol trisphosphates [35–37]). However, the aspects of junctional communication affecting intercellular propagation of effects induced by ionizing radiation remain undefined. To further understand the role of intercellular communication in the responses to ionizing radiation, normal human AG1522 skin fibroblasts and adenocarcinoma (HeLa) human cells with inducible functional expression of connexin26 (Cx26) or connexin32 (Cx32) were exposed to graded doses of different types of ionizing radiation. Whereas AG1522 fibroblasts express several connexins that form functional channels, HeLa cells have near complete lack of endogenous connexins [38]. Therefore, the expression of single connexins in HeLa cells renders them ideal for the generation of crucial information on the role of permeability of junctional channels composed of different connexins in the stress responses of irradiated cell populations. Whereas certain junctional channels may primarily propagate molecules that lead to amplification of the induced stressful effects, others may promote protective mechanisms [23, 24].

In earlier studies, we demonstrated that upregulation of connexins is part of the cellular stress response to ionizing radiation and other environmental agents [39]. Here, we

extend these studies and show that Cx26 and Cx32 differentially modulate the level of DNA damage, metabolic activity and clonogenic survival of HeLa cells exposed to different types of ionizing radiation. We also provide further evidence for the role of physical contact/direct intercellular communication in the radiation response of normal human cells.

## MATERIALS AND METHODS

### Cell culture

AG1522 normal human diploid skin fibroblasts were obtained from the Genetic Cell Repository at the Coriell Institute for Medical Research. Cells at passage 10–12 were grown in Eagles' Minimum Essential Medium (MEM) (CellGro) containing 12.5% (vol/vol) heat inactivated (30 min at 56°C) fetal bovine serum (FBS) (Sigma), supplemented with 4 mM L-alanyl-L-glutamine (CellGro), 100 U/ml penicillin and 100 µg/ml streptomycin (CellGro). They were maintained in 37°C humidified incubators in an atmosphere of 5% CO<sub>2</sub> (vol/vol) in air. AG1522 cells express several connexins, including Cx26, Cx32 and Cx43, and functionally communicate with other cells by gap junctions [39]. For experiments with confluent cultures, the cells were seeded at numbers that allowed them to reach the density-inhibited state within 5 days. They were then fed twice on alternate days, and experiments were initiated 48 h after the last feeding. Under these conditions, 95–98% of cells were in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. In the case of experiments with sparse cultures, the cells were seeded 48 h prior to irradiation at a density that allowed them to be ~40% confluent and not in contact with each other at the time of irradiation. At 24 h before irradiation, they were incubated with MEM supplemented with 0.5% FBS to enrich the population with cells in G<sub>0</sub>/G<sub>1</sub> phase. To compare the effects on confluent and sparse cultures, confluent cells were similarly fed with medium at 0.5% FBS.

To investigate the role of connexins in the radiation response, HeLa cells that express specific connexins were used. HeLa cells expressing Cx26 or Cx32 were generated by transfecting parental cells (BD Biosciences, Palo Alto, CA, USA) with vectors containing rat Cx26 or Cx32 cDNA [40]. The expressed connexins form junctional channels that discriminate between communicated signaling molecules [40]. Cells destined for irradiation by protons, γ rays or iron ions were plated in 25 cm<sup>2</sup> polystyrene flasks, and cells for α-particle-irradiation were seeded in stainless steel dishes (36-mm internal diameter) with 1.5 µm-thick replaceable polyethylene terephthalate (PET) bottoms at a seeding density of ~1.5 × 10<sup>5</sup> cells/dish. The cells were grown at 37°C in humidified incubators, in an atmosphere of 5% (vol/vol) CO<sub>2</sub> in air, in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, 4 mM L-Alanyl-L-Glutamine (CellGro), 5% (vol/vol) Tet system

approved FBS (BD Biosciences, San Jose, CA), 200 µg/ml Hygromycin B (Invitrogen, Carlsbad, CA), and 100 µg/ml Geneticin (GIBCO, Carlsbad, CA) (DMEM complete medium). To induce Cx26 or Cx32 expression, the cells were incubated with 1 µg/ml doxycycline (BD Biosciences, San Jose, CA) on Day 3 after seeding; they were then fed on Day 4, and experiments were started 24 h later when the cultures were confluent. The ability of the cells to communicate through gap junctions was routinely verified by the parachute assay or scrape-loading technique, as we have described [40]. Further, HeLa cells expressing Cx26 or Cx32 form functional channels with contiguous AG1522 normal human cells, as illustrated in Fig. 1. To assess functional gap junction coupling, donor cells are typically loaded with a junction-permeable fluorescent dye (e.g. calcein-AM) and a cell tracker fluorescent dye (e.g. CellTracker Orange CMTMR). Labeled and unlabeled cells are then mixed and cultured at confluent density. After 2–3 h, if gap junctions form between donor and receiver cells, calcein will spread into receiver cells, and then laterally through gap junctions into adjacent cells. The donor cell is identified by the CMTMR (which is restricted to the donor cell). Degree of coupling is reflected in the number of instances of dye spread to receiver cells, and by the number of other receiver cells to which the dye has spread. We have repeatedly shown irradiated and unirradiated HeLa cells that express connexin functionally communicate with each other.

### Irradiations

Cells were exposed to  $\gamma$  rays (linear energy transfer (LET)  $\sim 0.9$  keV/ $\mu\text{m}$  in liquid water) from a  $^{137}\text{Cs}$  source (JL Shepherd Mark I, San Fernando, CA) at a dose rate of 1.3 Gy/min. For  $\alpha$ -particle-irradiation, they were exposed at 37°C to a 7.4 MBq  $^{241}\text{Am}$ -collimated source housed in a helium-filled plexiglass box at a mean absorbed dose rate of 2 cGy/min. Irradiation was carried out from below, through the PET base, with  $\alpha$  particles of a measured mean energy of 3.7 MeV (0.92 MeV/u) with Full Width at Half Maximum of 0.5 MeV. The LET corresponding to a mean energy of 3.7 MeV is  $\sim 109$  keV/ $\mu\text{m}$  in liquid water. The source was fitted with a photographic shutter to allow accurate delivery of the specific radiation dose. Microscopic examination of pits etched in CR-39 plastic after a 1-min exposure showed no source hot- or cold-spots [41]. Experiments with 1000 MeV protons and 1000 MeV/u iron ions were performed at the NASA Space Radiation Laboratory (NSRL) located at Brookhaven National Laboratory, Upton NY. Description of the facility and radiation beam characteristics can be found at <http://www.bnl.gov/medical/nasa/LTSF.asp>. The tissue culture flasks, with adherent cells, were placed in the plateau region of the Bragg curve and irradiated at a dose-rate of 0.5 Gy/min. They were positioned orthogonal to the beam such that the irradiating particles impacted first the plastic of the culture

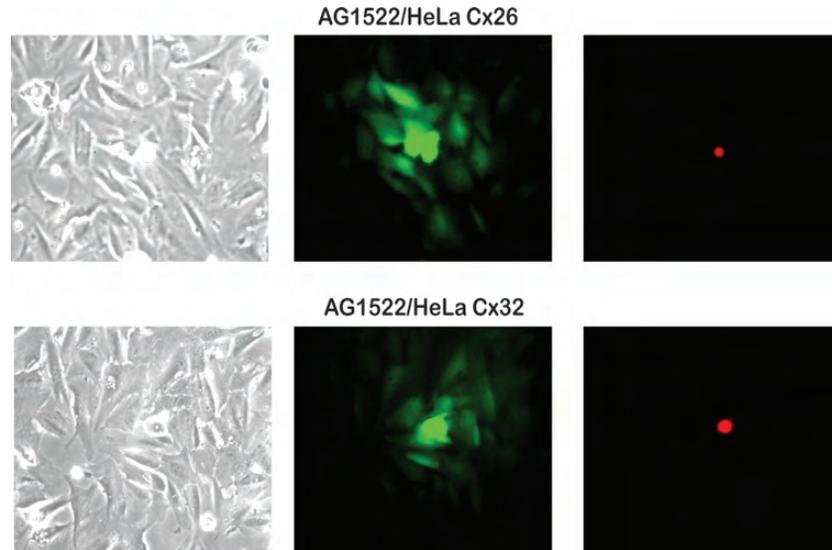
vessel, followed by the adherent cells and then the growth medium. At the place where they were positioned, the LET of protons was estimated to be  $\sim 0.2$  keV/ $\mu\text{m}$ , and that of 1000 MeV/u iron ions to be  $\sim 151$  keV/ $\mu\text{m}$  in liquid water. At 3–6 h before the radiation exposure, the flasks were filled to capacity with 37°C growth medium supplemented with 0.5% FBS that was equilibrated at pH 7.4. This ensured that during the irradiation, temperature fluctuations were attenuated, and the cells were immersed in medium when in the vertical position, alleviating changes in osmolarity and partial oxygen tension that affect their responses to ionizing radiation [42, 43]. In all cases, control cells were handled in parallel but were sham-irradiated. A detailed description of the fraction of cells whose nuclei were traversed by an average of one or more particle tracks according to the method given by Charlton and Sephton [44] is found in our earlier publication [13]. At absorbed doses of 0.8 or 1.0 Gy from 3.7 MeV  $\alpha$  particles or 1000 MeV/u iron ions, respectively, 100% of the cells in the exposed populations would be traversed through the nucleus by an average of one or more particle tracks [13, 14]. In the case of confluent cultures exposed to 4 Gy of 1000 MeV protons or 4 Gy of  $^{137}\text{Cs}$   $\gamma$  rays, every cell in the population is uniformly traversed by thousands of proton tracks or electron tracks resulting from  $\gamma$ -irradiation.

### Clonogenic survival

Cell survival curves were generated by a standard colony formation assay. Confluent cells were trypsinized within 5–10 min after irradiation and were suspended in DMEM complete medium supplemented with 20% conditioned medium. The conditioned medium was harvested from HeLa cells that were actively grown for 48 h. The cells were counted, diluted and seeded in 60-mm diameter dishes at numbers estimated to give about 80 to 100 clonogenic cells per dish. Four replicates were done for each experimental point, and the experiments were repeated at least twice. After an incubation of 8–10 days, the plates were rinsed with phosphate buffered saline, fixed in ethanol, stained with crystal violet, and macroscopic colonies were then counted. Survival values were corrected for the plating efficiency, which ranged from 50–70%.

### Micronucleus formation

Micronuclei, a form of DNA damage, were evaluated by the cytokinesis block technique [45]. After treatments, confluent cells were subcultured, and  $\sim 3 \times 10^4$  cells were seeded in chamber flaskettes (Nalgene Nunc, Rochester, NY) in the presence of 1.5 µg/ml cytochalasin B (Sigma, St Louis, MO) and incubated at 37°C. After 48 h (for HeLa cells) or 72 h (for AG1522 fibroblasts), the cells were rinsed in PBS, fixed in ethanol, stained with Hoechst 33342 solution (1 µg/ml PBS, Molecular Probes, Eugene, OR), and viewed with a fluorescence microscope. At least 1000 cells/treatment were



**Fig. 1.** Transfer of calcein (green dye; junction permeable) from HeLa cells expressing Cx26 or Cx32 to AG1522 cells. HeLa cells were loaded with calcein and CMTMR (orange dye; junction impermeable) and mixed with AG1522 cells at a ratio of 1:99. By 2 h, calcein was communicated to neighboring cells. Left panel: light microscopy image showing confluent culture density.

examined, and only micronuclei in binucleated cells were considered for analysis. At the concentration used, cytochalasin B was not toxic to HeLa cells.

### Metabolic activity

Briefly,  $\sim 5 \times 10^4$  cells/ml derived from control or irradiated cultures were suspended in DMEM complete medium and seeded in standard 24-well culture plates. At 72 h after seeding, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) was added to a 2 mM concentration, and the cells were incubated for a further 4 h. Reduction of MTT by succinate dehydrogenase, a component of complex II of the electron transport chain, generates formazan, a blue crystal product that reflects the energetic stage of mitochondria and indicates cell viability [46]. At the end of the 4 h incubation, the cells were harvested and rinsed with PBS. The formazan crystals were then dissolved in dimethyl sulfoxide (DMSO) and the optical density at 560 nm was measured.

### Statistical analyses

Poisson statistics were used to calculate the standard error associated with the percentage of cells with micronuclei over the total number of cells scored. Pearson's  $\chi^2$  test was used to compare treatment groups with the respective controls. Statistical analyses of clonogenic survival and MTT-reduction activity measurements were carried out using the Student's *t* test. A *P* value of  $< 0.05$  was considered statistically significant. Experiments were repeated two to five times, and standard errors of the means are indicated

on the figures when they are greater than the size of the data point symbols. Unless otherwise indicated, the data shown are from pooled experiments.

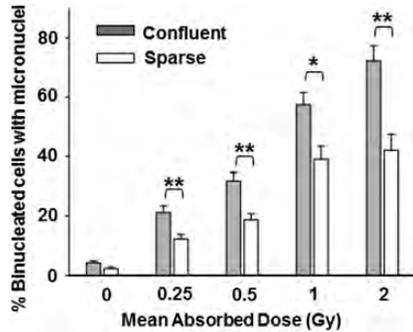
## RESULTS

### Gap junction permeability and cellular responses to ionizing radiation

In earlier studies, we showed that incubating confluent normal human AG1522 cells, which express several connexins, including Cx26, Cx32 and Cx43, with inhibitors of gap junctions attenuates the DNA damage and proliferative failure induced in these cells by energetic  $\alpha$  particles or iron ions [13, 14]. In further support of the role of direct intercellular communication in response to ionizing radiation, exposure of confluent AG1522 cultures (capable of forming gap junctions) or sparse cultures (not capable of forming gap junctions) to graded doses of 1000 MeV/u  $^{56}\text{Fe}$  ions resulted in significantly different levels of chromosomal damage. The data in Fig. 2 depict micronucleus formation, a form of chromosomal damage that arises mainly from DNA double-strand breaks, in irradiated confluent or sparse cultures that have been incubated for 3 h following irradiation and subsequently subcultured and incubated for 72 h to assay for micronuclei. Relative to sparse cells, the fraction of micronucleated cells originating from confluent cultures exposed to mean absorbed doses in the range of 0.25 to 2 Gy was significantly enhanced ( $P < 0.004$ ).

To gain insight into the effects of specific types of junctional channels in responses to ionizing radiation, we tested

the effects of expression of Cx26 or Cx32 in HeLa cells devoid of endogenous connexins. Following doxycycline-induction of connexin expression, the transfected cell lines form functional junctional channels, verified by dye transfer between coupled cells [40]. Confluent cell cultures, with or without induced expression of connexins, were exposed to graded doses of 3.7 MeV (0.92 MeV/u)  $\alpha$  particles, 1000 MeV/u  $^{56}\text{Fe}$  ions, 1000 MeV protons or  $^{137}\text{Cs}$   $\gamma$  rays.

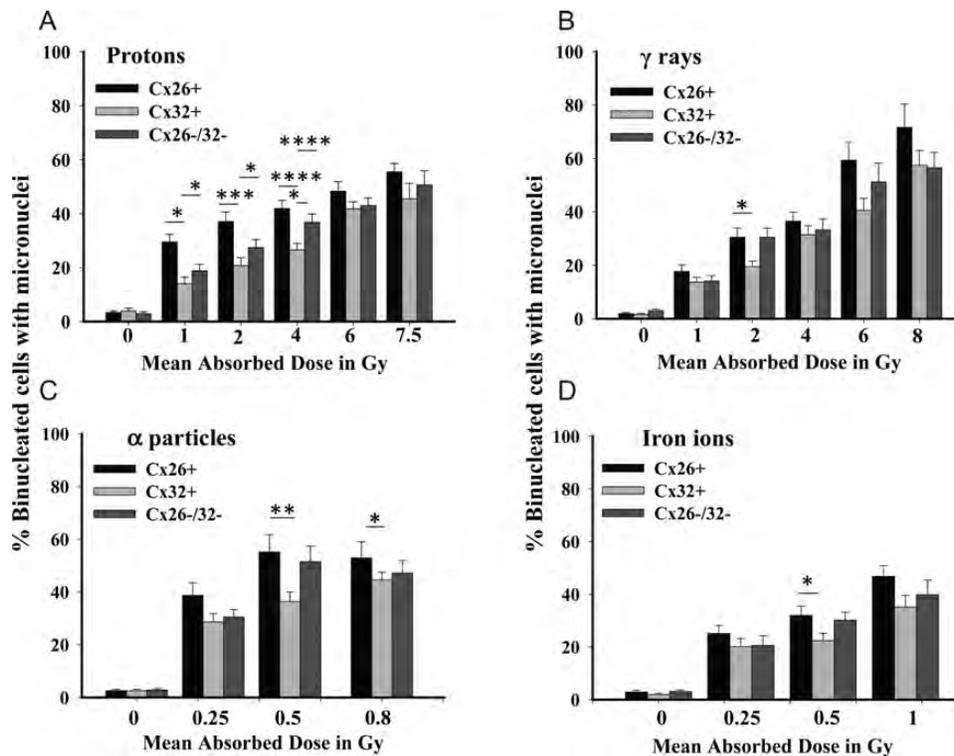


**Fig. 2.** Micronucleus formation in AG1522 normal human cells from confluent or sparse cultures exposed to 1000 MeV/u  $^{56}\text{Fe}$  ions followed by 3 h incubation at 37°C. \* $P < 0.001$ , \*\* $P < 0.004$ .

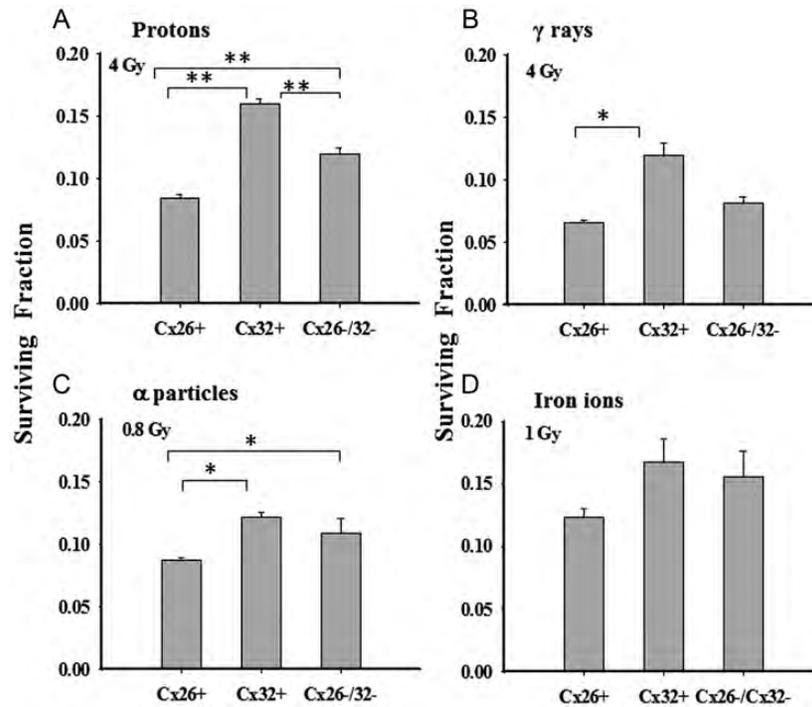
Within 10 min after irradiation, the cells were harvested and subcultured to evaluate micronucleus formation.

The results show a general trend of greater levels of DNA damage in Cx26-expressing cells than in Cx32-expressing cells from both low (protons,  $\gamma$  rays) or high ( $\alpha$  particles, iron ions) LET radiations (Fig. 3). The most significant differences were at the intermediate levels of absorbed dose, possibly reflecting compromised DNA repair due to cellular toxicity at the higher doses. The most prominent differential modulating effect of the two connexins occurred in the cells exposed to 1000 MeV protons. Relative to irradiated cells that express Cx32, greater fractions of cells with micronuclei were observed in irradiated cultures of HeLa cells that express Cx26 ( $P < 0.002$  at 2 Gy of 1000 MeV protons;  $P < 0.05$  at 2 Gy of  $^{137}\text{Cs}$   $\gamma$  rays;  $P < 0.001$  at 0.5 Gy of 3.7 MeV  $\alpha$  particles;  $P < 0.05$  at 0.5 Gy of 1000 MeV/u  $^{56}\text{Fe}$  ions). Interestingly, there was no statistically significant difference in micronucleus formation between Cx32-expressing cells and the connexin-null cells, with the exception of the Cx32-expressing cells exposed to 1–4 Gy of 1000 MeV protons.

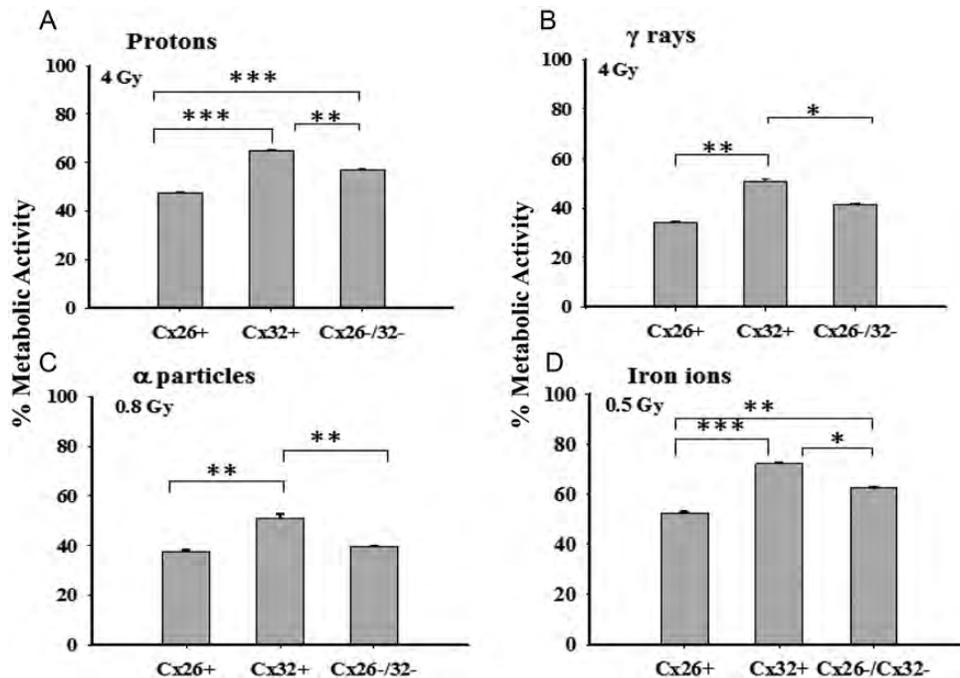
The data in Figs 4 and 5 illustrate the modulating effect of Cx26 and Cx32 expression on the clonogenic survival and metabolic activity of irradiated HeLa cells, respectively.



**Fig. 3.** The effect of expressing connexin26 or connexin32 in HeLa cells devoid of endogenous connexins on micronucleus formation following exposure to ionizing radiation. Confluent cells were exposed to graded doses of (A) 1000 MeV protons, (B)  $^{137}\text{Cs}$   $\gamma$  rays, (C) 3.7 MeV  $\alpha$  particles, or (D) 1000 MeV/u iron ions. The cells were subcultured for micronucleus formation assay within 5–10 min after irradiation and incubated for a further 48 h. \* $P < 0.05$ , \*\* $P < 0.006$ , \*\*\* $P < 0.002$ , \*\*\*\* $P < 0.001$ .



**Fig. 4.** The effect of expressing connexin26 or connexin32 in HeLa cells devoid of endogenous connexins on their clonogenic survival following exposure to ionizing radiation. Confluent cells were exposed to (A) 1000 MeV protons, (B)  $^{137}\text{Cs}$   $\gamma$  rays, (C) 3.7 MeV  $\alpha$  particles, or (D) 1000 MeV/u iron ions. The cells were processed for the survival assay within 5–10 min after irradiation. \* $P < 0.05$ , \*\* $P < 0.005$ .



**Fig. 5.** The effect of expressing connexin26 or connexin32 in irradiated HeLa cells devoid of endogenous connexins on their metabolic activity, as evaluated by the 'MTT-reduction assay'. Confluent cells were exposed to (A) 1000 MeV protons, (B)  $^{137}\text{Cs}$   $\gamma$  rays, (C) 3.2 MeV  $\alpha$  particles, or (D) 1000 MeV/u iron ions, and assayed within 5–10 min after irradiation. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.001$ .

Confluent cells were exposed to either 4 Gy of  $^{137}\text{Cs}$   $\gamma$  rays, 0.8 Gy of 3.7 MeV  $\alpha$  particles, 4 Gy of 1000 MeV protons or 1 Gy of 1000 MeV/u iron ions. Each radiation exposure resulted in approximately equivalent (5–15%) clonogenic survival in parental HeLa cells. Within 5–10 min after irradiation, the cells were trypsinized and assayed for clonogenic survival and metabolic activity.

The clonogenic survival studies showed that HeLa cells that express Cx26 were more sensitive to the lethal effects of the radiations than Cx32-expressing cells, consistent with the micronucleus formation data (Fig. 3). The connexin-dependent sensitizing effect was evident for all types of radiation (48% greater for protons, 46% greater for  $\gamma$  rays, 28% greater for  $\alpha$  particles, 25% greater for iron ions) (Fig. 4). Also consistent with the micronucleus formation data, expression of Cx32 exerted a sparing effect for the proton irradiation only.

The effects of the expression of the connexins on metabolic activity, as measured by the MTT assay, was highly correlated with the effects on clonogenic survival (Fig. 5). Where survival was enhanced, the MTT assay showed enhanced metabolic activity compared with controls, and in all but one case ( $\alpha$  particles) where survival was decreased, the MTT values were lower. In the case where there was no significant effect on survival (HeLa-Cx26 cells exposed to  $\gamma$  rays), there was no significant effect on metabolic function. Although the differences in clonogenic survival between iron ion-irradiated Cx-26- or Cx32-expressing cells and control cells were not statistically significant (Fig. 4D), the differences in metabolic activity were significant (Fig. 5D). Together, the clonogenic and metabolic survival results are generally in good agreement with the micronucleus formation results described in Fig. 2.

## DISCUSSION

The spread or mitigation of toxic effects of radiation is expected to have important consequences for radiotherapy. Several studies have shown that gap junction intercellular communication is an important mediator of non-targeted responses of confluent cells to low [2, 47] or high [13, 14, 16] doses/fluences of ionizing radiation. However, in these studies, the cells probably expressed several connexins, and the physiological functions of the cells or their responses to stress were likely to have been influenced by the combined molecular permeability properties of the junctional channels of the different connexins. For example, AG1522 normal human skin fibroblasts, which manifest significant radiation-induced bystander effects [1, 2], express Cx26, Cx32 and Cx43 (and perhaps other connexin) channels. Thus, their overall cellular responses to radiation may mask differential effects mediated by different types of connexin channels.

The results of the experiments reported in this study further support the role of direct contact between cells in

amplifying the effects of ionizing radiation on exposed cellular populations (Fig. 2). Importantly, they reveal the effects on induction of DNA damage, clonogenic survival and metabolic activity that can be attributed to specific connexins. This study revealed for the first time that different connexins can have opposing effects regarding irradiation-induced chromosomal damage and cellular nonviability. Together, the data show that whereas expression of Cx26 enhances irradiation-induced stressful effects, expression of Cx32 mitigates the stress. The results suggest that the molecules communicate through Cx26 or Cx32 channels differentially, affecting these endpoints (Figs 3–5). The effects of the connexins may be due to intercellular communication through gap junctions, but they may also involve roles of connexins that are independent of channel functions (reviewed in [48]). Connexin proteins interact with cytoskeletal proteins, components of adherens and tight junctions as well as with  $\beta$ -catenin, kinases and phosphatases [48]. Characterizing the interaction of Cx26 and Cx32 with other proteins in control and irradiated cells, together with identifying the molecules communicated through these channels under normal homeostatic or stress conditions would increase our understanding of the mechanisms underlying the differential effects of these connexins in the radiation response.

The current study examined cellular outcomes due to connexin expression that are determined during or shortly after irradiation; this connexin-specificity is the key finding reported here. It is striking that the different effects of specific connexins are evident so rapidly (5–10 min). The rapid and differential effects of the connexins on chromosome damage and metabolic rate define the initial conditions that the cells must deal with and respond to at later times. Such longer-term responses will necessarily derive from the initial connexin-specific responses. The importance of the (different, connexin-specific) initial effects is demonstrated by our previous studies showing that the chromosomal damage and potentially lethal effects enhanced by junctional communication persist strongly for hours after irradiation [13, 14]. Extension of these studies to investigate the differential effects of Cx26 and Cx32 on specific DNA repair processes would reveal important information that could be exploited in radiotherapy of tumors that express these connexins.

The data in Fig. 4 show that the magnitude of the effects of the different connexins on enhancement or reduction of clonogenic survival is a function of the character of the ionizing radiation. That is, relative to irradiated control cells, Cx26 had no significant effect on clonogenic survival following  $\gamma$ - or iron ion-irradiation, but decreased survival following the other types of irradiation. By the same token, Cx32 was ineffective in enhancing survival following  $\gamma$ - or iron ion-irradiation, but was effective in response to the other forms. The basis for these differential responses may

be due to the track structure (i.e. LET) of the radiations. That is, high LET particles, such as  $\alpha$  particles and energetic iron ions, generate substantially more reactive chemical species per traversal than do low LET radiations such as  $^{137}\text{Cs}$   $\gamma$  rays (reviewed in [49]). In fact, the differences due to track structure may be somewhat hidden in these studies by the high mean absorbed doses. Experiments conducted at lower doses and low dose-rates may more sensitively reveal the short- and long-term effects due to the LET of the radiations. In biological materials, iron ion-irradiation produces secondary radiations due to fragmentation of the iron ion itself, and these secondary particles could exert effects that modulate signaling events mediated by connexins. However, at the high doses used in this study, it is unlikely that effects due to the secondary radiations generated upon impact of the primary iron ions with the biological material would have had distinct effects that could have significantly altered the magnitude of the observed cellular responses.

### CONCLUSION

In summary, our studies may be useful in radiotherapy and in formulation of countermeasures to the harmful effects of radiation. While they suggest a critical role for the permeability of junctional channels in enhancing or mitigating the immediate toxic effects of radiation, they do not preclude a role for other mechanisms such as secreted factors. Crosstalk between soluble factors in the cellular micro-environment and junctional channels may play a role [50–53]. Expansion of the studies to include sparse and confluent irradiated cultures of HeLa cells expressing Cx26 or Cx32, as was done with AG1522 cells (Fig. 2), would shed light on whether these connexins mediate the observed responses through a role in junctional communication or other effects.

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