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Intercellular Communication Amplifies Stressful Effects in High-Charge, High-Energy (HZE) Particle-Irradiated Human Cells

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Gap junction intercellular communication/Cell killing/Potentially lethal damage repair/DNA damage/ Linear energy transfer of space radiation.

Understanding the mechanisms that underlay the biological effects of particulate radiations is essential for space exploration and for radiotherapy. Here, we investigated the role of gap junction intercellular communication (GJIC) in modulating harmful effects induced in confluent cultures wherein most cells are traversed by one or more radiation tracks. We focused on the effect of radiation quality (linear energy transfer; LET) on junctional propagation of DNA damage and cell death among the irradiated cells. Confluent normal human fibroblasts were exposed to graded doses of 1 GeV protons (LET ~0.2 keV/μm) or 1 GeV/u iron ions (LET ~151 keV/μm) and were assayed for clonogenic survival and for micronucleus formation, a reflection of DNA damage, shortly after irradiation and following longer incubation periods. Iron ions were ~2.7 fold more effective than protons at killing 90% of the cells in the exposed cultures when assayed within 5–10 minutes after irradiation. When cells were held in the confluent state for several hours after irradiation, substantial potentially lethal damage repair (PLDR), coupled with a reduction in micronucleus formation, occurred in cells exposed to protons, but not in those exposed to iron ions. In fact, such confluent holding after exposure to a similarly toxic dose of iron ions enhanced the induced toxic effect. However, following iron ion irradiation, inhibition of GJIC by 18-α-glycyrrhetinic acid eliminated the enhanced toxicity and reduced micronucleus formation to levels below those detected in cells assayed shortly after irradiation. The data show that low-LET radiation induces strong PLDR within hours, but that high-LET radiation with similar immediate toxicity does not induce PLDR and its toxicity increases with time following irradiation. The results also show that GJIC among irradiated cells amplifies stressful effects following exposure to high-, but not low-LET radiation, and that GJIC has only minimal effect on cellular recovery following low-LET irradiation.

INTRODUCTION

In the last two decades, substantial evidence has shown that ionizing radiation induces biological responses by mechanism(s) that are independent of nuclear traversal by charged particles. Biological changes, including genetic alterations, were shown to occur in a greater number of cells than expected when mammalian cell cultures were exposed to low fluences of energetic particles that target only a small fraction of the cells in the exposed population. Likewise, bystander effects have also been noted in co-cultures of irradiated and unirradiated cells, and in cell populations exposed to growth media harvested from irradiated cultures.[1–3] However, the intercellular propagation of stressful effects in cultures exposed to radiation doses that result in the targeting of most of the cells in the population with one or more radiation tracks, and the role of gap junction communication in the propagation, has not been explicitly studied; the underlying mechanisms remain unclear and are likely to depend on radiation quality (linear energy transfer; LET).

Whereas the ionization events produced by fast electrons ejected from molecules in cells exposed to high-energy X or γ rays are well separated in space, those produced by heavy charged particles occur in dense columns along the particle

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Abbreviations: Gap junction intercellular communication (GJIC); Potentially lethal damage repair (PLDR); Linear energy transfer (LET); 18-α-glycyrrhetinic acid (AGA); High charge and high energy (HZE).

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trajectory.[4,5] Depending on the physiological state of the cell, these radiation-induced bursts of reactive events may alter the cellular redox environment, modify signaling cascades and biochemical reactions, and cause differential long-term effects in the irradiated cells.[6] These effects may be further modulated by intercellular communication among the irradiated cells.

Recently, we have shown that holding α particle-irradiated normal human fibroblasts in the confluent state for various periods of time after irradiation enhanced lethality and the expression of DNA damage.[7] Here, we extend these studies and investigate the effect of intercellular communication on the modulation of the stress induced in normal human fibroblasts exposed to particulate radiations found in deep space, namely low-LET protons and high-LET iron ions. Characterizing the role of the cross-talk among cells exposed to different types of ionizing radiation may contribute to understanding the effects of radiation quality in the enhancement or mitigation of the induced detrimental effects. The information gained may help in the management of space radiation health risks during extended missions. In fact, the limited knowledge about the biological effects of and the response to space radiation has been considered the single most important factor limiting the prediction of health risks associated with human space exploration.[8,9] In addition, the results are pertinent to radiotherapy,[10] as particle therapy with energetic protons or heavy ions (e.g. carbon ions) is increasingly being used in cancer treatment.[11–13]

Several mechanisms have been implicated in the spread of radiation-induced stressful effects in exposed cell cultures. They include perturbations of oxidative metabolism, direct and indirect modes of intercellular communication, physical contact and other factors, including modification of the constitutive ingredients of the milieu in which the cells are found.[14,15] Gap junctions linking contiguous cells were shown, by direct approaches, to mediate the propagation of stress between α particle-irradiated and non-irradiated cells.[16] Whether they contribute to the propagation of damaging or protective effects among proton or high charge and high energy (HZE) particle-irradiated cells has not been investigated.

MATERIALS AND METHODS

Cell culture

Low passage AG1522 normal human diploid skin fibroblasts were obtained from the Genetics Cell Repository at the Coriell Institute for Medical Research (Camden, NJ), and were cultured as we previously described.[16] The cells express connexin proteins and are proficient in gap junction communication.[17,18]

Irradiation

Confluent, density-inhibited AG1522 cells were cultured for experiments as previously described[13] and were exposed to graded doses from 1 GeV $^{1}H^{+}$ or 1 GeV/u $^{56}Fe^{26+}$ at the NASA Space Radiation Laboratory (NSRL) at the Brookhaven National Laboratory (Upton, NY, USA).[19] Description of the facility and radiation beam information can be found at http://www.bnl.gov/medical/nasa/LTSF.asp. The exposure times for iron ion-irradiation varied from 1 to 4 min depending on the dose. In case of proton-irradiation, the exposure times varied from 1 to 10 min. In all cases, control cells were handled in parallel with cells destined for irradiation but were sham-irradiated. The culture flasks were positioned perpendicular 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 was estimated to be 151 keV/μm for 1 GeV/u $^{56}Fe^{26+}$-irradiation and 0.2 keV/μm for 1 GeV $^{1}H^{+}$-irradiation. The flasks were filled to capacity, 3 to 6 h before the radiation exposure, with growth medium that was pH- and temperature-equilibrated. This ensured that during the irradiation, temperature fluctuations were attenuated and the cells were immersed in medium, which alleviates changes in osmolarity and partial oxygen tension, parameters that can greatly affect the radiation response.[20,21]

The dose absorbed as a result of a single particle traversal through the nucleus of an AG1522 cell (mean nuclear thickness: 1.2 μm[18]), and the percentage of cells traversed in an exposed culture, may be calculated using the terminology and methods given by Charlton and Sephton.[18] Briefly, the dose per traversal to the thin disk-shaped cell nucleus of the AG1522 cell is $d = (0.16)(\mathrm{LET})/A$, where A is the cross-sectional area of the cell nucleus. The units for d, LET, and A are Gy, keV/μm, and μm^2, respectively. Considering that the LET of 1 GeV protons or 1 GeV/u iron ions are ~0.2 and 151 keV/μm, respectively, and the mean nuclear area of an AG1522 cell[18] is 144 μm^2, the absorbed dose from a proton or an iron ion traversal would be 0.00022 Gy and 0.167 Gy, respectively. Alternatively, the absorbed dose from a particle traversal may be calculated using a straightforward calculation involving the nuclear mass (~173 pg, assuming a nuclear density of 1 g/cm^3) and the energy deposited during the particle traversal.

The fraction of cells f receiving exactly i traversals was calculated according to the equation $f = (D/d)^i \exp(-D/d)/(i!)$ where D is the mean dose to the cell population and d is the dose to an AG1522 cell from a proton or an iron ion traversal.[18] Thus, in AG1522 confluent cultures exposed to 0.1, 0.25 or 0.5 Gy from 1 GeV/u iron ions, ~44, 77 and 95% of the cells, respectively, would be traversed through the nucleus by an average of one or more particle tracks (Table 1). At doses of 1, 1.5 or 2 Gy, all the cells would be traversed on average by multiple particle tracks. In contrast, in confluent cultures exposed to 0.5, 1, 2, 4, 6 or 8 Gy from 1 GeV protons used in our experiments, every cell in the population

Table 1. Dosimetry parameters for confluent AG1522 cells irradiated with 1 GeV protons or 1 GeV/u iron ions

1 GeV protons (LET~0.2 keV/μm)				
Dose (Gy)	Fluence (particle/cm^2)	Unhit fraction	Hit fraction	Average hits (per cell nucleus)
0	0	0	0	0
0.5	1.6×10^9	0	1	2184
1.0	3.1×10^9	0	1	4369
2.0	6.3×10^9	0	1	8739
4.0	1.2×10^{10}	0	1	17478
6.0	1.9×10^{10}	0	1	26217
8.0	2.5×10^{10}	0	1	34956

1 GeV/u iron ions (LET~151 keV/μm)				
Dose (Gy)	Fluence (particle/cm^2)	Unhit fraction	Hit fraction	Average hits (per cell nucleus)
0	0	0	0	0
0.1	4.1×10^5	0.561	0.439	0.58
0.25	1.0×10^6	0.235	0.765	1.45
0.5	2.0×10^6	0.055	0.945	2.90
1.0	4.1×10^6	0.003	0.997	5.80
1.5	6.2×10^6	0	1	8.68
2.0	8.3×10^6	0	1	11.58

is traversed by an increasing respective number of tracks (Table 1) that effect a rather uniform irradiation of the population.

Clonogenic survival

Survival curves were generated by a standard colony formation assay. Briefly, confluent cell cultures were trypsinized within 5–10 min after irradiation or after incubation periods at 37°C of 3 or 5 h, which normally allow repair activity and/or commitment for permanent arrest in the cell cycle to occur,[22] or 24 h when usually most of radiation-induced DNA damage is repaired and/or commitment to reproductive inactivation happens.[23] Following dissociation, the cells were suspended in growth medium, counted, diluted, and seeded in 10-cm dishes at numbers estimated to result in ~150 to 200 clonogenic cells per dish. After an incubation of 12 to 14 days, the plates were rinsed with phosphate buffered saline (PBS), fixed in ethanol, stained with crystal violet, and colonies consisting of 50 cells or more were counted as survivors. Survival values were corrected for the plating efficiency, which ranged from 20 to 30%. Each graph in RESULTS is representative of two to five separate experiments, and the results are reported as means ± standard deviation. Comparisons between treatment groups and controls were performed using the Student's t test. A p value of 0.05 between groups was considered significant.

Micronucleus formation

The fraction of micronucleated cells in the exposed cultures was measured by the cytokinesis block technique.[24] Briefly, irradiated confluent cell populations and their respective controls were subcultured, ~3×10^4 cells were seeded in chamber flaskettes (Nalgene Nunc, Naperville, IL) and allowed to grow in the presence of 2 μg/ml cytochalasin B (Sigma, St. Louis, MO). Following 72 h incubation at 37°C, the cells were rinsed in PBS, fixed in ethanol, stained with Hoechst 33342 solution (1 μg/ml PBS) (Cat. No. H-3570, Molecular Probes, Eugene, OR), and viewed under a fluorescence microscope (Dialux20, Leitz, Wetzlar, Germany). At least 1000 cells were examined for each data point in each experiment, and only micronuclei in binucleated cells were considered for analysis. At the concentration used, cytochalasin B was not toxic to AG1522 cells. Each graph in RESULTS is representative of at least 2 separate experiments, and Poisson statistics was used to calculate the standard errors associated with the percentage of micronucleated cells in the total number of binucleated cells. The latter ranged between 40 and 50% of the cells in the population. Comparisons between treatment groups and respective controls were performed using the Pearson's χ^2-test. A p value of 0.05 between groups was considered significant.

Inhibition of gap junction communication

18-α-glycyrrhetinic acid (AGA) (Sigma), a reversible inhibitor of gap junction communication, was dissolved in 99.5% dimethyl sulfoxide (DMSO) and added to cell cultures at a concentration of 50 μM, 30 min prior to irradiation. The cells were incubated in the presence of the drug until they were trypsinized. Control cell cultures were incubated with the dissolving vehicle (0.25% DMSO). Clonogenic survival results were corrected for the plating efficiency of sham-treated cells incubated with AGA, which resulted in slight toxicity for incubation periods greater than 5 h.

RESULTS AND DISCUSSION

The objective of this study was to investigate whether the stressful effects of radiation found in space and used in radiotherapy were influenced by direct cell-to-cell communication among the irradiated cells. To test this hypothesis, confluent, density-inhibited AG1522 cells that functionally communicate through gap junctions were exposed to graded doses from two types of space radiation [1 GeV protons (LET ~0.2 keV/μm) or 1 GeV/u iron ions (LET ~151 keV/μm)]. Within 5–10 min after exposure or following 3 and 24 h incubation at 37°C, the cells were subcultured and assayed for clonogenic survival and micronucleus formation, a

reflection of DNA damage.[24] As expected, high-LET iron ions were more effective than low-LET protons at inducing cell killing (Fig. 1A). When clonogenic survival was measured shortly after irradiation, a dose of 4 Gy from energetic protons was required to produce 90% killing of the exposed cells, whereas a dose of 1.5 Gy from iron ions yielded the same effect (Fig. 1A). When compared to cells exposed, in parallel, to ^{137}Cs γ rays (data not shown), the relative biological effectiveness (RBE) of iron ions and protons, estimated at the 10% survival level, were ~2.7 and 1.0 respectively.

At a mean dose of 1.5 Gy from 1 GeV/u iron ions, each cell is traversed on average by ~8 particle tracks. In contrast, in cell cultures exposed to 4 Gy from 1 GeV protons, each cell is traversed by ~17400 particle tracks (Table 1). These data illustrate the severity of the damaging effects of the dense ionizations and excitations produced along the tracks of energetic iron particles. The bursts of reactive oxygen species (ROS) and nitrogen species (RNS) in and around these

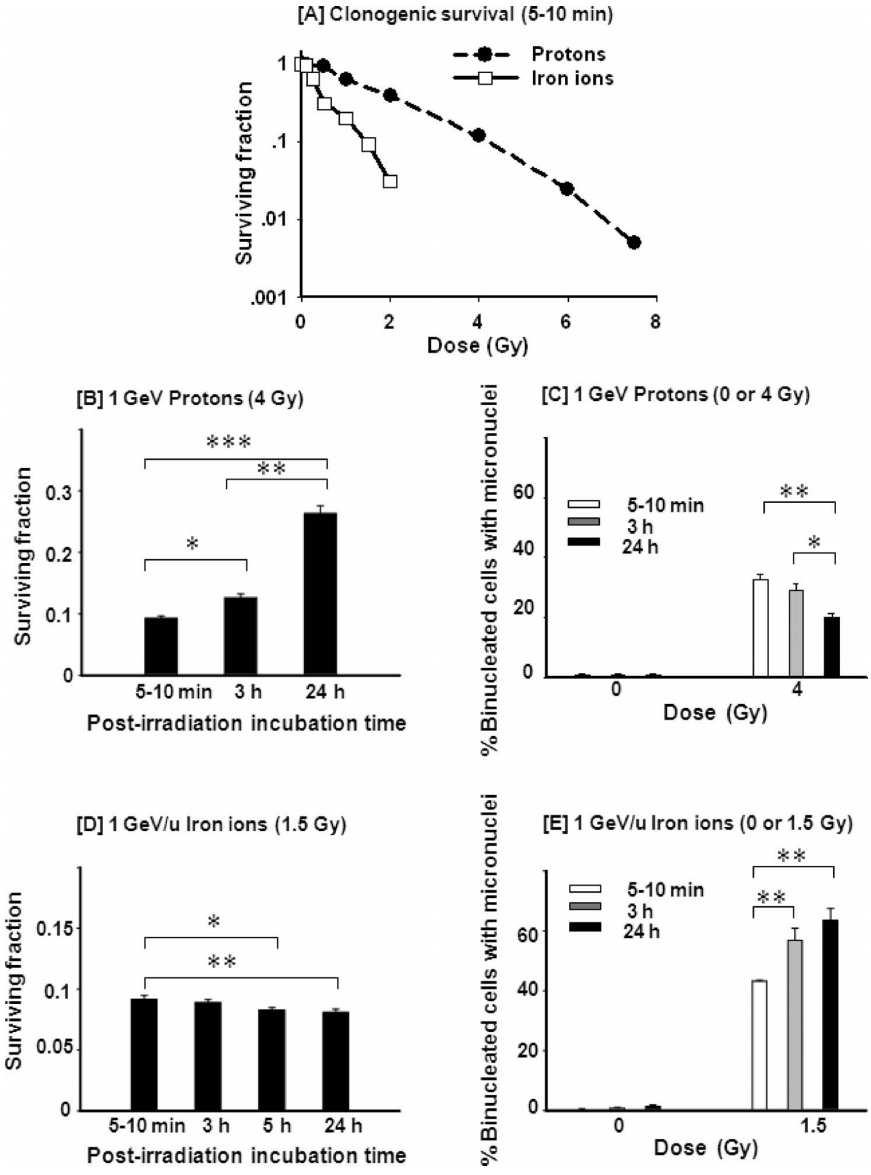

Fig. 1. Modulation of stressful effects in confluent AG1522 normal human fibroblasts exposed to energetic protons or iron ions as a function of time after irradiation. Clonogenic survival of AG1522 cells exposed to 1 GeV protons or 1GeV/u iron ions and subcultured for the assay within 5–10 min after irradiation *(Panel A)*. Clonogenic survival (*Panel B*) and micronucleus formation (*Panel C*) in cell cultures exposed to 0 or 4 Gy from 1 GeV protons and held in confluence for various times prior to subculture. Clonogenic survival (*Panel D*) and micronucleus formation (*Panel E*) in cell cultures exposed to 0 or 1.5 Gy from 1 GeV/u iron ions and held in confluence for various times prior to subculture. (*: $p < 0.03$; **: $p < 0.01$; ***: $p < 0.0001$).

iron radiation tracks, as well as in the intercellular matrix alter the cellular redox environment, may modify signaling cascades and normal biochemical reactions, generating damage to cellular molecules and organelles that is far more extensive than the damage produced along proton tracks.[5,6)]

Next, we investigated the modulation of radiation-induced damaging effects - survival and micronucleus formation - during the first few hours after exposure. Confluent holding of AG1522 cells exposed to 4 Gy from protons for 3 to 24 h prior to subculture resulted in significant PLDR that increased as a function of the length of the post-irradiation incubation period (Fig. 1B). Relative to cells assayed within 5–10 min after proton-irradiation, clonogenic survival increased by ~35% ($p < 0.05$) and 180% ($p < 0.0001$) when cells were assayed following 3 and 24 h incubation, respectively. Thus, the sparing effect was enhanced as a function of the post-irradiation incubation time. In contrast, incubation of confluent cells exposed to an isosurvival dose of 1.5 Gy from iron ions for similar periods, did not result in PLDR, but rather decreased survival (Fig. 1D). Following incubation periods of 5 and 24 h, survival decreased by ~12%.

It is well established that DNA double-strand breaks are the major lethal event in irradiated cells.[25)] Thus, it is possible that the lack of PLDR in iron ion-irradiated cells may be due to non-repairable DNA damage in these cells. Micronuclei arise predominantly from DNA double-strand breaks; therefore, we examined their formation in proton and iron ion-irradiated cells that were held in confluence for various times after irradiation.

In the proton-irradiated cells, the post-irradiation PLDR correlated with decreased micronucleus formation, showing a 10% ($p < 0.32$) and a 38% ($p < 0.01$) decrease at 3 h and 24 h, respectively (Fig. 1C). This suggests that the DNA damage in response to proton irradiation is repairable over a period of hours, and that the PLDR could be due to this repair.

In contrast, in the iron ion-irradiated cells there was a substantial increase in micronucleus formation over the same period (Fig. 1E); 30% ($p < 0.01$) at 3 h and 46% ($p < 0.01$) at 24 h post-irradiation. Therefore, it appears that the type of damage that occurs with iron ion radiation not only inhibits post-irradiation repair of damaged DNA, but actually sets into motion additional mechanisms of DNA damage that develop over hours. We note that the extent of micronucleus formation is greater than the observed decrease in survival. The full toxic effects of the DNA damage that develops over many hours may require longer times to become evident. The next experiments investigated the possible role of GJIC in these effects.

In previous studies, we and others have shown that GJIC mediates the propagation of stressful effects from α particle-irradiated to contiguous non-irradiated cells.[26–28)] Gap junctions are dynamic structures that are critical for diverse physiological functions. By allowing direct intercellular transfer of cytoplasmic molecules, they provide a powerful pathway for direct molecular signaling between cells.[29)] Therefore, we hypothesized that GJIC may contribute to the propagation of PLDR and/or stressful effects among the irradiated cells during confluent holding. Stress-inducing molecules propagated through gap junction channels may have resulted in the enhancement of DNA damage in the irradiated cells and prevented PLDR. To test this hypothesis, and further elucidate the role of LET in expression of PLDR, confluent cells were exposed, in the presence or absence of the gap junction inhibitor AGA, to 1.5 Gy from 1 GeV/u iron ions or 4 Gy from 1 GeV protons that results in a similar survival level (10%). The cells were then held in confluence at 37°C for 5–10 min or 5 h prior to subculture.

The data in Fig. 2A confirm those in Fig. 1B in showing significant ($p < 0.0001$) PLDR in proton-irradiated cells. They also show that incubation with AGA slightly ($p < 0.03$) attenuated lethality during the 5 h confluent holding period. Thus, there was a small effect of GJIC on cell survival, but without an effect on micronucleus formation (Fig. 2C). In contrast, incubation with AGA prevented the decrease (~12%) in survival ($p < 0.03$) that occurred in cells exposed to a mean dose of 1.5 Gy from iron ions and held in confluence for 5 h (Fig. 2B), and even allowed a small amount of PLDR ($p < 0.01$) to occur. These sparing effects on cell killing correlated with decreases in micronucleus formation (Fig. 2D). Inhibition of GJIC resulted in a decrease ($p < 0.01$) in the fraction of micronucleated cells when the irradiated confluent cell populations were subcultured 5–10 min after exposure; when the subculture occurred at 5 h after exposure, incubation with AGA suppressed the enhancement in DNA damage that typically occurred during confluent holding ($p < 0.0001$) and attenuated ($p < 0.03$) the fraction of micronucleated cells to a level below that observed when cells were assayed 5–10 min after irradiation. The data at 5–10 min suggest that, in high-LET-irradiated cells, GJIC allows rapid propagation of signals that produce DNA damage. The effects at 5 h indicate that this effect continues over time and is inhibited by inhibition of GJIC. The difference in micronucleus formation at 5–10 min and at 5 h in the presence of AGA could reflect an underlying DNA repair process previously masked by the propagation of DNA damaging signals through gap junctions in response to iron ion irradiation.

Confluent-holding of proton-irradiated cells in the presence of AGA resulted in a decrease ($p < 0.0001$) in the fraction of cells with micronuclei when cells were assayed shortly (5–10 min) after exposure (Fig. 2C). This may reflect the complexity of proton irradiation, which can result in some amount of secondary high-LET particle generation (low energy neutrons)[30)] that could lead to a small degree of propagation of stressful effects through gap junction channels. However, incubation in the presence of AGA for 5 h did not result in further decrease (Fig. 2C), suggesting that

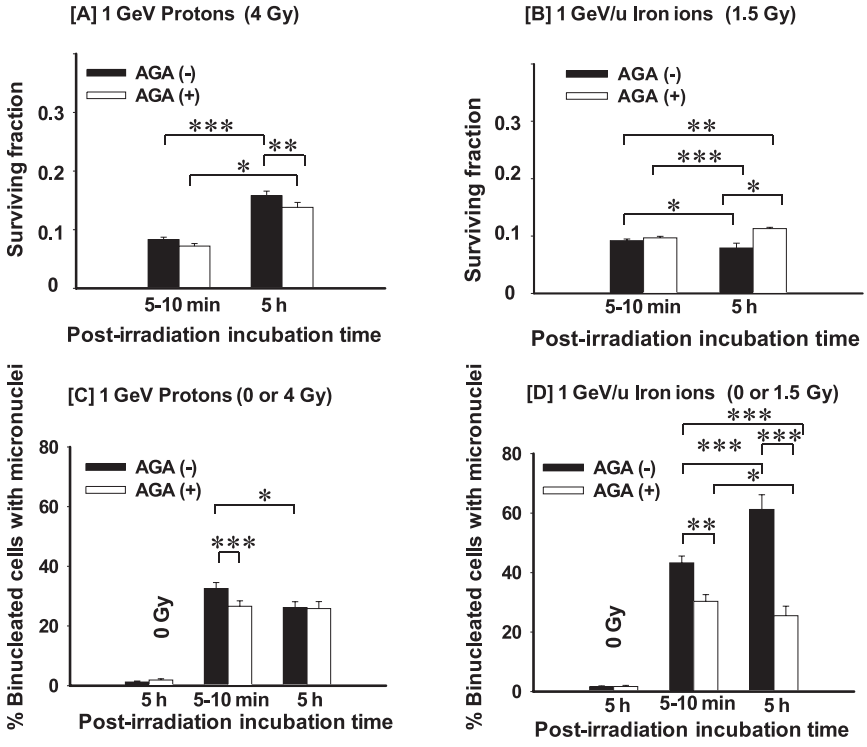

Fig. 2. The role of gap junction intercellular communication in the propagation of stressful effects among energetic proton- or iron ion-irradiated confluent AG1522 cells. Clonogenic survival of cells exposed to 0 or 4 Gy from 1 GeV protons (*Panel A*), or 0 or 1.5 Gy from 1 GeV/u iron ions (*Panel B*) and held in confluence at 37°C for 5–10 min or 5 h prior to subculture in the absence (■) or presence (□) of the gap junction inhibitor 18-α-glycyrrhetinic acid (AGA). *Panels C* and *D* describe the fraction of micronucleated cells in the proton- or iron ion-irradiated cultures described in *Panels A* and *B*, respectively. (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$).

repair of DNA damage in response to proton radiation does not involve a prominent role for GJIC. AG1522 cells express at least one type of connexin channel other than connexin43. It is possible that certain channels promote protective effects that may be masked by stressful effects mediated by other connexin channels. The use of mammalian cells in which specific connexins can be expressed in the absence of endogenous connexins[31] would shed light on the role of junctional channel permeability in the biological processes that occur in confluent cultures during the post-irradiation period.

Taken together, our studies extend the seminal findings of Tobias, Blakely and colleagues, which showed that exposure to HZE particles results in negligible PLDR.[32,33] They also support previous findings that GJIC is a critical mediator of bystander effects induced in cell populations exposed to HZE particles wherein a small proportion of the cells is irradiated.[27,34] They show that GJIC enhances toxic and clastogenic effects in cell cultures where every cell is targeted by an iron ion. While the results are relevant to our understanding of the biological effectiveness of protons and HZE particles that astronauts encounter during prolonged space travel,[35] the occurrence of these effects following exposure to HZE particles used in cancer therapy may enhance therapeutic outcome. The absence of PLDR in HZE-irradiated cells would be also relevant in the scheduling of fractionated regimens.

To summarize, this study highlights the importance of the character of particulate radiations in the propagation of biological effects.[14,15] Its expansion to investigate the nature and amount of the molecules communicated through gap junctions should increase our knowledge of the biological effects of proton- and HZE particle-induced cellular responses. The propagation of molecules that enhance oxidative stress in HZE particle-irradiated cells may damage DNA repair proteins and perturb oxidative metabolism, which may account for the observed increased toxicity over time after irradiation.

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