Université de Sherbrooke

THE ROLE OF INTERCELLULAR COMMUNICATION AND OXIDATIVE METABOLISM IN THE PROPAGATION OF IONIZING RADIATION-INDUCED BIOLOGICAL EFFECTS

by

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Acronyms, abbreviations and symbols

γ  Gamma
α  Alpha
δ  Delta
p  Probability of an event
s  Second
min Minute
h  Hour
μm Micrometer
AGA 18-α-glycyrrhetinic acid
ATM Ataxia-telangiectasia mutated
ATP Adenosine triphosphate
cGy Centigray
Cx Connexins
DNA Deoxyribonucleic acid
DSB Double strand break
keV Kilo-electronvolt
LET Linear energy transfer
MeV Mega-electronvolt
fs Femtosecond
GeV Giga-electronvolt
GJIC Gap junction intercellular communication
GPX Glutathione peroxidase
Gy Gray
kDa Kilo-dalton
HNE Hydroxynoneal
HR Homologous recombination
HZE High charge and high energy particles
IR Ionizing radiation
MN Micronucleus formation
NHEJ Non-homologous end joining
OER Oxygen enhancement ratio
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Abstract

Coordinated interactions of specific molecular and biochemical processes are likely involved in the cellular responses to stresses induced by different ionizing radiations with distinctive linear energy transfer (LET) properties. Here, we investigated the roles and mechanisms of gap junction intercellular communication and oxidative metabolism in modulating cell killing and repair of potentially lethal damage (PLDR) in confluent AG1522 human fibroblasts exposed to 1 GeV protons (LET~0.2 keV/\mu m), $^{137}$Cs $\gamma$ rays (LET~0.9 keV/\mu m), $^{241}$Am $\alpha$ particles (LET~122 keV/\mu m) or 1 GeV/u iron ions (LET~151 keV/\mu m) at doses by which all cells in the exposed cultures are irradiated. As expected, $\alpha$-particles and iron ions were more effective than protons and $\gamma$ rays at inducing cell killing. Holding $\gamma$- or proton-irradiated cells in the confluent state for several hours after irradiation promoted increased survival and decreased chromosomal damage. However, maintaining $\alpha$-particle or iron ion-irradiated cells in the confluent state for various times prior to subculture resulted in increased rather than decreased lethality, and was associated with persistent DNA damage and increased protein oxidation and lipid peroxidation. Inhibiting gap junction communication with 18-$\alpha$-glycyrrhetinic acid or by knockdown of connexin43, a constitutive protein of junctional channels in these cells, protected against the toxic effects expressed in these cells during confluent holding. Up-regulation of antioxidant defense by ectopic over-expression of glutathione peroxidase, protected against cell killing by $\alpha$-particles when cells were analyzed shortly after exposure. However, it did not attenuate the decrease in survival during confluent holding. Together, these findings indicate that the damaging effect of $\alpha$ particles results in oxidative stress, and the toxic effects in the hours following irradiation are amplified by intercellular communication, but the communicated molecule(s) is unlikely to be a substrate of glutathione peroxidase.

To further understand the role of GJIC, we tested the effect of specific connexin channel permeabilities on radiation-induced cell killing and induction of DNA damage. We used human adenocarcinoma (HeLa) cells in which specific connexins can be expressed in the absence of endogenous connexins. When exposed to protons, $\gamma$ rays, $\alpha$ particles, or iron ions, connexin26 and connexin43 channels mediated the propagation of toxic effects among irradiated cells; in contrast, connexin32 channels conferred protective effects.

Collectively, these studies provide a novel mechanistic understanding of the molecular events that mediate the fate of cell populations exposed to different types of ionizing radiation. They show that the LET of the radiation significantly impacts these events. The enhancement of cell killing in the hours after exposure of tumor cells to high charge and high energy particles and or $\alpha$ particles support the use of these particles in cancer radiotherapy. Characterization of the molecules that are communicated through junctional channels from tumor to normal cells would help formulate countermeasures to protect normal tissues during radiotherapy. Future in vivo research would contribute to validating these concepts.
Résumé

Les interactions coordonnées des processus moléculaires et biochimiques sont probablement impliquées dans la réponse au stress cellulaire induit par des rayonnements ionisants de transfert d'énergie linéaire (TEL) différent. Ici, nous avons étudié le rôle des jonctions de type gap et le métabolisme oxydatif dans la modulation de la mort cellulaire et la réparation des dommages potentiellement létaux (PLDR) dans des cultures de confluences de fibroblastes humains AG1522. Ces cultures ont été exposées à des protons d'énergie 1 GeV (TEL~0,2 keV/μm), des rayons γ de 137Cs (TEL~0,9 keV/μm), des particules α de²⁴¹Am (TEL~122 keV/μm) ou des ions fer d'énergie 1 GeV/u (TEL~151 keV/μm) et à des doses où toutes les cellules exposées sont irradiées. Comme attendu, les particules α et les ions fer ont été plus à même d'induire la mort cellulaire que les protons et les rayons γ. Le maintien des cellules à confluence pendant plusieurs heures après irradiation aux rayons γ et aux protons favorise la survie cellulaire et diminue les dommages chromosomiques. En revanche, le fait de maintenir dans un état de confluence des cellules traversées par des particules α pour des temps donnés différents aboutit à un accroissement de la mort cellulaire, ce qui a été associé à une augmentation des lésions de l'ADN, à l'oxydation des protéines et à la peroxydation lipidique. D'autre part, l'inhibition des jonctions gap par l'acide 18-α-glycyrrhétinique ou la diminution de l'expression de la protéine connexine 43, protéine constitutive des canaux jonctionnels de ces cellules, ont un effet protecteur contre les effets toxiques des radiations. Une sur-régulation des protections anti-oxydantes par une surexpression anormale de la glutathion peroxydase protège les cellules contre les effets toxiques lorsque celles-ci sont analysées peu après l'irradiation. Toutefois, cela n'a pas atténué la diminution de la survie lors du maintien de cellules à l'état de confluence. L'ensemble de ces données indiquent que les dommages induits par les particules α résultent du stress oxydant et que ces effets toxiques sont amplifiés par la communication intercellulaire dans les heures suivant l'irradiation. Cependant, la (les) molécule(s) transmise(s) n'est probablement pas un substrat de la glutathion peroxydase.

Pour mieux comprendre le rôle des jonctions gap, nous avons testé l'effet de la perméabilité d'une connexine (Cx) spécifique sur la mort cellulaire radio-induite et l'induction de dommages de l'ADN. Nous avons utilisé des cellules issues d'adénocarcinome humain (HeLa) dans lesquelles des connexines spécifiques peuvent être exprimées en l'absence des connexines endogènes. Suite à l'exposition aux protons, aux rayons γ, aux particules α ou aux ions fer, nous avons constaté que les canaux formés de Cx26 et ceux formés de Cx43 jouent un rôle dans la propagation des effets toxiques parmi les cellules irradiées tandis que les canaux formés de Cx32 confèrent des effets protecteurs.

Collectivement, ces études apportent une compréhension mécanistique nouvelle des événements moléculaires qui interviennent dans le devenir des populations de cellules exposées à différents types de radiations ionisantes. Elles montrent que le TEL des radiations peut avoir des répercussions importantes sur ces événements. L'induction de la mort cellulaire des cellules tumorales dans les heures suivant l'exposition à des radiations de haut TEL ou de particules α est en faveur de l'utilisation de ces particules en radiothérapie. La caractérisation des molécules transmises des cellules tumorales aux cellules normales via les canaux jonctionnels permettrait de formuler des mesures pour protéger les tissus sains durant la radiothérapie. D'autres recherches sont nécessaires pour étudier la pertinence de ces conclusions in vivo et valider ces concepts.
I. Introduction

Ionizing radiation (IR) is an effective method of cancer radiotherapy, diagnostic radiology, and nuclear medicine (SAUNDERS et al., 1985; BLAKELY and KRONENBERG, 1998; HALL and GIACCIA, 2006; DURANTE and LOEFFLER 2010). It consists of particulate or electromagnetic types with low- or high-linear energy transfer (LET) properties (TUBIANA et al., 1990; HALL and GIACCIA, 2006). The ionization and excitation events produced as a result of cellular traversal by different types of IR are dependent on the energy and mass of the ionizing particle. LET is defined as the energy transferred per unit length of the track (TUBIANA et al., 1990; HALL and GIACCIA, 2006). Extensive studies indicate that radiation-induced biological effects are dependent on LET (ELKIND 1984; TODD et al., 1985; GOODHEAD et al., 1993; TSURUOKA et al., 2005; HAMADA et al., 2006; FRANKENBERG et al., 2006; WHALEN et al., 2008). In comparing the biological effects of different radiations, it is customary to use X-rays as the standard. The relative biological effectiveness (RBE) of high LET radiation such as α particles, neutrons, or heavy ions is significantly greater than that of low LET radiation such as high energy protons (TUBIANA et al., 1990; BLAKELY and KRONENBERG, 1998; HALL and GIACCIA, 2006). Various biological end points such as cell killing, chromosome aberration, induction of DNA damage, mutation induction, cell transformation and change in gene expression support the LET dependence of radiation effects (see, for example: YANG et al., 1985; HEI et al., 1988; KASTAN et al., 1991; CHEN et al., 1993; BELYAKOV et al., 1999; KAWATA et al., 2004; GUIDA et al., 2005; DESAI et al., 2005; DING et al., 2005; WHALEN et al., 2008; TSURUOKA et al., 2008, Autsavapromporn et al. 2011). However, the mechanisms underlying LET effects remain unclear.
Recently, human exposure to IR has been on the increase. In addition to exposure to background radiation, humans are exposed to radiation from industrial applications, fallouts from weapons testing and significantly from medical applications such as diagnostic radiology or nuclear medicine procedures. In addition, with the expansion of the space program and the initiation of long-term space flights, there is great concern by the National Aeronautics and Space Administration (NASA) in the biological effects of high charge (Z) and high energy (E) ions known as HZE particles (e.g., iron ions) and of high energy protons (CUCINOTTA et al., 2006).

Cell traversal by a single HZE or α particle results in the deposition of a large amount of energy along the particle tracks, with the potential of producing clustered DNA damage (up to 25 lesions per cluster) and damage to other molecules such as proteins and lipids. In contrast, cellular exposure to comparable doses from low LET radiation (e.g. γ rays or energetic protons) generates sparse ionizations that result in clusters with a maximum 10 lesions (CUCINOTTA et al., 1998; SUTHERLAND et al., 2001; SEMENNENKO and STEWART, 2004). Therefore, it is important to understand how a biological response is produced by low- or high-LET radiations and how the effect is processed in cells.

Recent evidence has suggested that gap junction intercellular communication (GJIC) and oxidative metabolism are involved in the propagation of stressful effects from irradiated to non-irradiated bystander cells in an exposed population to α particles (AZZAM et al., 1998, 2001, 2002, 2003, 2004; Zhou et al., 2001; SHAO et al., 2003b). The involvement of these mechanisms in the propagation of signaling events among irradiated cells has not been investigated previous to this project. Gap junctions are dynamic intercellular membrane channels that are critical for diverse physiological function implicated in the control of cell homeostasis, proliferation and death. They allow the direct exchange of small molecules (≈1 kDa) that are well above the size of
most secondary messengers between adjacent cells. They are composed of connexins (Cx), which are members of a large family of proteins. Different Cxs form channels with specific permeability properties (KOREEN et al., 2004; KING and BERTRAM, 2005; SHAO et al., 2007; HARRIS and LOCKE, 2008) and play a critical role in cellular responses to IR (AZZAM et al. 1998, 2001, 2003). The nature of the signaling molecule(s) communicated through gap junction channels linking irradiated cells with bystander cells remains unknown.

Two mechanism of transmission of molecules among irradiated cells and between irradiated and unirradiated neighbor cells have been proposed. They consist either in diffusible factors excreted into the cell culture medium or factors that are directly transmitted by GJIC (AZZAM et al., 1998, 2001, 2002, 2003, 2004; MOTHERSILL and SEYMOUR, 1997, 1998, 2001). It is thought that IR-induced formation of reactive oxygen species (ROS) is the messenger that triggers damage to cellular constituents, including proteins, lipids and DNA. It contributes to the biological effects of both low- or high-LET radiation. Though a burst of excess ROS is initially produced at the time of irradiation and is believed to persist for only microseconds or less (SPITZ et al., 2004), radiation-induced oxidative stress on the cell may be prolonged due to persistent long-term effects on oxidative metabolism (AZZAM et al., 2001, 2004). Exposure to IR may affect mitochondrial and membrane oxidases (Burdon, 1996) leading to excess ROS production, and may also disrupt antioxidant activity. ROS produced at the time of irradiation or subsequently as a result of perturbations in oxidative metabolism modulates the expression of signaling pathways in irradiated cells, regulates intercellular communication, including GJIC, as well as cell growth, differentiation and apoptosis (HARRIS and LOCKE, 2008; UPHAM and TROSKO, 2009). However, the mechanisms by which intercellular communication and oxidative metabolism contribute to low- or high-LET radiations
have not been clearly elucidated. A better understanding of the mechanisms of radiobiological effects of low- or high-LET radiation on human cells and tissues is of significance to radiation therapy and radiation protection.

I.1. Physics of radiation biology

I.1.1. Ionizing radiation

Energetic particles or electromagnetic ionizing radiations (IR) transfer their energy when they interact with matter, thus causing ionization (i.e., emission of electron from atom) or excitation (i.e., an interaction that transfer energy, but does not completely displace an electron). Examples of electromagnetic radiation are X or γ rays. The latter rays consist of a spectrum of waves, like other electromagnetic radiations that are non-ionizing such as radio waves, microwaves, infrared, visible light, and ultraviolet light. However, X and γ rays are distinctly characterized by their short wavelengths, high frequency, and high energy.

Particulate ionizing radiations, on the other hand, include energetic electrons, protons, α particles, neutrons, and heavy charged ions. Like X and γ rays, particulate radiations induce significant biological effects when they traverse living matter. However, depending on the specific physical characteristics, such as energy and mass, of each type of particulate radiation, the concentration of induced biochemical effects in the traversed matter varies due to unique patterns of energy deposition and ionization events. Unlike the sparse ionization events produced by X rays, γ rays, and highly energetic electrons, certain charged particles such as α particles produce dense ionization columns along the particle path. This is due to the vast difference (~8,000 fold) in the charge-to-mass ratio of α particles and electrons. Therefore, heavy charged
elements mainly cause clustered DNA damage that results in DNA double strand breaks.

I.1.2. Linear energy transfer

Radiation quality or linear energy transfer (LET) is a term used to describe the different ionization densities produced by IR along the track of the irradiating particle. LET is a measure of the ionization density, and the LET concept is defined as the energy transferred per unit length of particle track, in keV/μm. Typically, X or γ rays are considered low LET (sparsely ionization) radiations, while energetic neutrons, protons and heavy charged particles are high LET (densely ionization) radiations (reviewed in HALL and GIACCIA, 2006). Note that the demarcation value between low- and high-LET radiations is at about 10 keV/μm (PODGORSAK, 2005).

Exposure to IR results in the deposition of energy events that lead to DNA damage. The level of DNA damage is believed to increase with increasing LET values of the radiation. Condensed energy deposition results in clusters of ionizing events. Consequently, these ionization clusters can yield numerous lesions in DNA and the site of such lesion is termed clustered DNA damage. High LET radiation is believed to produce high yield of such damage (NIKJOO et al., 2001; reviewed in PODGORSAK, 2005; HALL and GIACCIA, 2006; LEHNERT 2007).

Track structure depends greatly on LET. A high LET radiation such as α particles will have a thicker and shorter particle track compared to that of low LET radiations such as protons at the same LET (Fig. 1). Another issue is the secondary electrons (δ rays). Often, the energy deposited in the medium by high LET radiations such as carbon, neon and iron ions is not considered in calculation of LET. However, δ
rays are of low LET radiation and have a torturous track path. Therefore, in reality, certain high LET radiations will have combined high- and low-LET radiation components to its tracks (CUCINOTTA et al., 1998; MUROYA et al., 2006).

Figure 1. Projections over the XY plane of track segments calculated (at ~10^{-13} s) for (a) \(^1\text{H}^+\) (0.15 MeV), (b) \(^4\text{He}^{2+}\) (1.75 MeV/nucleon), (c) \(^{12}\text{C}^{6+}\) (25.5 MeV/nucleon), and (d) \(^{20}\text{Ne}^{10+}\) (97.5 MeV/nucleon) impacting ions. Ions are generated at the origin and along the Y axis in liquid water under identical LET conditions (~70 keV/\mu m). Dots represent the energy deposited at points where an interaction occurred. From MUROYA et al. (2006).

I.1.3. Relative biological effectiveness

The concept of relative biological effectiveness (RBE) has evolved because of the availability of different types of IR that produce a different degree of damage. This is due to the fact that the LET for each type of radiation is different. RBE is the ratio of the doses of low and high LET radiations that would give the same radiobiological effect. RBE is dependent on LET, dose, dose rate and the biological endpoint investigated. Generally, RBE increases with LET, mainly due to track structure. High
LET radiation is more effective at inducing biological damage that is less repairable than low LET radiation. In general, the RBE of radiation increases with its LET up to a value of about 100 keV/μm and above this value starts to decline due to energy deposition in excess of that needed to cause the biological effect (over kill) (Fig. 2). At an LET of 100 keV/μm, IR can most efficiently produce double-strand breaks by a single track (reviewed in HALL and GIACCIA, 2006; LEHNERT 2007).

Figure 2. Diagram illustrating why ionizing radiation with a LET of 100 keV/μm has the greater RBE for cell killing, mutagenesis, or oncogenic transformation. From HALL and GIACCIA (2006).

1.2. Biological effects of ionizing radiation

1.2.1. The actions of ionizing radiation on DNA

When cells are exposed to IR, the induced biological effects result mainly from damage to the DNA, which is considered to be the most critical target molecule within a cell. The damage to DNA can be inflicted by two processes: direct action and
indirect action of radiation (Fig. 3). Due to their unique inherent physical properties and energy deposition patterns, particulate radiations cause biological changes mainly by *directly* damaging critical targets in the cells like DNA. Alternately, electromagnetic radiation interacts with other atoms or molecules in the cell, especially water to produce free radicals (*e.g.* hydroxyl, superoxide radicals) and other reactive species that go on to damage critical targets in the vicinity; therefore, they cause cellular damage largely by an *indirect* manner (reviewed in HALL and GIACCIA, 2006; LEHNERT 2007). Ultimately, these direct and indirect effects of IR produce biological and physiological alterations in the cell or organism that manifest in seconds to even decades after irradiation. This thesis will further explore the mechanisms underlying the biological effects electromagnetic and particulate radiations in human cells.

Figure 3. Mode of action of radiation on a cell. In direct action, an electron resulting from absorption of an incident photon enters the nucleus and ionizes or excites the DNA. In indirect action, the ejected electron interacts with water to produce an OH radical, which diffuses to and reacts with the DNA. From SELMAN (1983).
1.2.2. DNA damage and biological effects

The most frequent types of DNA damage produced are base and sugar modifications, single- and double-strand breaks, sites of base loss, tandem lesions, DNA-DNA and DNA-protein crosslinks, and various clustered lesions. The so-called "locally multiply damaged sites" (also termed "clusters of damaged sites" or more conveniently "clustered lesions") refer to the formation of two or more lesions in close proximity along the DNA (including different combinations of all possible singly damaged sites produced in opposite strands or in the same strand within about 10-20 base pairs separation) by a single radiation track (WARD, 1988; GOODHEAD, 1994; BOUDAÏFFA et al., 2000). These clustered types of lesions, whose complexity depends on the LET of the radiation, result from the spatial heterogeneity of the energy deposition events that follow the passage of radiation through matter.

Whereas high LET radiation can cause clusters and dense DNA damages and the direct action is dominant process, the indirect action is dominants for low LET radiation (about two third of the damages) which can cause only sparse DNA damages. Moreover, high LET radiation leads to damage with higher complexity, which can be extremely hazardous to the cells involved. Both high and low LET radiations cause dose-dependent lesions in DNA (Table 1), including single strand breaks (SSB) and double strand breaks (DSB). As this thesis and other studies demonstrate, for the same total absorbed dose, high LET radiation is more damaging to cells than low LET radiation.

Among the DNA lesions, the DSB is the most serious and potentially the most lethal. The DNA SSB are of lesser biological significance as they can be repaired easily and accurately using an opposite template strand. Cells that experience DNA SSB can thus carry out normal cellular functions following DNA repair. In contrast,
DNA DSB is complex and requires a cohort of DNA repair proteins and a multitude of signalling events. The DSBs are mainly repaired by two processes. Homologous recombination (HR) which is error-free but occurs in S and G2 phase of the cell cycle, or non-homologous end joining (NHEJ) which is the common mechanism for rejoining DNA DSB in mammalian cells and occurs at all phases of the cell cycle; however, it is error-prone (BURMA and CHEN, 2004; BURMA et al., 2006).

Failure of cells to repair DNA damage correctly may contribute to mutagenesis and/or genome instability that can lead to carcinogenesis, aging, inherited disease, and cell death (see, for example: BECKER and SEVELLA, 1993; BREEN and MURPHY, 1995; CADET et al., 1997; WALLACE, 1998; SUTHERLAND et al., 2000; O’NEILL, 2001; HALL and HEI, 2006; VON SONNTAG, 2006).

**Table 1.** Estimation of the number of early physical and biochemical changes that occur when mammalian cells are irradiated with 1 Gy of low LET radiation. From GOODHEAD (1984).

<table>
<thead>
<tr>
<th>Initial physical damage</th>
<th>Select biological damage</th>
<th>Selected cellular effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionization in the cell nucleus</td>
<td>~1000,000</td>
<td>Lethal events</td>
</tr>
<tr>
<td>Ionization directly in DNA</td>
<td>~2000</td>
<td>Damaged bases</td>
</tr>
<tr>
<td>Excitation directly in DNA</td>
<td>~2000</td>
<td>Damaged sugar</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA-protein cross-links</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA SSB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA DSB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromosome aberrations</td>
</tr>
</tbody>
</table>
I.2.3. Cellular responses to DNA damage

As described above, IR induces many types of DNA lesions, of which DNA DSB are the most deleterious. The spectrum of DNA lesions is recognized by DNA-damage-response pathways. As shown in Figure 4, cells respond to DSB through the action of systems that detect the DNA lesion and then trigger various downstream events. These systems can be viewed as classical signal-transduction cascades in which a 'signal' (DNA damage) is detected by a 'sensor' (DNA damage binding protein) that then triggers the activation of a 'transducer' system (protein kinase cascade), which amplifies and diversifies the signal by targeting a series of downstream 'effectors' of the DNA-damage response. This DNA-damage response pathway is extremely sensitive and selective; it is triggered rapidly and efficiently by a low number of and maybe just one DNA DSB, and must remain inactive under other conditions (KASTAN et al., 1991; reviewed in LEHNERT, 2007; JACKSON and BARTEK, 2009).

Figure 4. DNA damage response pathway. DSB are recognized by different sensor proteins, which transmit the signal to a series of downstream effectors molecules through a transduction cascade, to activate signalling mechanisms for cell-cycle arrest and induction of repair, or cell death if the damage is irreparable. From LEHNERT (2007).
The early activation and recruitment to the damage sites of the protein kinase Ataxia-Telangiectasia Mutated (ATM) play a major role in signalling the DSB response pathways in mammalian cells. In response to DNA damage ATM kinase is rapidly auto-phosphorylated and in turn phosphorylates various substrates involved in cell cycle regulation or maintenance of genomic instability (SHILOH, 2003; KASTAN and BARTEK, 2004; LAVIN et al., 2005). ATM is most renowned for its regulation of DSB-induced cell cycle arrests that include G1/S, intra G2, and G2/M arrests (SHILOH, 2001; KURZ et al., 2004). ATM also contributes to the regulation of apoptosis in response to DSB (BROWN et al., 2009). ATM deficiency leads to the human cancer predisposition and neurodegenerative syndrome Ataxia Telangiectasia (AT). At the cellular level, ATM deficiency is manifested by increased sensitivity to IR (BARZILIAI et al., 2002; reviewed in HALL and GIACCIA, 2006).

Inactive ATM exists as a dimer that undergoes autophosphorylation on serine 1981 in a response to DSB to become an active monomer (BAKKENIST et al., 2003). Recent data suggest that ATM is recruited and activated at sites of DSB (ANDEGEKO et al., 2001). Activated ATM is also intimately linked with numerous substrates (Fig. 5). Downstream substrates for ATM include Murine double minute-2 (MDM2), Tumour suppressor gene 53 (TP53 or p53), two serine/threonine protein kinase (CHK1 or CHK2), Breast cancer type 1 susceptibility protein (BRCA1), Nibrin (NBS1), nonrecepter protein tyrosine kinases (c-Abl) and Stress-active protein kinases (SAPK), which are involved in cell cycle progression, DNA repair or apoptosis. Collectively, these proteins function as key regulator of the DNA damage response and a clear interdependency exist among them as inactivation of any renders cells hypersensitive to DSB (DE TOLEDO et al., 2000, PETRINI et al., 2003; SEDELNIKOVA et al., 2003; SHILOH, 2003; reviewed in LEHNERT, 2007).
Currently, there is substantial evidence that ATM acts upstream of p53 in a signal transduction pathway initiated by IR. ATM has intrinsic protein kinase activity and phosphorylates p53 at serine-15 in response to DNA damage (BANIN et al., 1998; CANMAN et al., 1998). Furthermore, ATM, p53 and a p53 downstream effector, the cyclin-dependent kinase inhibitor 1A (CDKN1A), known also as p21\textsuperscript{Waf1}, have been implicated in the cell cycle arrest (G1) that occurs in human fibroblast cells exposed to low or high LET radiations (DE TOLEDO et al., 2000; AZZAM et al. 2000). However, a comprehensive characterization of these effects in cells exposed to high LET radiation has not been as established. It may be dependent on many factors, such as cell line, types of IR, and dose. For example, relative to low LET X, human TK6 lymphoblastoid cells exposed to iron ions (LET \textsim 1000 keV/\mu m) expressed substantially greater inhibition of S-phase progression as a result of higher frequency clustered DNA damage blocking DNA replication (GOTO et al., 2002). In this project, we use ATM/p53/p21\textsuperscript{Waf1} signaling events to characterize the propagation of stress among irradiated cells and between irradiated and neighboring non-irradiated bystander cells.
Figure 5. ATM is activated in response to DSB and signals the presence of DNA damage by phosphorylating downstream targets including p53. Downstream effectors of p53 are p21/clin1 and 14-3-3σ. P21 inhibits the activity of cdk2/clin E and 14-3-3σ inhibits the activity of cdc2/clin B causing cell-cycle arrest, which is also mediated by activation of Chk1 and Chk2. c-Abl activates SAPK for transcriptional regulation of stress-response gene. Other proteins (BRCA1, NBS1) are involved in DNA repair. From LEHNERT (2007).

I.2.4. Ionizing radiation-induced reactive oxygen species

I.2.4.1. Radiolysis of water

Water is the most predominant molecule in living organisms (about 80% of the mass of a living cell in water) (SELMAN, 1983; reviewed in LEHNERT, 2007), therefore, a major proportion of the radiation energy deposited will be absorbed in cellular water and one must understand the radiolysis of water to understand the early stages in the complicated chain of radiobiological events that follow the passage of radiation and ultimately lead to the observation of a biological response. In this context, IR provokes the decomposition reaction of water producing a variety of ROS or
reactive nitrogen species (RNS) in the cellular environment. These species are likely to be major contributors to the induction of chemical modifications and changes in cells that may subsequently act as triggers of biological damage or signalling effects (MUROYA et al., 2006; MEESUNGNOEN, 2007).

The interaction of IR with water causes ionization and excitation of the water molecules. The sequence of events that occurs chronologically during water radiolysis is usually divided into three characteristic stages: (i) the physical stage (~$10^{-15}$ s), (ii) the physicochemical stage (~$10^{-12}$ s), and (iii) the nonhomogeneous chemical stage (~$10^{-6}$ s) (PLATZMAN, 1958). For example, following exposure to low LET radiations such as X- or γ-rays, the water decompositions are $e_{aq}^-$ (hydrated electron), $H^+$, $OH^-$, $H^+$ (hydrogen atom), 'OH (hydroxyl radical), $H_2$ (molecular hydrogen), $H_2O_2$ (hydrogen peroxide), $HO_2^-/O_2^-$ (hydroperoxyl/superoxide anion radicals, $pK_a = 4.8$) etc., (the time scale of events that occur in the radiolysis of water is shown in Fig. 6). In contrast, under high LET radiations, the general trend is that by increasing the LET of the radiation, the lower the free-radical is (e.g., $e_{aq}^-$, 'OH, and $H^+$) and the higher the molecular (e.g. $H_2$) primary yields are. This behavior is explained by the increased intervention of biradical reactions as the local concentration of radicals along the track of the radiation increases (AUTSAVAPROMPORN et al., 2007; MEESUNGNOEN, 2007).
Figure 6. Time scale of events in the low LET radiolysis of water. From MEESUNGNOEN (2007)
1.2.4.2. Oxidative stress and antioxidant defence

To consider the radiolysis of water, IR leads to the formation of ROS (e.g., \( \cdot OH, H_2O_2, O_2^- \)) or RNS (e.g., \( \cdot NO \)) that are believed to persist for milliseconds or less. A high level of ROS/RNS that might result from disruption in the balance between oxidant production and cellular antioxidant defence produces a state of oxidative stress, which leads to oxidative damage to biomolecules such as DNA, proteins and lipids that contributes to the radiobiological effect of IR. For example (Fig. 7), the oxidation of polyunsaturated fatty acids in membrane induced by ROS is called lipid peroxidation (ALBANESE and DAINIAK, 2003).

Organisms have protective systems against cellular oxidative stress. Central to these systems is endogenous antioxidant enzymes. Superoxide dismutases (Mn-SOD in mitochondria and Cu-Zn-SOD in the cytosol), glutathione peroxidase (GPX) and catalases (CAT) constitute primary enzymatic defence system. Mn-SOD or Cu-Zn-SOD interact with \( O_2^- \) to form \( H_2O_2 \) and \( O_2 \). CAT catalyzes the dismutation of \( H_2O_2 \) to \( H_2O \) and \( O_2 \). GPX catalyze the reduction of \( H_2O_2 \) to \( H_2O \) using glutathione (GSH) as substrate (HALLIWELL and GUTTERIDGE, 1985; RILEY, 1994; LEHNERT, 2007).

There is much evidence to support the concept that exposure to IR results in the formation of ROS/RNS within minutes of exposing cells to ionizing radiation, which causes oxidative damage to biomolecules. For example, in the dose range between 50-400 cGy from \( \gamma \) rays, the amount of ROS/RNS detected per cell increased with dose and was accompanied by a decrease in the level of GSH (MORALES et al., 1998). For high LET \( \alpha \) particles, the intracellular production of ROS/RNS was 50-fold greater than the extracellular production and the effect was inhibited by diphenyleneiodonium (DPI), an inhibitor of flavoproteins, suggesting the involvement of plasma membrane-
bound NADPH oxidase and possible other enzymes (e.g. nitric oxide synthases) containing flavoproteins (NARAYANAN et al., 1997).

Figure 7. Oxidative and antioxidant systems in mammalian cells. Superoxide anion \( (O_2^-) \) is produced in cytosol and mitochondria. Two molecules of \( O_2^- \) rapidly dismutase, either spontaneously or via superoxide dismutases (SOD) to dioxygen and hydrogen peroxide \( (H_2O_2) \), the latter permitting flux of ROS between cellular compartments. \( H_2O_2 \) can be enzymatically metabolized to \( O_2 \) and \( H_2O \) by a number of different enzyme systems or converted to the hydroxyl radical ('OH), which is extremely reactive, via a chemical reaction catalyzed by transition metals. From LEHNERT (2007).

1.2.4.3. Effects of ionizing radiation on the cell membrane

The physicochemical structure of biological membrane makes them peculiarily susceptible to oxidative damage, and consequently a target of IR-generated ROS. In this context, the generation of ROS inducing protein and lipids modifications seems to be most possible mechanism representing an alternative target to DNA in radiation induced cell damage. For a long time, the biological membranes have been considered
as inactive semi-permeable lipid bilayers consisting of amphipathic phospholipids, sterol and proteins allowing the segregation of molecules in different cell compartments. Polyunsaturated lipids of the plasma membrane contain double bonds between some of their carbon atom that are susceptible to attack by 'OH and O₂. These radicals act on polyunsaturated fatty acids and irradiation of plasma membrane lipids in the presence of O₂ results in lipid peroxidation (HALLIWELL and GUTTERIDGE, 1985; LEHNERT, 2007; CORRE et al., 2010).

Lipid peroxidation causes cell damage by its decomposition in breakdown toxic products that are bifunctional aldehydes. These aldehydes can act as bioactive molecules in physiological and/or pathological conditions. They can affect and modulate several cell functions at very low and non toxic concentrations including signals transduction, gene expression, cell proliferation and other cell responses. The most abundant aldehyde that has been identified is 4-Hydroxynoneal (4-HNE). This toxic product has been reported to be involved in cell cycle control, mutagenesis and the regulation of expression of a multitude of gene (FENG et al., 2004; LEONARDUZZI et al., 2004). Furthermore, carbonyl derivatives of proteins are also formed by the interaction of protein amino acid side chains with lipid peroxidation products including 4-HNE. Carbonyl derivatives of proteins constitute suitable biomarkers of ROS-mediated protein oxidation (STADTMAN, 2006). Covalent modifications of proteins due to radiation-induced ROS may serve as an indicator of oxidative stress induced by IR (AUTSAVAPROMPORN et al., 2011 BUONANNO et al., 2011)

I.2.5. Repair of radiation damage

When mammalian cells are exposed to IR, two phenomena have been observed: sublethal damage repair (SLDR) and potentially lethal damage repair (PLDR). In
Figure 8, SLDR or split-dose recovery is characterized by restoration of the shoulder on the survival curve and is measured as the increase in survival when the time interval between two dose fractions from X rays is increased to allow for repair of radiation damage. Compared to low LET X rays, a smaller component of SLDR is associated with high LET neutrons. SLDR is largely completed in about 1-2 h, but it may be longer in the late-responding normal tissues in vivo. (ELKIND and SUTTON, 1960; ELKIND, 1984).

![Figure 8](image)

**Figure 8.** The fraction survival response of 10T1/2 cells, derived from mouse embryo, exposed to equal of dose from X rays or neutron. The X rays were generated by 50 kV set at 1500 rad/min and the dose rate of neutron is 37.8 rad/min (PE; plating efficiency). From ELKIND (1984).

Experiments involving fractionation survival curves determined at various times after a conditioning radiation dose, and repeat doses after intervals long enough
for full repair, as well as experiments with a variety of different types of cells irradiated in vitro and/or in vivo led to the following general conclusions.

- Low LET radiation kills mammalian cells as a result of a damage accumulation process.
- Surviving cells following a low LET exposure are sublethally damaged.
- Cells rendered hypoxic by a change in ambient conditions rapid enough to avoid metabolic consequences due to sustained hypoxia are fully able to repair sublethal damage (ELKIND and SUTTON, 1960).
- Qualitatively, normal cells and tumour cells have similar properties with respects to the accumulation and the repair of sublethal damage.
- A smaller component of sublethal damage accumulation is associated with a high LET radiation, compared to low LET radiation. Accordingly, cells repair less SLD after high LET exposures (NGO, et al., 1979) (e.g. results in Fig. 8).
- The repair of sublethal damage reflects the repair of DNA breaks before they can interact to form lethal chromosome aberrations.

PLDR is characterized by a change in the survival curve slope and is measured by the increase in survival in the post irradiation period as the cells are treated under different conditions such as incubation at suboptimal temperature, in minimal medium, treatment with inhibitors of protein synthesis, or holding in density-inhibition (confluent) state (PHILLIPS, and TOLMACH, 1966; LITTLE, 1973; RAJU et al., 1977). Examples are shown in Figure 9 where cells in stationary phase cultures are held in a particular metabolic state in G₁ that favours the efficient repair of PLD lesion. Conditions which allow the cells to leave this state and progress into S favour fixation of damage and reduced survival (LITTLE, 1973). However, this situation is less clear
with cells exposed to high LET radiation such as α particles. This is often explained by the hypothesis that repair mechanism is less effective or non-functional with lesions generated by densely ionizing radiations. The experiments in this thesis were designed to characterize the mechanisms underlying defective PLDR in human cells exposed to high LET radiations.

The relevance of PLD to radiotherapy became much more obvious when it was shown that it occurs in irradiated human tumours (HAHN et al, 1974). It has been suggested that the radioresistance of certain types of human tumours is linked to their ability to repair PLD; that is, radiosensitive tumours repair PLD inefficiently, but radioresistant tumours have efficient mechanisms to repair PLD (HALL and GIACCIA, 2006).

Figure 9. The fraction survival response of confluent human fibroblast AG1522 cells exposed to γ rays (4 Gy) or α particles (0.85 Gy) and held in confluent at 37°C prior to subculture. From AZZAM et al., (2000).
1.3. Targeted and non-targeted effects of ionizing radiation

This thesis examines targeted and non-targeted effects of IR, and the following is a brief synopsis of the progress that occurred in this field in the past two decades.

1.3.1. Targeted effects

It has been long considered that the important deleterious effects of IR result from the deposition of energy in the cell nucleus causing damage to genomic DNA, the critical radiation-sensitive "volume" or primary target for IR-induced effects. Thus, IR-induced cell death, by mitotic failure and/or apoptosis, is attributed to failure to repair DNA damage. Upon repair of DNA damage, the progeny of an irradiated cell would be expected to be normal, but if the damage is misrepaired or unrepaired, the progeny of irradiated cells would be expected to show IR-induced genetic changes in all descendant cells (i.e., the change is clonal). As malignant transformation is generally regarded as being initiated by a gene mutation or a chromosomal aberration, the initiating lesion for malignant transformation has been similarly attributed to DNA damage in the directly irradiated cells (LEA, 1946; LETT et al., 1961; MARSHELL et al., 1970; LITTLE, 2003; HALL and GIACCIA, 2007; reviewed in LEHNERT, 2007).

The evidence implicating the DNA, as the sensitive target for IR-induced damage is supported by the following:

- The viscosity of DNA in vitro was found to decrease by exposure to X rays, an effect they attributed to a reduction in the molecular weight of the DNA and, by implication, the introduction of DNA strand breaks (TAYLOR et al., 1948).

- The sensitivity of cell nuclei to the lethal effects of X rays were 2-4 times greater than for cytoplasmic irradiation (ORD and DANIELLI, 1956).
In contrast to radioisotopes incorporated into the cytoplasm, radioisotope incorporated into DNA cause cell killing (MUNRO, 1970; WARTERS et al., 1978).

IR-induced DNA damage as the cause of cell death is supported by the finding that incorporation of thymidine, particularly the halogenated pyrimidines into DNA increases radiation sensitivity (SZYBALSKI, 1974).

DNA DSB plays an important role in the production of mutagenic lesions by IR (LEENHOUTS and CHADWICK, 1978; LITTLE, 1991).

Chemical agents that interfere with DNA repair processes affect cell survival following exposure to IR (WALDREN and RASKO, 1978).

The levels of chromatid and chromosomal aberrations following IR correlate well with cell killing (NATARAJAN et al., 1980).

The relative abilities of cells to repair DNA damage relate closely with cell survival (THACKER and WILKINSON, 1995).

I.3.2. Non-targeted effects

Recently, the traditional dogma of radiation biology that IR-induced deposition of energy in the nucleus of an irradiated cell is the sole cause of adverse consequences has been challenged by observations in which effects of IR arise in cells that are not themselves irradiated, but are in the vicinity of irradiated cells. The bystander effect has been considered to refer to the occurrence of biological effects in non-irradiated cells as a result of exposure of other cells to IR (NAGASAWA and LITTLE, 1992; MOTHERSILL and SEYMOUR, 1997, 1998, 2001; MOTHERSILL et al., 2001; AZZAM et al., 1998, 2001, 2002, 2003, 2004; SAWANT et al., 2001; LITTLE et al., 2002; SHAO et al., 2002, 2003a, 2003b, 2008a, 2008b; MITCHELL et al., 2004;
ZHOU et al., 2000a, 2000b, 2002, 2004; HU et al., 2006; PRISE and O'SULLIVAN, 2009). Several protocols have been used to detect IR induced bystander effects: cultures consisting of sparse-or density-inhibited cells were exposed to low fluences of α particles generated from conventional broad-or microbeam irradiators, the transfer of medium from irradiated onto non-irradiated cells, and radiolabeled cells were mixed with non-labeled cells and assembled in multicellular clusters (NAGASAWA and LITTLE, 1992; MOTHERSILL and SEYMOUR, 1997, 1998; AZZAM et al., 1998, 2001; BISHAYEE et al., 1999; SAWANT et al., 2001; HOWELL and BISHAYEE, 2002).

The discovery of bystander effects can be traced back to the early 1950's (PARSON et al., 1954). But, these effects were first identified clearly in 1992 (Fig. 10) when NAGASAWA and LITTLE (1992), found that when only 0.1-1% of the cell population was traversed by low fluences of α particles, 20-40% of the cells in the exposed population had chromosomal damage in the form of sister chromatid exchanges (SCE). These results indicated that the target of genetic damages by α particles was much larger than the nucleus or in fact than the cell itself. These results were subsequently confirmed by others using the same endpoint in human fibroblast cells (DESPANDE et al., 1996). Subsequently, experiment using gene expression as an endpoint have indicated that signals for stressful effects are transmittable from irradiated to bystander cells in response of exposure of cell populations to low fluence α particles irradiation (AZZAM et al., 1998, 2001, 2002, 2003, 2004; LITTLE et al., 2002).

An increasing amount of data from IR-induced bystander effect studies has led to the proposal that damage-inducing signals can be transmitted from irradiated to non-irradiated cells, leading to a variety of biological effects. Bystander responses include SCE formation, gene mutation, micronucleus formation, cell cycle arrest,
chromosomal instability, up-regulation of stress responsive proteins and cell killing (NAGASAWA and LITTLE, 1992; AZZAM et al., 1998, 2001, 2002, 2003, 2004; ZHOU et al., 2000a, 2000b, 2002, 2004; HALL and HEI, 2003). All these biological endpoints are related to genetic damage in bystander cells. However, some responses such as apoptosis or radio-adaptive responses have also been reported to occur in a bystander manner. These highlight the possibility of beneficial bystander effects (IYER and LEHNERT, 2002; COATES et al., 2004; ZHOU et al., 2004; DE TOLEDO and AZZAM, 2006; PRISE and O’SULLIVAN, 2009; RZESZOWAKA-WOLNY, et al., 2009; SJOSTEDT and BEZAK, 2010). Bystander effects have been demonstrated for both low- and high-LET radiations, but they are usually more prominent for high LET radiations, particularly α particles with LET of ~100 keV/μm.

**Figure 10.** Induction of sister chromatid exchanges (SCE) by low doses of α particles. (A) Induction of SCE by irradiation with plutonium-238 α particles. (B) Distribution of frequencies of SCE among individual cells. A, nonirradiated cells; B, 0.31 mGy; C, 2.45 mGy. The hatched bars in the histograms represent α particles induced SCE while
the open bars represent background frequencies of SCE based on the distribution observed in nonirradiated cells. From NAGASAWA and LITTLE (1992).

I.3.3. Mechanisms

The mechanisms underlying the propagation of stressful or protective effects from irradiated to non-irradiated bystander cells are not fully understood. Though, research to date has provided some hints. In particular, some evidence suggests that multiple signal transduction pathways are involved. Gap junction intercellular communication (GJIC), secreted diffusible factors, and oxidative metabolism have been proposed to mediate radiation-induced bystander effects (AZZAM et al., 1998, 2001, 2002, 2003; MOTHERSILL and SEYMOUR, 1997, 1998, 2001; IYER and LEHNERT, 2002; SHAO et al., 2003a, 2003b, 2007; DE TOLEDO and AZZAM, 2006; ZHOU et al., 2000a, 2000b, 2002, 2004). Two major pathways will be briefly described below.

I.3.3.1. Role of intercellular communication

The first one is GJIC, which is a cell-to-cell gap junction's route and requires the irradiated and non-irradiated cells to be in physical contact. Figure 11 shows gap junctions which are dynamic structures that are critical for diverse physiological function (YAMASAKI et al., 1995; YAMASAKI and NAUS, 1996; TROSKO and RUCH, 1998). By allowing direct intercellular transfer of cytoplasmic molecules, they provide a powerful pathway for direct molecular signalling between cells. Each of the ~20 isoform of connexins (Cx) forms channels with distinct permeability properties (HARRIS, 2001). The channels are formed by two apposed subunits called hemichannels or connexons, one contributed by each cell. Each hemichannel is a hexamer of connexin and can be composed of more than one connexin isoform. Though the properties of channels formed by each isoform differ, connexin pores are
generally considered to allow permeation by molecules up to ~1 kDa, well above the size of most second messengers (WILLECKE et al., 2002). Connexin channels have been shown to be highly selective among molecular permeants. The selectivity among cytoplasmic permeants is not simply on the basis of size or charge. Although connexin channels are permeable to second messengers (HARRIS, 2001), different connexins form channels with different selectivities for second messengers (NEESSEN et al., 2000; GOLDBERG et al., 2002; BEDNER et al., 2006). For example, ATP, ADP, AMP, glutamate and glutathione are significantly more permeable through junctional Cx43 than Cx32 channels. On the other hand, adenosine and inositol triphosphate (IP3) are more permeable through Cx32 than through Cx43 channels.

![Figure 11. The structure of a gap junction. From YAMASAKI and NAUS (1996).](image)

Evidence for the involvement of GJIC in propagation of targeted and non-targeted effects has been derived from studies with high- or low-LET radiations. The involvement of GJIC was confirmed by the modulation of stress responsive proteins.
(e.g., p53 and p21\textsuperscript{Waf1}) and the induction of micronucleus formation in GJIC-proficient cells in confluent, density-inhibited cultures of human fibroblasts exposed to low fluences of α particles (AZZAM et al., 1998, 2001, 2002, 2003). Treatment with gap junction inhibitors such as lindane resulted in a marked reduction of cells expressing stress responsive proteins (Fig. 12). These findings indicate that the inhibition of gap junctions by lindane had a significant effect on reduction of these responses, which may have resulted from changes in gap junction permeability and Cx43 expression (GUAN et al., 1995; GUAN and RUCH, 1996; AZZAM et al., 1998; KE et al., 2005). Other studies have also showed that irradiation of even 10% of confluent human-hamster hybrid A\textsubscript{LJ} cells with a single α particle per cell through the nucleus results in a mutant yield similar to that observed when all cells in the population are irradiated. This effect was significantly eliminated by chemical inhibition of gap junction mediated intercellular communication, or when exposed cells expressed a dominant negative Cx43 vector (ZHOU et al., 2001b).

Other experiments identified the importance of the propagation distance of α particle-induced bystander effects. When confluent human skin fibroblasts were exposed to low fluence α particles, the stress-responsive protein p21\textsuperscript{Waf1} was induced in bystander cells within 100 μm from irradiated cells. The mean propagation distance ranged from 20 to 40 μm around the intranuclear α particle impact point, which corresponds to a set of 30 affected cells. Interestingly, the increased level of p21\textsuperscript{Waf1} expression was inhibited by pretreatment with the gap junction inhibitor 18-α-glycyrrhetinic acid (AGA). Therefore, this result indicated that GJIC is a critical contributor to propagation of toxic effect in bystander cells (GAILLARD et al., 2009). Other studies have locally irradiated sections of three-dimensional human skin constructs with helium ions from a microbeam, and 72 h later examined apoptotic and
micronucleated cells at different distances away from the irradiated plane. They found that a significant number of damaged cells were up to 1 mm away from the targeted region (BELYAKOV et al., 2005).

Little is known concerning the stressful signal that may be transferred among irradiated cells or between irradiated and bystander cells via gap junctions. The connexin proteins, which may form the gap junction channels, allow ions, secondary messengers and small molecules to pass freely between cells, and modification of these proteins by phosphorylation can open or close the connexin pores. Whether specific signal molecules are transmitted between cells or the junctions are specifically opened/closed as part of the bystander response needs to be addressed.

![Figure 12](image)

**Figure 12.** Gap junction intercellular communication mediates the radiation-induced stressful effects from irradiated to non-irradiated cells. (A) Western blot analysis of p21$^{\text{Waf1}}$ expression in α particle-irradiated AG1522 human fibroblasts in the presence or absence of gap junction inhibitor (lindane, DDT, or dieldrin). (B) In situ immunofluorescence detection of p21$^{\text{Waf1}}$ expression in non-irradiated lindane-treated and irradiated confluent AG1522 cells exposed to 0.3 Gy α particles in the presence or absence of lindane. From AZZAM et al. (2001).
The second mechanism is through soluble factors secreted from irradiated cells into the culture medium. A large number of studies have shown that bystander responses occur when bystander cells are incubated with culture medium harvested from irradiated cells (MOTHERSILL and SEYMOUR, 1997, 1998, 2001; IYER and LEHNERT, 2000a, 2000b; MOTHERSILL et al., 2001; YANG et al., 2005). Thus, no physical contact between irradiated and bystander cells occurs. The observation of bystander effects under such conditions suggested that cell-to-cell contact via GJIC was not necessarily needed and implied that GJIC was not the only pathway mediating bystander effects. These two pathways, however, are not necessarily exclusive of each other. Moreover, not all cell types can produce bystander signals, and not all cell types would respond to these signals (Table 2).

**Table 2.** Cloning efficiencies for four cell lines that received a 5 Gy dose of irradiation directly or medium from cell cultures irradiated with 5 Gy. From MOTHERSILL and SEYMOUR (1997). (%SF is the survival fraction as a percent of the control plating efficiency, HaCAT; human keratinocytes, PC-3; human prostate carcinoma, MSU-1; human fibroblasts, SW 48; human colon carcinoma, Med.; medium).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control cloning efficiency</th>
<th>%SF 5 Gy (medium from cells)</th>
<th>%SF (medium no cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaCAT</td>
<td>22.5±0.91</td>
<td>14.9±0.71</td>
<td>61.3±4.1</td>
</tr>
<tr>
<td>PC-3</td>
<td>19.9±1.75</td>
<td>11.9±1.21</td>
<td>80.3±5.2</td>
</tr>
<tr>
<td>MSU-1</td>
<td>19.2±0.14</td>
<td>57.1±7.3</td>
<td>123.1±18.4</td>
</tr>
<tr>
<td>SW 48</td>
<td>13.2±1.07</td>
<td>1.8±0.5</td>
<td>10.36±1.3</td>
</tr>
<tr>
<td>MSU med. to</td>
<td>25.8±1.73</td>
<td>-</td>
<td>111.49±5.8</td>
</tr>
<tr>
<td>HaCAT cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSU-1 cells</td>
<td>17.2±0.29</td>
<td>-</td>
<td>1.69±1.45</td>
</tr>
</tbody>
</table>
However, the released factor causing the IR-induced bystander effects has yet to be elucidated. The factors leading to such effects appeared to be released by irradiated cells within the first few hours after exposure. It was suggested that the released factor may be a protein as it was labile when heated but stable when frozen; cytokines or other factors that act to increase intracellular levels of ROS/RNS in bystander cells have been considered as candidates (MOTHERSILL and SEYMOUR, 1997, 2001; IYER and LEHNERT, 2000a, 2000b).

Apoptosis has been reported to be a significant pathway of cell death induced by exposure to bystander factors (LYNG et al., 2000; BELYAKOV et al., 2001). Calcium is an important signalling molecule and changes in intracellular calcium modulate cell functions and can lead to apoptosis (CLAPHAM 1995). Increase in calcium concentration has been shown to cause mitochondria ROS formation and loss in mitochondrial membrane potential in bystander cells recipient of medium from irradiated cells (LYNG et al., 2002). More recent experiments examined the effect of dilution of the irradiated cell conditioned medium (ICCM) on the bystander effect. Results indicated that the effect of ICCM from the different cell lines reached a plateau at different dilutions, which correlated with inherent radiosensitivity of the cells investigated. These finding strongly point to a chemical-mediated activation of a signaling molecules and implicates ROS/RNS in the response (RYAN et al., 2008). These studies suggested a possible link between the bystander response and genomic instability.

More recently, the effect of α particle-irradiated medium on bystander responses was studied by a novel approach utilizing cells plated on either one or both sides of double-mylar dishes. It has been suggested that secreted transforming growth factor-beta 1 (TGF-β1) in the medium of α-particles irradiated cultures may have a role in mediating the bystander response (ZHOU et al., 2002). In contrast to the latter
studies, it was also shown that α-particle irradiation of culture medium devoid of cells caused the generation of SCE-inducing factors; such factors, however, were short-lived (LEHNERT et al., 1997). In both situations, the supernatant from irradiated cells or irradiated medium caused the induction of SCE in non-irradiated cells to the same extent observed with direct α particle-irradiated cells. Interestingly, both of short lived medium-and cell-derived SCE-inducing effects were inhibited by SOD, suggesting that ROS are involved in these responses.

1.3.3.2. Role of oxidative metabolism

It is well established fact that normal oxidative metabolism is key endogenous generator of ROS/RNS (FINKEL, 2000; SPITZ et al., 2000). A disruption of the balance between oxidant production and antioxidant defense results in a state of oxidative stress that can promote several pathological conditions, including degenerative senescence and cancer (FINKEL and HOLBROOK, 2000). Oxidant encompass a variety of chemical species, among which some are highly unstable (e.g., \( \cdot \text{OH} \) and \( \text{O}_2^- \)) and other relatively longer lived and widely diffusible (e.g., \( \text{H}_2\text{O}_2 \) and \( \text{\cdot.NO} \)) (HALLIWELL and GUTTERIDGE, 1985). The endogenous targets of oxidants are diverse and include nucleic acids, proteins, and lipids.

An indication that ROS were involved in the induction of SCE in bystander cells present in cell cultures exposed to very low fluences of α particles was suggested when the bystander response was inhibited by SOD (LEHNERT et al., 1997). Although the exact mechanism of bystander effects remain to be elucidate, a wealth of evidence suggests that ROS are potentially involved in the signal transmission. It is thought that irradiated cells secrete some factors that induce elevation of intercellular ROS in bystander cells. Subsequent study carried out by AZZAM et al., (2002) showed that α particle-induced metabolic ROS is also involved in the activation of
signalling pathways in bystander cells. Interestingly, H$_2$O$_2$ and O$_2^{-}$ formed by metabolic processes as a result of exposing confluent cells to α particles were shown to participate in inducing the stress responsive proteins p53 and mitogen activated protein kinase (MAPK) in bystander cells (Fig. 13).

![Western blot analysis of p21$^{\text{Waf1}}$ expression levels in α particle-irradiated AG1522 human fibroblasts in the presence or absence of antioxidant enzymes. (A) SOD, (B) catalase. From AZZAM et al. (2002).](image)

Further evidence that supports the role of ROS in inducing DNA damage in bystander cells was derived when antioxidant enzymes significantly reduced the excess formation of micronuclei in confluent human fibroblast cells exposed to α particles (AZZAM et al., 2002). A disproportionate increase in the fraction of cells with micronuclei in cultures exposed to 1 or 2 cGy of α particles was significantly reduced when the exposed cultures were perincubated with SOD, catalase or diphenyliodonium (DPI), an inhibitor of flavin-containing oxidase enzymes such as NAD(P)H oxidase. In agreement with a reduction in the frequency of micronucleated cells, treatment with DPI also reduced the accumulation of p53 and p21$^{\text{Waf1}}$ immunoreactive protein in
bystander cells (AZZAM et al., 2002). Other studies have demonstrated that an increased concentration of malonaldehyde (MDA) and a decrease of GPX activity together with changed levels of isoenzyme of SOD, in the mitochondria and cytoplasm of non-irradiated mega-colonies of Me45 melanoma cells grown in the neighbourhood of irradiated colonies (PRZYBYSZEWSKI et al., 2004).

In contrast to the above studies whereby stress responses were observed in non-irradiated cells, a cell growth-related bystander response was observed in cells recipient of supernatant from α particle-irradiated cells (IYER and LEHNERT, 2000). Such a response also led to the upregulation of ROS in bystander cells and was mediated by the redox-activated TGF-β1 cytokine (IYER and LEHNERT, 2000a, 2000b).

Oxidative stress has also been implicated in toxic bystander effects observed in other media transfer experiments (LYNG et al., 2000, 2001; MOTHERSILL et al., 2000). Treatment of irradiated cultures with the antioxidant, L-lactate and L-deprenyl, or with drugs that inhibit collapse of mitochondrial membrane potential, inhibited the cytotoxic effects on non-irradiated cell of conditioned medium from the irradiated cells (MOTHERSILL et al., 2000; LYNG et al., 2001). *NO is another factor that mediated these effects, but the exact mechanism remains unknown. Experiments showed that *NO participation in the medium-mediated bystander effects on cell killing and induction of DNA damage depends on the LET of the radiation (SHAO et al., 2002). Studies on bystander cells neighbouring cells irradiated with α particles found that *NO was a crucial signalling molecule determining the appearance of DSB in bystander cells (HAN et al., 2007). Furthermore, SHAO et al., (2008a, 2008b) showed that both *NO and TGF-β1 may be involved in signalling radiation-induced bystander effects when individual glioblastoma cells were irradiated with α particles and then co-
cultured with non-irradiated cells in the presence of an inhibitor of \(^{15} \text{NO}\) synthase or of anti-transforming growth factor \(\beta 1\) (TGF-\(\beta 1\)) antibody. However the involvement of \(^{15} \text{NO}\) in response to IR is cell type specific and not all glioblastoma cells respond to radiation by accumulation of inducible \(^{15} \text{NO}\) synthase (MATSUMOTO et al., 2000).

In experiments that mimic \textit{in vivo} conditions whereby cells are arranged three dimensionally, Roger Howell's laboratory at the New Jersey Medical School used a multicellular cluster model consisting of mixture of differing ratios of cells with or without tritiated thymidine incorporated into their DNA. In this arrangement, the short range of the \(^3\text{H}\) \(\beta\) particles caused irradiation only of the cells with the label. Howell's experiments revealed greater than expected decrease in survival in the cell mixture than could be accounted for by only the lethal effects of radiation in the labelled cells. The effect could be reduced by treating the cell mixture with a gap junction inhibitor. A hydroxyl radical scavenger did not prevent the effect (BISHAYEE, et al., 1999).

More recent studies showed how the bystander response manifests in complex \textit{in vivo} systems. Mothersill's group at McMaster University, in Canada, showed that gill tissue from X ray-treated trout induced bystander signals in recipient non-irradiated trout tissues. The proteomic changes associated with the bystander effect differed from those associated with direct radiation exposure (SMITH et al., 2007). Another recent work demonstrated oncogenic bystander radiation effects in mouse cerebellum. Authors reported bystander tumour induction in cerebellum of radiosensitive \textit{Pathched-1} (Ptch) heterozygous mice after X rays exposure of the other parts of the body. They also provided evidence supporting the role of gap junction intercellular communication in transmission of bystander signals to the central nervous system (MANCUSO, et al., 2008).
Hypothetical bystander messenger(s)

Numerous studies have attempted to elucidate the nature of signals that elict DNA damage and other effects in bystander cells, but the messengers that propagate stressful effects from irradiated to bystander cells are still not fully identified. Two mechanisms of transmission from irradiated to bystander cells have been proposed as described above. A bystander messenger can be either a soluble factor excreted into the cell culture medium from the irradiated cells or a factor that is directly transmitted by GJIC between hit and non-hit cells (MOTHERSILL and SEYMOUR, 1998, 2001; GROSOVSKY, 1999).

Based on this distinction, it can be speculated that at least two types of bystander messengers might be involved. A primary messenger is emitted by targeted cells; it travels through gap junctions, is likely water soluble and unlikely a protein. One suitable candidate could be long-lived organic radicals capable of transferring through gap junctions. Such radical could have lifetimes of up to 20 hours (KOYAMA, et al., 1998). Among other candidates for GJIC mediated primary bystander messengers are calcium ions (LYNG, et al., 2001), IP3 and cAMP (HARRIS 2001), which are important secondary messengers involved in calcium metabolism.

Secondary bystander messengers should be long-lived, more stable, most likely emitted by activated not directly traversed cells. These might be medium-borne factors such as lipid hydroperoxides (LEHNERT. 1991), ceramide (HAIMOVITZ-FRIEDMAN, et al., 1994), 5-hydroxytryptamine, glycine, nicotine (POON, et al., 2007). Other evidence supports a role of cytokines such as TNF-α (RAMESH, et al., 1996) or Interleukin-1 and Interleukin-8 (KHAN, et al., 1998).

There is a range of possible candidate for bystander effect mediation which are medium borne and could be either primary or secondary messengers. ROS and RNS
that have been proposed as possible signals involved in bystander responses are H₂O₂ and, "NO (MATSUMOTTO et al., 2000; AZZAM et al. 2002, 2003, 2004; SHAO et al., 2008a, 2008b). Furthermore, it is likely that a combination of signalling pathways is involved in mediating bystander responses.


- Multiple pathways likely
- Cell-cell communications through gap junctions and/or secretion of a cytotoxic factor into the medium is thought to be involved in the transduction of the bystander signal. Oxidative metabolism has been shown to be important in both mechanisms.
- The bystander effects appear to be particularly important at low doses.
- TP53 gene functions need not be involved in the process.
- In confluent cultures, gap junction-mediated cell-to-cell communications is predominant. Molecules of a size <1 kDa are likely implicated.
- In sparse cultures, secreted signalling molecules, particularly those that complex with ROS/RNS are involved.
- It is not clear if ROS/RNS are only the initiating signalling event that triggers other downstream, more stable secondary signalling pathways.
- The cell to cell contact is not required to induce bystander responses in non-targeted cells after low LET radiation. Whereas propagation of bystander signal(s) via GJIC is more likely to be induced by high LET radiation.

- Intercellular signalling molecules implicated in these effects are ROS/RNS, long lived radical (e.g. \(^{15}\text{NO}\)), cytokines (e.g. TGF-\(\beta\)1, TGF-\(\alpha\) and Interleukins 1 and 8), small molecules like amino acid and biogenic amines (e.g., 5-hydroxytryptamine, glycine and nicotine).

- Intracellular signalling molecules implicated in these effects: MAPK and their downstream proteins [e.g., nuclear factor kappa-light-chain-enhancer of activated B cells (NF\(\kappa\)B), Raf-1 kinase, extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), p38 mitogen-activated protein kinase], protein kinase C (PKC) isoforms, p53, p21\(^{\text{Waf1}}\), ATM, ATR and DNA-dependent protein kinase (DNA-PK).

- Understanding the mechanisms and signalling pathways induced in bystander cells may lead to novel therapeutic approaches involving targeted radiotherapy regiments. For example, turning on cytotoxic bystander response in tumor cells may improve the efficacy of targeted radiation approaches or combined gene therapy. It is also possible that normal tissues may be protected by turning off certain bystander responses.
I.4. Objectives of the research project

The goal of this research project is to investigate the biological and molecular aspects of two inter-related fundamental processes: The roles and mechanisms of gap junction intercellular communication and oxidative metabolism in determining human cellular responses to IR with different LET properties. Particularly, this study focuses on the communication of signalling events between irradiated cells. Depending on the radiation type, communication of stressful or protective effects among the targeted cells may amplify or mitigate the damage initiated by the physical and chemical changes induced by the initial traversal of cells by IR tracks. The occurrence of such events would significantly affect the response to IR at the tissue/system levels.

I.4.1. Hypothesis

We hypothesize that the structure and permeability properties of gap junction channels determine in an LET-dependent manner, the magnitude and nature of biological effects propagated among irradiated cells, and these events are modulated by endogenous oxidative metabolism.

I.4.2. Experimental strategy

The nature of cells used in experiments is of relevance to understanding mechanisms. We will use confluent, density-inhibited (95-98% in G0/G1 phase) normal human diploid fibroblasts (AG1522) at passage 9-13 that express connexin (Cx) proteins (Cx26, Cx32, and others). We will also use confluent (G1 phase) human adenocarcinoma (HeLa cells) in which specific connexin can be expressed in the absence of endogenous connexins. We have a number of unique stable HeLa cell lines with selective inducible expression of functional Cx26 or Cx32. The expressed connexins form junctional channels that discriminate among communicated signaling
molecules. HeLa cells expressing connexins also form functional channels with AG1522 cells.

Cells will be irradiated by 1 GeV protons (LET ~0.2 keV/μm) and 1 GeV/u iron ions (LET ~151 keV/μm) at the NASA Space Radiation Laboratory (NSRL) radiation facility at Brookhaven National Laboratory (BNL). Exposure to $^{137}$Cs γ rays (LET ~0.9 keV/μm) or 3.2 MeV α particles (LET ~122 keV/μm) will be performed at the New Jersey Medical School Cancer Center.

In this study, we propose to investigate the following aims:

1. To investigate the role of intercellular communication in the propagation of induced biological effects among human cells exposed to different types of IR and to identify specific connexin channel permeabilities that correlate with intercellular propagation of toxic or protective effects.

2. To further elucidate the role of oxidative metabolism in the propagation of radiation-induced effects among human cells exposed to low- or high-LET radiations.
II. Article No. 1

In this chapter, we report our preliminary study regarding the role of intercellular communication and oxidative metabolism in the propagation of toxic or protective effects in human cells exposed to low LET \( \gamma \) rays or high LET \( \alpha \) particles.

This work is presented in the following article, entitled: "The Role of Gap Junction Communication and Oxidative Stress in The Propagation of Toxic Effects among High-Dose \( \alpha \)-Particle-Irradiated Human Cells", by Narongchai Autsavapromporn, Sonia M. de Toledo, John B. Little, Jean-Paul Jsy-Gerin, Andrew L. Harris and Edouard I. Azzam.

This article is published in Radiation Research, Vol. 175, Pages 347-357 (2011).
Abstract

We investigated the roles of gap junction communication and oxidative stress in modulating potentially lethal damage repair in human fibroblast cultures exposed to doses of α particles or γ rays that targeted all cells in the cultures. As expected, α particles were more effective than γ rays at inducing cell killing; further, holding γ-irradiated cells in the confluent state for several hours after irradiation promoted increased survival and decreased chromosomal damage. However, maintaining α particle-irradiated cells in the confluent state for various times prior to subculture resulted in increased rather than decreased lethality, and was associated with persistent DNA damage and increased protein oxidation and lipid peroxidation. Inhibiting gap junction communication with 18-α-glycyrrhetinic acid or by knockdown of connexin43, a constitutive protein of junctional channels in these cells, protected against the toxic effects in α particle-irradiated cell cultures during confluent holding. Upregulation of antioxidant defense, by ectopic overexpression of glutathione peroxidase, protected against cell killing by α particles when cells were analyzed shortly after exposure. However, it did not attenuate the decrease in survival during confluent holding. Together, these findings indicate that the damaging effect of α particles results in oxidative stress, and the toxic effects in the hours following irradiation are amplified by intercellular communication, but the communicated molecule(s) is unlikely to be a substrate of glutathione peroxidase.

Keywords: α particles, Potentially lethal damage repair, Gap junction communication, Connexin43, Oxidative stress, Linear energy transfer.
Réssumé

Nous avons étudié le rôle des jonctions communicantes (jonction de type gap, GJIC) ainsi que le métabolisme oxydatif dans la modulation de la réparation des dommages potentiellement létaux dans des cultures de fibroblastes humains exposées à des doses de particules α ou de rayons γ où toutes les cellules sont touchées. Comme on pouvait s'y attendre, les particules α ont été plus à même d'induire la mort cellulaire que les rayons γ ; de plus, le maintien des cellules à confluence pendant plusieurs heures après irradiation aux rayons γ a favorisé la survie clonogénique tout en diminuant les lésions chromosomiques. Toutefois, le maintien des cellules irradiées par des particules α à l'état de confluence selon différents temps d'incubation avant le repiquage a abouti à l'aggravation de la létalité plutôt qu'à une atténuation, ce qui a été associé à la persistance des dommages de l'ADN ainsi qu'à l'accroissement de l'oxydation des protéines et de la peroxydation lipidique. L'inhibition des jonctions gap par l'acide 18-α-glycyrrhétinique ou la diminution de l'expression de la connexine 43, protéine constitutive des canaux jonctionnels de ces cellules, ont protégé les cultures de cellules à confluence contre les effets toxiques exprimés au cours de la période d'incubation post-irradiation par des particules α. Une sur-régulation des protections anti-oxydantes par une surexpression anormale de la glutathion peroxydase a prévenu la mort cellulaire quand les cellules ont été analysées juste après l'exposition ; cependant cela n'a pas atténué la diminution de la survie clonogénique lorsque les cultures étaient maintenues à confluence. L'ensemble de ces résultats indiquent que les effets néfastes des particules α résultent dans le stress oxydant et que les effets toxiques dans les heures suivant l'irradiation sont amplifiés par la communication intercellulaire, mais la (les) molécule(s) communiquée(s) n'est probablement pas un substrat de la glutathion peroxydase.

Mots-clés : particules α, réparation des dommages potentiellement mortels, communicantes jonction gap, connexine 43, stress oxydatif, transfert d'énergie linéaire
1. Introduction

It has been over four decades since it was shown in cultured human cells that radiation-induced lethal damage can be attenuated by appropriate postirradiation conditions (1). Holding X-irradiated cells in the confluent, density-inhibited state for several hours after irradiation significantly enhanced their survival (2). It has been proposed that the protective effect is due to the repair of potentially lethal damage (PLD) (3). Radiation-induced PLD repair was correlated with a loss of chromosomal aberrations, sister chromatid exchanges, and a decrease in giant cell formation (4-6). Significantly, potentially lethal damage repair (PLDR) was observed, in vivo, in solid tumor cells after X-irradiation (7). Although DNA repair has been implicated in the cellular processes leading to PLDR (8-11), the exact mechanism(s) remain unclear.

Most PLDR studies have been performed in mammalian cells exposed to X or γ rays (12); fewer studies investigated this phenomenon in cells exposed to high linear energy transfer (LET) radiations (13-15). The studies generally revealed lack of increased survival when cultured cells were held in quiescence for various periods of time at 37°C following exposure to α particles or energetic heavy ions (14, 16). High LET radiations induce complex DNA damage and are capable of more efficient cell killing than low LET X and γ rays (17, 18). Although high LET-induced DNA damage can be repaired, albeit with slower kinetics than DNA damage induced by low LET radiation (19, 20), such repair does not promote increased survival during the post-irradiation incubation period (13).

In recent studies, we have observed that incubation of α particle-irradiated normal human cells at 37°C for various times prior to subculture results in decreased clonogenic survival rather than a mere lack of effect (21). A similar decrease in survival during post-irradiation incubation can be also noted in results previously
obtained by Raju et al. (14). These data suggest that mechanisms other than DNA repair, or that can adversely affect DNA repair, may contribute to the observed effect.

Twenty years ago, Trosko and colleagues proposed that the modulation of intercellular communication plays a major role in the response to ionizing radiation (22, 23). Furthermore, they postulated that redox-modulated events and intercellular communication act in concert to modulate radiation-induced changes in signal transduction (24). Consistent with these concepts, there has been substantial evidence from studies of cell cultures exposed to low fluences of α particles for the involvement of gap junction communication and oxidative metabolism in the propagation of stressful effects from irradiated to neighboring non-irradiated bystander cells (reviewed in (25, 26)). Here, we extend these studies and examine the involvement of these mechanisms in the propagation of stressful effects among irradiated cells, which leads to enhanced toxicity in confluent cell cultures exposed to doses of α particles in which every cell in the population is irradiated.

Gap junctions are dynamic structures that are critical for diverse physiological functions (27, 28). The intercellular channels that comprise gap junctions are formed by connexin proteins, and each of the ~20 isoforms of connexin forms channels with distinct permeability properties (27). By allowing direct intercellular transfer of ions and low molecular weight molecules, gap junctions provide a powerful pathway for molecular signaling between cells. Though the properties of channels formed by each isoform differ, in general, connexin pores are considered to allow permeation of small molecules (reviewed in (27)). Significantly, exposure to low- or high-LET radiation up-regulates and stabilizes connexin43, an effect that was associated with functional gap junction intercellular communication (GJIC) (29). Interestingly, several lines of evidence support the concept that junctional communication and oxidative metabolism
are inter-related (30). Redox-modulated transcription factors were shown to activate connexin43 expression in irradiated cells (31).

There is a strong connection between generation of reactive oxygen species (ROS) and damage that follows radiation exposure. Though a burst of excess ROS is initially produced at the time of irradiation and is believed to persist for only microseconds or less (32), radiation-induced cellular oxidative stress may be prolonged due to persistent effects on oxidative metabolism (33). Exposure to ionizing radiation may affect mitochondrial and membrane oxidases (34-36) leading to excess ROS production, and may also disrupt antioxidant activity. In this report, the involvement of oxidative stress and junctional communication in enhancing toxicity in α particle-irradiated human cells is investigated by direct approaches whereby gap junction communication is down-regulated by knock-down of connexin43, a major constitutive protein of junctional channels in skin cells, and antioxidant potential is increased by ectopic over-expression of glutathione peroxidase.

2. Materials and Methods

Cell culture

AG1522 normal human skin fibroblasts were obtained from the Genetics Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Stock cultures were routinely maintained in a 37°C humidified incubator in an atmosphere of 5% CO₂ in air, and cells in passage 10 to 13 were used in experiments. Cells destined for γ-irradiation were seeded in 25 cm² polystyrene flasks, and cells for α particle-irradiation were grown in stainless steel dishes (36 mm internal diameter) with 1.5 μm-thick replaceable polyethylene terephthalate (PET) foil bottoms at a seeding density of ~1.2 \times 10^5 cells/dish. The cells were subsequently fed on days 5, 7 and 9 with Eagle's
minimal essential medium supplemented with 12.5% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Experiments were started 48 h after the last feeding in confluent cultures where 95-98% of the cells were in Go/G1 as determined by autoradiographic measurements of [3H]-thymidine uptake or flow cytometry. Synchronization of cells in Go/G1 by confluent, density inhibition of growth eliminates complications in interpreting the survival results, as radiation sensitivity changes at different phase of the cell cycle (37). Importantly, this protocol maximizes interactions among the cells.

Culturing AG1522 cells that were loaded with calcein-AM together with non-loaded cells on either PET or polystyrene showed that cells grown on these substrates communicate with each other via gap junctions, as was verified by the transfer of calcein dye from the loaded to the unloaded cells and the prevention of the transfer when the cells were incubated with gap junction inhibitors (data not shown).

Irradiations

Cells were exposed to γ rays (LET = 0.9 keV/μm, (38)) from a 137Cs source (J.L. Shepherd Mark I, San Fernando, CA) at a dose rate of 1.3 Gy/min. For irradiation with α particles, cells were exposed at 37°C in a 5% CO2 atmosphere to a 0.0002 Ci 241Am-collimated source housed in a helium-filled Plexiglas box at a dose rate of 2 cGy/min. Irradiation was carried out from below, through the PET base, with α particles with an average energy of 3.2 MeV (LET = 122 keV/μm, (39)) at contact with the cells. The source was fitted with a photographic shutter to allow accurate delivery of the specific radiation dose (40). In all cases, control cells were handled in parallel with cells destined for irradiation but were sham-irradiated.

The absorbed dose received by a single α particle traversal through the cell nucleus (mean nuclear thickness: 1.2 μm (41)), and the percentage of cells traversed
may be calculated using the terminology and methods given by Charlton and Sephton (42). Briefly, the dose per traversal to the thin disk-shaped cell nucleus of the AG1522 cell is \( d = (0.16)(\text{LET})/A \), where \( A \) is the cross-sectional area of the cell nucleus. The units for \( d \), LET, and \( A \) are Gy, keV/\( \mu \text{m} \), and \( \mu \text{m}^2 \), respectively. Considering that the LET of 3.2 MeV \( \alpha \) particle is 122 keV/\( \mu \text{m} \) and the mean nuclear area of an AG1522 cell is 144 \( \mu \text{m}^2 \) (41), the absorbed dose from an \( \alpha \) particle traversal would be 13.5 cGy. Alternatively, a value of \(-17.9\) cGy may also be derived for the absorbed dose from a particle traversal 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 (~193 keV, assuming a range over which the particle stops of 19.9 \( \mu \text{m} \) (35) and a continuous slowing-down of the particle).

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 an \( \alpha \) particle traversal (42). Thus, in an AG1522 confluent culture exposed to mean doses of 10, 50 or 80 cGy from 3.2 MeV \( \alpha \) particles, 50, 86 and 99% of the cells, respectively, would be traversed through the nucleus by an average of one or more particle tracks.

**Cell survival**

To measure PLDR, survival curves were generated with AG1522 cells exposed to \( \gamma \) rays or \( \alpha \) particles by standard colony formation assay. Confluent cell cultures were trypsinized within 5-10 min after irradiation or after various incubation periods at 37\(^\circ\)C. The cells were suspended in growth medium, counted, diluted, and seeded in 100 mm dishes at numbers estimated to give about 150 to 200 clonogenic cells per dish. Four replicates were done for each experimental point, and the experiments were repeated 2 to 5 times. After an incubation of 12 to 14 days, the plates were rinsed with
PBS, fixed in ethanol, stained with crystal violet, and colonies consisting of 50 cells or more were scored under low magnification with an Olympus dissecting microscope. Survival values were corrected for the plating efficiency, which ranged from 20 to 30%.

**Micronucleus formation**

The frequency of micronucleus formation was measured by the cytokinesis block technique (43). After treatments, confluent cells were subcultured, and ~3x10^4 cells were seeded in chamber flasks (Nalgene Nunc, Rochester, NY) in the presence of 2 µg/ml cytochalasin B (Sigma, St. Louis, MO) and incubated at 37°C. After 72 h, the cells were rinsed in PBS, fixed in ethanol, stained with Hoechst 33342 solution (1 µg/ml PBS), and viewed under a fluorescence microscope. At least 1000 cells/treatment were examined, and only micronuclei in binucleated cells were considered for analysis. At the concentration used, cytochalasin B was not toxic to AG1522 cells.

**Western blot analyses**

Following treatments, the cells were harvested by trypsinization, pelleted, rinsed in PBS, repelleted, and lysed in chilled radio-immune precipitation assay (RIPA) buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 50 mM NaF, 5mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with protease inhibitor cocktail (Sigma) and sodium orthovanadate (1 mM). The extracted proteins were fractionated by SDS-PAGE and submitted to immunoblotting. Anti-phospho-TP53 (serine 15) (no. 9284) from Cell Signaling (Boston, MA), anti-p21^waf1 (no. 05-345) and anti-4-hydroxynonenal (4-HNE) (no. LV1462895) from Millipore (Billerica, MA) and anti-connexin43 (no. c6219) from Sigma were used in the analyses. Secondary antibodies conjugated with horseradish peroxidase and the enhanced chemiluminescences systems from GE Healthcare (Piscataway, NJ) were used for
protein detection. Luminescence was determined by exposure to X ray film, and densitometry analysis was performed with an EPSON scanner and National Institutes of Health Image J software (NIH Research Services Branch, Bethesda, MD). Staining of the nitrocellulose membranes with Ponceau S (Sigma) or reaction of goat anti-rabbit immunoglobulin G (sc 2030, Santa Cruz Biotechnology, Santa Cruz, CA) with a protein of ~30 kD was used to verify equal loading of samples (loading control).

Inhibition of gap junction communication

18-α-glycyrrhetinic acid (AGA) (Sigma), a reversible inhibitor of gap junction communication was dissolved in DMSO and added to cell cultures at a concentration of 50 μM at 30 min prior to irradiation. The cells were incubated in the presence of the drug until they were harvested 1, 3 or 5 h later. Under this protocol, AGA did not alter the plating efficiency of unirradiated cells, but inhibited cell coupling. Control cell cultures were incubated with the dissolving vehicle.

GJA1 small interfering RNA silencing

A pool of 4 siRNAs capable of targeting gja1 mRNA that codes for connexin43 (Cx43) was from Thermo Scientific Dharmacon (Lafayette, CO) (ON-TARGETplus SMARTpool siRNA J-011042-05, J-011042-06, J-011042-07, and J-011042-08). Scrambled siRNA Duplex was included as control. Briefly, 10^5 cells suspended in 75 μL electroporation buffer were transfected with Cx43-siRNA at a concentration of 50 pM by electroporation in 0.1 cm electrode gap cuvettes using a Gene Pulser Xcell™ system (Bio-Rad, Hercules, CA). The cells received two 900 V pulses of 0.07 ms duration with 5 s intervals between the pulses. A total of 0.5 x 10^6 cells per experiment were transfected. Following transfection, the cells were diluted in growth medium and treatments were performed 72 h later when the cells were confluent and the level of connexin43 was decreased by 85.3 ± 1.5% as verified by Western blot analyses.
Vectors and cell transduction with glutathione peroxidase

Replication-defective recombinant adenovirus type 5 with the E1 region substituted with the human genes encoding glutathione peroxidase (AdGPX) was obtained from ViraQuest (North Liberty, IA). The infectious units of the adenovirus were typically at $1 \times 10^{10}$ PFU/ml. At the time of infection, the growth medium was replaced with serum-free fresh medium, adenovirus was added to a multiplicity of infection (MOI) of 100, and cells were incubated for 24 h. They were then fed fresh medium and were used for experiments 24 h later. Total glutathione peroxidase activity was measured by the spectrophotometric method of Lawrence and Burk (44) using cumene hydroperoxide as the substrate. Typically, GPX activity was increased by ~3-fold in AG1522 cells transduced with AdGPX. Cells transduced with empty vector served as control.

Protein oxidation

Protein carbonyl levels, an index of protein oxidation (45), were determined by immunoblotting using the oxyblot assay kit from Millipore (Temecula, CA). Briefly, samples containing 20 µg protein extracted from whole cell lysates were derivatized with 2,4-dini-trophenylhydrazine (DNPH) to the corresponding 2,4-dinitrophenylhydrazone (DNP). DNPH-derivatized protein samples were separated by SDS-PAGE, blotted onto nitrocellulose membranes, reacted with anti-dinitrophenylhydrazone antibody and visualized by standard immune technique.

Statistical analysis

The statistical significance in measurements of the fraction of micronucleated cells was determined using Chi-square analysis. Statistical analyses of clonogenic survival measurements were carried out using Student's $t$ test. A $p$ value of less than
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.

3. Results

Potentially lethal damage repair in γ and α particle-irradiated human cells and its correlation with induced DNA damage and prolonged oxidative stress

Most studies of PLDR following α particle irradiation have been performed in rodent or transformed human cells. Here, we used AG1522 human diploid fibroblasts in the confluent state to maximize cell-cell interactions, and compared, in parallel studies, the extent of PLDR in these cells following exposure to graded doses from 3.2 MeV α particles (LET ~122 keV/μm) or 137Cs γ rays (LET ~0.9 keV/μm). The cells were trypsinized to examine clonogenic survival within 5-10 min after exposure or after a 3 h incubation period at 37°C. As expected, the data in Figures 1A and 1B show that α particles are more effective per unit dose than γ rays at inducing cell killing. Whereas a dose of 80 cGy from α particles reduced survival to the 10% level when cells were assayed shortly after exposure, a dose of 4 Gy from γ rays yielded the same effect showing that the relative biological effectiveness (RBE) of α particles compared to γ rays under those conditions is ~5, which is consistent with previous findings (40, 46). When γ-irradiated cells were assayed for clonogenic survival following 3 h incubation, a significant increase in survival was observed at all the doses tested, indicating the occurrence of PLDR (Fig. 1A). In contrast, a decrease rather than increase in survival was observed, in parallel experiments, when cells were held in confluence for 3 h following α particle-irradiation (Fig. 1B). The results therefore
show that during the incubation period, radiation-induced toxic effects were enhanced rather than attenuated. Relative to γ rays, the RBE of α particles, calculated at the 10% survival level, was ~12.5 when cells were assayed for survival 3 h after irradiation.

Similar to clonogenic survival (Figs. 1A and 1B), when AG1522 cell populations were γ-irradiated (1, 4 or 8 Gy) and held in confluence for 3 h prior to subculture to assay for DNA damage in the form of micronuclei, a significant decrease ($p<0.05$) in the fraction of micronucleated cells was observed when compared to the results obtained in cell populations that were sub-cultured shortly after exposure (Fig. 1C). In contrast, following α particle irradiation (10, 50 or 80 cGy), relative to cell cultures assayed shortly after exposure, there was a slight increase rather than decrease in the fraction of micronucleated cells when cell cultures were held in confluence for 3 h (Fig. 1D), consistent with previous findings (13). The greatest increase ($p < 0.05$) occurred following exposure to the low mean dose of 10 cGy at which only 50% of cells in the exposed culture are traversed by a particle track.

We also examined, by Western blot analyses, the phosphorylation of serine 15 in TP53, a marker of DNA damage (47), in irradiated cells that were harvested within 5-10 min or 3 h after exposure. Whereas P-TP53 (serine 15) level was decreased by 3-fold following a 3 h incubation of cells exposed to 4 Gy from γ rays, it was decreased by 2-fold in cells exposed to an isosurvival dose of 80 cGy from α particles (Fig. 1E). Relative to γ-irradiated cells, these data indicate a greater level of persistent DNA damage in α particle-irradiated cells held in confluence for 3 h; this damage may be expressed in forms other than micronuclei.

Consistent with the enhanced toxicity expressed during the 3 h incubation period after α particle irradiation (Figs. 1B and 1D), an increase in protein carbonylation and lipid peroxidation was also observed (Figure 2). This increase
reflects enhanced oxidative stress that likely results from excess ROS generation caused by perturbed oxidative metabolism. The representative data in Figure 2 show 2-3-fold increases in carbonylation and 4-hydroxynonenal (HNE)-modification in certain proteins in α particle-irradiated cells during confluent holding.

Role of GJIC in propagation of stressful effects among α particle irradiated cells

To gain insight into the mechanism(s) underlying the enhanced toxicity during the incubation period following α particle irradiation (Fig. 1B), we investigated whether intercellular communication among irradiated cells is involved in the observed enhancement in lethal effects. To this end, confluent AG1522 cells were exposed to 80 cGy from α particles in the presence or absence of the gap-junction inhibitor AGA. Parallel cultures were exposed to 4 Gy from γ rays that results in isosurvival level and treated similarly with AGA as the α particle-irradiated cultures. The drug (50 μM) was added 30 min prior to irradiation and remained until the cells were harvested for the clonogenic survival assay either shortly (5-10 min) after exposure or after 1 to 5 h incubation periods. At 50 μM, AGA effectively inhibited, in AG1522 cells, the transfer through gap junctions of calcein in co-culture studies (data not shown) or Lucifer Yellow (48) as verified by the scrape-loading and dye transfer assay (49); it resulted in no or slight toxicity. Whereas treatment with AGA, did not significantly affect survival of γ-irradiated cells during the post-exposure incubation periods, it prevented the decrease in survival that is observed in control α particle-irradiated cells (Figs. 3A and 3B). In the presence of AGA, survival of α particle-irradiated cells held in confluence for 1, 3 or 5 h prior to subculture was similar to survival of cells assayed shortly after irradiation.

Consistent with the above finding, the fraction of micronucleated cells was decreased in confluent cultures exposed in the presence of AGA to 80 cGy from α
particles, and held in quiescence in the presence of the drug for periods of time up to 3 h ($p < 0.01$) (Fig. 3D). In contrast, AGA did not alter micronucleus formation in γ-irradiated cells that were assayed shortly or 3 h after irradiation. Together, the data in Figure 3 support the involvement of GJIC in the propagation, specifically among α particle-irradiated cells, of induced stressful events. They suggest that molecules with differential effects, or different amounts of the same molecule(s), may be propagated via gap junctions among cells exposed to γ rays or to α particles, respectively.

To investigate the role of GJIC in the propagation of stress among α particle-irradiated cells by a more direct approach, we used AG1522 cells in which the expression of connexin43 was decreased by siRNA. Compared to scrambled siRNA-treated cells (Scr), transfection with connexin43 siRNA (Cx43-siRNA) reduced the level of the protein by ~85% (Fig. 4A) in 72 h after transfection, a time when the experiments were performed and the cells were confluent. The morphology, cloning efficiency and colony size distribution of Scr and Cx43-siRNA-transfected AG 1522 unirradiated cells were similar (data not shown). However, upon exposure to 80 cGy from α particles, the induction of p21wafl, a downstream effector of the DNA damage and stress responsive protein p53 (50), was attenuated in Cx43-siRNA-irradiated cells (1.5-fold versus 2.3 fold increase in Scr cells) (Fig. 4A), suggesting reduced overall stress in the exposed cells. Importantly, compared to Scr cells, incubation at 37°C for 3 h post-exposure to 80 cGy, resulted in ~ 22% increase ($p < 0.0001$) in clonogenic survival in cells from cultures treated with Cx43-siRNA (Fig. 4B), which correlated with a decrease ($p < 0.03$) in the fraction of micronucleated cells (Fig. 4C) and down-regulation of p21wafl (Fig. 4A). These data strongly support the involvement of connexin43-mediated intercellular communication in the propagation of stressful effects among α particle-irradiated cells.
Oxidative metabolism and the collective response of normal human cells to α particle-irradiation

Several studies have shown that oxidative metabolism participates in short and long term effects of ionizing radiation (33, 51). Notably, it mediates the propagation of stressful effects from α particle-irradiated to neighboring bystander cells (52, 53). Here, we investigated whether it also mediates the propagation of stress among irradiated cells that incur major oxidative stress from α particle traversal (32). To this end, we measured clonogenic survival and micronucleus formation in high dose-irradiated confluent AG1522 cells where glutathione peroxidase (GPX) had been ectopically overexpressed and in their respective controls. The GPX enzyme converts hydrogen peroxide (H₂O₂), a product of dismutation of superoxide radicals by the superoxide dismutases, to water (54). Though a burst of excess ROS is initially produced at the time of irradiation and is believed to persist for only microseconds or less (33), radiation-induced oxidative stress on cells may be prolonged due to persistent long-term effects on oxidative metabolism. To assess the role of metabolically generated ROS in the cellular response to radiation, we harvested cells for analyses following 3 h incubation after irradiation, a time during which propagation of signaling molecules leading to greater toxicity among irradiated cells occurs, and compared the results with effects measured within 5-10 min after exposure.

Similar to the decrease in survival observed in control cells exposed to α particles and held in confluence for 3 h (Fig. 1B), the data in Figure 5A show that exposure to 80 cGy followed by 3 h incubation of cells transduced with empty vector also results in reduced survival when compared to cells assayed within minutes after irradiation. Cells transduced with a vector expressing GPX were more radioresistant (p < 0.0005) than empty vector-transduced cells that were assayed within minutes after
irradiation, indicating that oxidizing species contribute to the lethal effects of α particle-irradiation. However, unlike the inhibition of connexin43-mediated GJIC, over-expression of GPX did not alleviate the decrease in clonogenic survival that occurred in control cells during the post-irradiation incubation period (Fig. 5A). These data therefore suggest that the molecule(s) communicated among high dose-irradiated cells enhance toxicity in these cells (Fig. 2), but themselves may not be oxidizing species that GPX acts upon. Alternatively, the oxidative stress induced by 80 cGy from α particles may saturate antioxidant defenses, as most cells in the exposed cultures would be traversed on average by ~6 particles. The specific energy deposited in the directly hit area is expected to be very large (55) and would result in an absorbed dose of ~13 to 18 cGy in an irradiated AG1522 cell (42), which causes massive oxidative ionization events that the overexpressed GPX could not entirely ameliorate. Thus, residual long-lived and long-range reactive species (56, 57) may still be able to diffuse through junctional channels to enhance cell death.

The data in Figure 5B (average of 4 experiments) describe the effect of overexpressed GPX on micronucleus formation in α particle-irradiated cells held in confluence for 5-10 min or 3 h prior to subculture. They show that the decrease in survival observed during confluent holding of empty vector-transduced cells exposed to 80 cGy (Fig. 5A) does not correlate with an increase in micronucleus formation, which is similar to the data described in Fig. 1D. In AdGPX transduced cells, the frequency of micronucleated cells in cultures exposed to 80 cGy and assayed shortly after irradiation was significantly lower than in empty vector-transduced cells exposed to the same dose of radiation indicating radioprotection. Confluent holding of these cells for 3 h after irradiation resulted in slight decrease in micronucleus formation.
4. Discussion

Characterizing biological effects in cells exposed to different types of ionizing radiation and understanding the underlying mechanisms is relevant not only to issues in radiotherapy and radiation protection, but also to basic knowledge of the cellular responses to stress, particularly oxidizing and clastogenic stresses. Extensive data have shown that the deposition of radiation energy into cells can cause damage to all cellular macromolecules and, depending on dose, could result in serious injury to the traversed cells (58). However, cells employ various strategies for detecting damage and repairing it (59). Holding cells in the confluent density-inhibited state after irradiation, or maintaining them in growth factors-depleted medium was shown to influence the fraction of cells that survive the irradiation because of the repair of PLD (2, 3). Although PLDR has been extensively studied over decades, the molecular and biochemical events mediating its expression remain incompletely understood, particularly for cells exposed to high LET radiations. Such studies would have important implications to radiotherapy, as high LET α and high charge/high energy particles are being increasingly used in cancer treatment (60, 61). Understanding the biological effects that occur shortly or a few hours after exposure to such particles may help potentiate their therapeutic efficacy and clarify the associated risks to irradiated, or bystander, normal tissues adjacent to the tumor target. Furthermore, the results of this study, although using high doses of radiation, are pertinent to our understanding of signaling events mediating low dose effects that are relevant in radiation protection, as humans may be exposed to significant doses of α or high charge and high energy particles during specialized activities such as mining and or prolonged space travel, respectively.
By using human fibroblasts exposed to γ rays, a low LET radiation, or α particles, a high LET radiation, we have shown that holding α particle-exposed cells in the confluent state for several hours after irradiation, results in decreased (Fig. 1B) rather than the increased cell viability that occurs in γ-irradiated cells (Fig. 1A). After 3 h of confluent holding, α particle-irradiation was over 12 times more effective than γ-irradiation at inducing cell killing (Fig. 1); in contrast, when survival is measured shortly after irradiation, an RBE of 5 is deduced at the 10% survival level. Significantly, our data indicate that gap junction communication mediates the propagation of events that lead to the increased toxic effects seen with α particle irradiation. Treatment of cells with a gap junction inhibitor (Fig. 3B) attenuated the enhanced lethal effect: When cells were irradiated and held in confluence in the presence of 18-α-glycyrrhetinic acid, a sparing of the enhanced toxicity was observed, and survival was similar to that measured shortly after irradiation (Fig. 3B). Clonogenic survival was not however increased as in γ-irradiated cells that were held in confluence after irradiation. The sparing effect was associated with a decrease in micronucleus formation (Fig. 3D). Interestingly, the decrease in the fraction of micronucleated cells was observed in cell populations that were sub-cultured for the assay shortly after exposure, suggesting that the gap junction-mediated propagation of events leading to increased lethality in α particle-irradiated cell cultures occurs rapidly after exposure. In contrast, treatment of γ-irradiated cells with AGA resulted in no remarkable effect.

As chemical inhibitors may not be necessarily specific in their effect, we investigated the role of GJIC in the propagation of lethal effects among α particle-irradiated cells by more direct approach. When cells transfected with Cx43-siRNA were exposed to an 80 cGy lethal dose of α particles and held in confluence for 3 h,
clonogenic survival was increased (22 ± 0.7%, \( p < 0.0001 \)) when compared with scrambled siRNA-transfected cells (Fig. 4B), and was associated with a decrease in micronucleus formation (Fig. 4C). Likely, the signaling molecules propagated through gap junctions act to induce lethality in cells in the exposed population that are traversed by a small number of tracks that fail to kill the cell when survival is measured shortly after irradiation. The deposition of energy from particulate radiation is known to occur in a non-uniform pattern (reviewed in (62)), and in AG1522 fibroblast cultures exposed to 80 cGy, ~1.6, 4.8, 9.5 and 14% of the cells would be traversed on average by 1, 2, 3 or 4 particle tracks, respectively (42). The communicated molecules may have induced processes that led to greater killing in these cells. In this context, it would be of interest to know how many \( \alpha \) particle traversals would kill an AG1522 cell. Together, our data are consistent with those of Jensen and Glazer (63) that showed greater cell killing by cisplatin in high density cell cultures consisting of gap junction proficient cells. They extend our previous findings and those of others showing that GJIC is an important mechanism that mediates the propagation of stressful effects from irradiated to non-irradiated cells in low fluence \( \alpha \) particle-irradiated cultures (64-66). Interestingly, relative to cells assayed shortly after irradiation, the data in Figure 1D show that a significant increase in micronucleus formation following a 3 h holding period occurred in cells from cultures exposed to an \( \alpha \) particle dose of 10 cGy in which 50% of the cells in the exposed population are bystanders.

The propagation of toxic effects among high dose \( \alpha \) particle-irradiated cells would be of significance in radio-immunotherapy with antibodies conjugated to \( \alpha \) particle emitters (67). Although loss of GJIC is widely regarded to correlate with tumorigenic phenotypes, there are exceptions. Specifically, substantial evidence indicates that increased level of connexin expression and of GJIC are correlated with
invasiveness, extravasation and metastasis in a variety of cancer cells. It has also been noted that primary tumors that are initially GJIC impaired become GJIC competent at the metastatic stage (68, 69). Thus, in those situations whereby tumors are treated by radio-immunotherapy with α particle emitters, GJIC may potentiate killing of both targeted and non-targeted cells in the tumor. Although the potentiating effect on cell killing observed in this study is small (Fig. 1B), the cumulative effect in therapeutic regimens involving repetitive administration of α particle emitters would become significant. For tumor cells with reduced GJIC, development of drugs and methods that can recover or increase GJIC may provide a new and potent way to enhance treatment of these tumors with high LET radiations. Thus, enhancement of GJIC by chemotherapeutic agents in tumor cells, coupled with radiotherapy using α particles, and the associated transmission of toxic compounds between cells in the irradiated tumor, would offer a therapeutic gain. By corollary, transmission of toxic effects from irradiated to neighboring normal bystander cells would pose a health risk if affected normal bystander cells undergo genetic changes but yet survive and become prone to neoplastic transformation.

In addition to the role of GJIC in enhancing the toxic effects of high fluence α particles, we investigated whether the increase in oxidative stress detected 3 h after irradiation (Fig. 2) contributes to the observed increase in cell killing (Fig. 1B). To this end, we measured clonogenic survival in α particle-irradiated cells in which the antioxidant GPX was ectopically over-expressed. Similar to the enhanced toxicity described in Figure 1B, holding empty vector-transduced cells in the confluent state for 3 h after exposure to a mean dose of 80 cGy resulted in significant decrease in survival (Fig. 5A). Ectopic overexpression of GPX significantly attenuated cell killing measured shortly after irradiation indicating that oxidative stress contributes to cell
killing in α particle-irradiated cells. In this context, it is of interest to note that the yield of H$_2$O$_2$ in irradiated cells is thought to increase with increasing LET (70). Thus, by more efficiently scavenging H$_2$O$_2$ in α particle-irradiated cells, overexpressed GPX would protect against chemical changes to cellular macromolecules that would be caused by H$_2$O$_2$ or by hydroxyl and superoxide radicals that result from its dissociation by the Haber-Weiss reaction (71). Interestingly however, holding GPX-transduced cells for 3h after α particle irradiation did not increase survival or decrease micronucleus formation over what was observed when cells were assayed shortly after irradiation (Figs. 4A and 4B). The latter results suggest that death-inducing or clastogenic factors other than, or in addition to, oxidizing species may be directly communicated through gap junctions to enhance cell killing of irradiated cells that would otherwise survive. Signaling events that lead to activation of nucleases may be involved.

Although the increase in lipid peroxidation and protein carbonylation observed in our studies during confluent holding of α particle-irradiated cells (Fig. 2) may be caused by excess ROS generated from an effect of the irradiation on oxidative metabolism, ROS generated at the time of the irradiation may have contributed to the effect. Whereas ~60 ROS per nanogram of tissue were estimated to be generated from a hit caused by $^{137}$Cs γ rays (67, 68) (i.e., ~10.4 ROS per cell nucleus, using a nuclear mass of ~173 pg, thus corresponding to a yield of about 1 ROS/100 eV), we can estimate that over 2000 ROS are generated from an α particle traversal, corresponding to a ROS concentration in the nucleus of ~19 nM. Such a ROS concentration can obviously cause extensive oxidative damage. The data in Figure 2, show an increase in 4-HNE adducts in proteins occurring within minutes after irradiation. Regardless, the net result is enhancement of cell killing that may be due to an effect of protein
carbonylation and lipid peroxidation on organelle structure and function (e.g. plasma membrane) (72) as well as DNA repair proteins and their accessories (73). Oxidative damage to proteins may render them prone to segregation and degradation. In this context, it is noteworthy that carbonylation is unreparable (74).

5. Conclusion

This study highlights the importance of radiation quality in the propagation of stressful effects among irradiated confluent cells. It reflects the advantages of using high LET radiotherapy in cancer treatment whenever appropriate. Enhancement of cell death by GJIC significantly contributes to the high RBE of α particles. Identifying the propagated factors that promote the death of irradiated cells would have obvious translational applications and would increase our understanding of radiation-induced signaling pathways. In addition, this study supports the importance of modifying biological factors and the time after irradiation at which the effect of dose and LET in the biological responses to ionizing radiation is evaluated. The latter parameters may greatly affect the biological effectiveness of a test radiation relative to γ rays.

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Figure 1: Potentially lethal damage repair in confluent AG1522 cells exposed to \(^{137}\)Cs \(\gamma\) rays or 3.2 MeV \(\alpha\) particles. Panel A: Clonogenic survival of AG1522 cells exposed to increasing doses of \(\gamma\) rays and assayed for survival within 5-10 min (●) or 3 h (■) after exposure. Panel B: Clonogenic survival of AG1522 cells exposed to increasing dose of \(\alpha\) particles and assayed for survival within 5-10 min (●) or 3 h (■) after exposure. Panel C: Fraction of micronucleated cells in control or \(\gamma\)-irradiated cultures held in confluence for various times after exposure. Panel D: Fraction of micronucleated cells in control or \(\alpha\) particle-irradiated cultures held in confluence for various times after exposure. Panel E: Western blot analyses of the phosphorylation of serine 15 in TP53 in \(\gamma\)-and \(\alpha\) particle-irradiated cells held in confluence for 3 h at 37°C after exposure to 4 Gy from \(\gamma\) rays or 80 cGy from \(\alpha\) particles. (*: \(p < 0.05\); ***: \(p < 0.0002\))
**Figure 2:** Oxidative stress in α particle-irradiated AG1522 cells. Confluent cells were exposed to 0 or 80 cGy; protein oxidation detected by quantifying carbonylation in modified proteins (*Panel A*), and lipid peroxidation measured through detection of 4-hydroxynonenal adducts (*Panel B*) were examined, by SDS-PAGE followed by immunoblotting in cells held in confluence at 37°C for 5-10 min or 3 h after exposure. Relative intensity refers to fold changes in carbonylation and 4-HNE adduct accumulation in proteins highlighted with an arrow.
Figure 3: Role of gap junction intercellular communication in the propagation of stressful effects among α particle-irradiated confluent cells: Effects of the gap junction inhibitor 18-α-glycyrrhetinic acid (AGA). Panel A: Clonogenic survival of AG1522 cells exposed to 0 or 4 Gy from γ rays in the presence (■) or absence (●) of AGA. The irradiated cell populations were subcultured to assay for survival within 5-10 min or after various holding periods at 37°C. Panel B: Clonogenic survival of AG1522 cells exposed to 0 or 80 cGy from α particles in the presence (■) or absence (●) of AGA. The exposed confluent cell populations were subcultured to assay for survival within 5-10 min or after various holding periods at 37°C. Panel C: Fraction of micronucleated cells in control or γ-irradiated cultures, and Panel D: Fraction of micronucleated cells in control or α particle-irradiated cultures treated as in Panels A or B, respectively. (**: p < 0.01; ***: p < 0.0003)
Figure 4: The effect of connexin43 knockdown in the propagation of stressful effects among α particle-irradiated cells. AG1522 fibroblasts were transfected with scrambled siRNA (Scr) or connexin43-siRNA (Cx43-siRNA); they were exposed to 80 cGy from α particles in the confluent state and harvested for analyses following 3 h incubation at 37°C. Panel A: Western blot analyses of connexin43 (Cx43) and p21wafl (CDKN1A) expression (The relative intensity was normalized against the respective loading control). Panel B: Clonogenic survival. Panel C: Micronucleus formation. (*: p < 0.03; ***: p < 0.0001)
Figure 5: The role of oxidative metabolism in the propagation of α particle-induced stressful effects. AG1522 cells were transduced with glutathione peroxidase (GPX) or empty adenovirus vector, exposed to 0 or 80 cGy from α particles that was followed by 5-10 min or 3 h incubation at 37°C. Panel A: Clonogenic survival. Panel B: Micronucleus formation. (*: p < 0.05; ***: p < 0.0005)
References


III. Article No. 2

To further confirm insight into the role of gap junction intercellular communication in the modulation of cell killing, induction of DNA damage and repair of potentially lethal damage, we extend here our study presented in Chapter II to several other types of space radiation such as protons and iron ions.

The complete work is reported in the following article, entitled: "Intercellular Communication Amplifies Stressful Effects in High-Charge, High-Energy (HZE) Particle-Irradiated Human Cells", by Narongchai Autsavapromporn, Sonia M. de Toledo, Manuela Buonanno, Jean-Paul Jay-Gerin, Andrew L. Harris and Edouard I. Azzam.

This article is in press in Journal Radiation Research (Japan).
Abstract

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 repair of 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-glycyrrhretinic 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 radiation. The results also show that GJIC among irradiated cells amplifies stressful effects following high, but not LET radiation, and that GJIC has only minimal effect on cellular recovery following low LET radiation.

Keywords: Gap junction intercellular communication / Potentially lethal damage repair / DNA damage / Linear energy transfer
Résumé

Comprendre les mécanismes qui sont à la base des effets biologiques des radiations particulaires est essentiel pour l'exploration spatiale et pour la radiothérapie. Nous avons étudié le rôle des jonctions GAP (GJIC) dans la modulation des effets nocifs induits dans les cultures confluentes dans lesquelles la plupart des cellules ont été traversées par une ou plusieurs particules. Nous nous sommes concentrés sur l'effet de la qualité du rayonnement (transfert d'énergie linéaire; TEL) sur la propagation des dommages de l'ADN et la mort cellulaire parmi les cellules irradiées. Des cultures confluentes de fibroblastes humains normaux ont été exposées à des doses graduées de protons d'énergie 1 GeV/u (TEL≈0,2 keV/μm), ou d'ions fer d'énergie 1 GeV/u (TEL≈151 keV/μm) afin d'étudier la survie clonogénique ainsi que la formation de micronoyaux, reflet de dommages de l'ADN, peu de temps après l'irradiation et après des périodes d'incubation plus longues. Les ions de fer ont été environ 2,7 fois plus efficaces que les protons à tuer 90% des cellules dans les cultures exposées lorsque le test de survie clonogénique a été initié dans les 5-10 minutes suivant l'irradiation. Lorsque les cellules ont été maintenues à confluence pendant plusieurs heures après l'irradiation, une réparation substantielle des dommages potentiellement létaux (PLDR) couplée avec une diminution de la formation de micronoyaux, a été observée dans les cellules exposées aux protons, mais celles exposées aux ions fer. En effet, le fait de maintenir les cellules à confluence après exposition à une dose similaire toxique d'ions de fer a accentué les effets toxiques induits. Cependant, l'inhibition des jonctions gap par l'acide 18-α-glycyrrhetinique a éliminé la toxicité accrue et la formation de micronoyaux a été réduite à des niveaux inférieurs à ceux détectés dans les cellules dosées peu de temps après l'irradiation. Les données montrent que le rayonnement à faible TEL induit de fortes PLDR en quelques heures, tandis que le rayonnement à TEL élevé, avec une toxicité immédiate semblable, ne provoque pas de PLDR et la toxicité augmente avec le temps après irradiation. Ces résultats suggèrent que les effets du stress induits par des radiations de haut TEL, et non de bas TEL, sont amplifiés par les communications intercellulaires parmi les cellules irradiées et que les GJIC ont un effet minime sur la récupération cellulaire après irradiation à faible TEL.
Mots-clés : communicantes jonction gap / réparation des dommages potentiellement mortels / dommages de l'ADN / transfert d'énergie linéaire
1. 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 \(\gamma\) rays are well separated in space, those produced by heavy charged particles occur in dense columns along the particle 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 \(\alpha\) 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.\(^8\text{-}^9\) 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.\(^11\text{-}^{13}\)

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.\(^14\text{-}^{15}\) Gap junctions linking contiguous cells were shown, by direct approaches, to mediate the propagation of stress between a 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.

2. 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. The cells express connexin proteins and are proficient in gap junction communication.

Irradiation

Confluent, density-inhibited AG1522 cells were cultured for experiments as previously described 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). Description of the facility and radiation beam information can be found at [http://www.bnl.gov/medical/nasa/LTSF.asp](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/\mu m for 1 GeV/u $^{56}$Fe$^{26+}$-irradiation and 0.2 keV/\mu m 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.

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

The fraction of cells 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, \( \sim 44, 77 \) 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 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. 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. Briefly, irradiated confluent cell populations and their respective controls were subcultured, ~3×10⁴ cells were seeded in chamber flasks (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, 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.
Comparisons between treatment groups and respective controls were performed using the Pearson's $\chi^2$-test. A $p$ value of $\leq 0.05$ between groups was considered significant.

**Inhibition of Gap Junction Communication**

18-$\alpha$-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 $\mu$M, 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.

3. **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 $\sim$0.2 keV/$\mu$m) or 1 GeV/u iron ions (LET $\sim$151 keV/nm)]. 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. 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 $\gamma$ 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 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 develops 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. 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. 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 Figure 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 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. 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.

Acknowledgment

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Figure 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 1 GeV/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)
Figure 2: Role of gap junction intercellular communication in the propagation of stressful effects among energetic proton or iron ion-irradiated confluent AG1522 cell cultures. 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. (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$)
Table 1: Dosimetry parameter for confluent AG1522 cells irradiated with 1 GeV protons or 1 GeV/u iron ions

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References


In this chapter, we investigate the effect of the permeability of specific connexins in the propagation of stressful or protective effect in human cancer cells exposed to different types of ionizing radiation whose LET varies from 0.2 to 151 keV/μm.

This work is presented in the following article, entitled: “Permeability of Connexin Channels Mediates Human Cells Response to Ionizing Radiation”, by Narongchai Autsavapromporn, Sonia M. de Toledo, Jean-Paul Jay-Gerin, Andrèw L. Harris and Edouard I. Azzam.

This article will submit to Journal of Radiation Research (in preparation).
Abstract

Direct intercellular communication between irradiated cells, is mediated by gap junction channels, which consists of connexin (Cx) proteins. Herein, the role of selective permeability of different connexin channels in irradiated cells has received considerable interest and recently we found that the involvement of Cx43 in the propagation of toxic effects in α-particle-irradiated cells. However, the exact contribution of other types of connexins still has not been elucidated. Here, we investigated the effects of specific connexin channel permeabilities in radiation-induced cell killing and induction of DNA damage with several biological endpoints such as clonogenic survival, metabolic activity and micronucleus formation. We used human adenocarcinoma (HeLa) cells in which specific connexins can be express in the absence of endogenous connexins. When exposed to protons, γ rays, α particles and iron ions at isosurvival dose levels, HeLa cells that express functional Cx26 junctionals were more sensitized from the effects of ionizing radiation than HeLa cells expressing Cx32. These results support that Cx26 channels mediated the propagation of toxic effects among irradiated cells; Cx32 channels conferred the protective effects. These findings open novel concepts that connexins would have translational implications in radiotherapy and the formulation of countermeasures against the toxic effects of ionizing radiation.

Keywords: Gap junction intercellular communication/ Connexin26 / Connexin32 / Linear energy transfer
Résumé

La communication intercellulaire directe entre les cellules irradiées est médiée par les jonctions communicantes (jonctions gap) qui se compose de connexines (Cx). Ici, le rôle de la perméabilité sélective de différents canaux dans les cellules irradiées a reçu un intérêt considérable et récemment, nous avons constaté la participation de la connexine 43 dans la propagation d'effets toxiques sur les cellules irradiées par des particules α. Toutefois, la contribution exacte des autres types de connexine n'a toujours pas été éclaircie. Ici, nous avons étudié les effets de la perméabilité spécifique de certaines connexine dans la mort cellulaire radio-induite et l'induction de dommages à l'ADN avec plusieurs paramètres biologiques tels que la survie clonogénique, l'activité métabolique et la formation de micronoyaux. Nous avons utilisé une lignée cellulaire humaine dérivée d'un adénocarcinome du col utérin (HeLa) dans lesquels les connexines spécifiques peuvent être exprimées en absence de connexines endogènes. Lorsqu'elles sont exposées à des protons, des rayons γ ou des ions de fer et à des doses comparables en terme de survie cellulaire, les cellules HeLa exprimant les canaux fonctionnels formés de Cx26 étaient plus sensibles aux effets des rayonnements ionisants que les cellules HeLa exprimant les canaux formés de Cx32. Ces résultats appuient le fait que les canaux formés de connexines 26 facilitent la propagation des effets toxiques parmi les cellules irradiées tandis que les canaux formés de connexines 32 confèrent, quant à eux, des effets protecteurs. Ces résultats ouvrent de nouvelles perspectives où les connexines pourraient avoir des implications en radiothérapie ainsi que la formulation de contre-mesure contre les effets toxiques des radiations ionisantes.

Mots-clés : communicantes jonction gap / connexine26 / connexine32 / transfert d'énergie linéaire
1. Introduction

Recent studies have indicated that the involvement of gap junction intercellular communication (GJIC), mediated by the connexin (Cx) proteins is involved in the propagation of stressful effects in among irradiated and between irradiated and unirradiated bystander cells varies with the linear energy transfer (LET) radiation or radiation quality. Various biological effects such as the cell killing, induction of DNA damage, up-regulation of stress-responsive proteins and mutation were shown to occur both in irradiated and unirradiated bystander cells. However, the involvement of these mechanisms in the propagation of signaling events among irradiated cells has not been investigated. Whereas the signals generated by irradiated cells that lead to cell death between irradiated cells would be beneficial, the communication of toxic or protective signaling molecules among irradiated cells will help in tailoring more effective novel strategies and treatment modalities for cancer therapy. Although the transmission of stressful or protective effects through gap junction channels mediated direct cell-to-cell communication among irradiated cells remains unknown. Whether the persistence of such effects may depend on the permeability of specific connexin channels.

Gap junctions are dynamic structures and intercellular membrane channels that are critical for diverse physiological function such as in the regulation of cell homeostasis, proliferation and death. By allowing direct exchange of small molecules (~1 kDa) and ions, well above the size of most secondary messengers between adjacent cells. They are comprised of two hemichannels or connexons, which are in turn formed by the oligomerization of six proteins subunits, termed connexins. To date, the connexin gene family consist of 21 members in human genome that are expressed in different tissues and have different selectivity related to the size and charge of
communicated molecules. For example, ATP, ADP, AMP, glutamate and glutathione are significantly more permeable through junctional Cx43 than Cx32 channels. On the other hand, adenosine and inositol trisphosphate (IP$_3$) are more permeable through Cx32 than Cx43 channels. This process of exchange of signaling molecules between neighbouring cells through gap junction proteins is termed gap junction intercellular communication.

Furthermore, GJIC plays a critical role in cellular response to ionizing radiation (IR). Previously, we found that Cx43-associated GJIC and oxidative metabolism have a significant role in mediating radiation-induced stressful effects in human cells exposed to low fluences of high LET $\alpha$ particles. Consistence with other investigators shows that the expression of Cx43 levels have been implicated in the cellular response to low LET $\gamma$ rays. Therefore, it is important to understand how a biological response from different connexin channels is induced by low- or high-LET radiation and how the effect is processed in cells. However, there are limited experimental data for the role of gap junction permeability in human cells exposed to low- or high-LET radiations.

In the present study, we further elucidate the role of specific connexin channel permeabilities that correlate with intercellular propagation of IR-induced effects in cultured cells. Specifically, we will identify the type of gap junction channels that correlates with the propagation of stressful or protective effects in HeLa cells exposed to low- or high-LET radiation. Clonogenic survival, micronucleus formation and metabolic activity were investigated in control or irradiated HeLa cells expressing Cx26 or Cx32 and their respective controls, exposed to energetic protons, $\gamma$ rays, $\alpha$ particles, or iron ions. Hence understanding of the communication of signaling events are modulated and transmitted by specific connexin proteins between irradiated cells.
will open possibilities for the clinical application in the treatment of cancer and may contribute to radiation protection.

2. Materials and Methods

Cell Culture

Human adenocarcinoma (HeLa) cells in which specific connexin can be expressed in the absence of endogenous connexin were kindly provided by Professor Andrew L. Harris at the UMDNJ - New Jersey Medical School (Newark, NJ). We have a number of unique stable HeLa cell lines with selective inducible expression of functional Cx26 or Cx32. The expressed connexins from junctional channels that discriminate among communicated signaling molecules.\(^{18}\) Cells destined for energetic protons, \(\gamma\) rays or iron ions were plated in 25-mm polystyrene flasks, and cells for \(\alpha\) particle-irradiation were grown in 36-mm stainless steel dishes with 1.5 \(\mu\)m-thick replaceable mylar bottoms at a seeding density of about \(\sim 1.5 \times 10^5\) cells/dish.\(^{19-20}\) The expression of Cx26 and Cx32 in HeLa cells upon incubation with 1 \(\mu\)g/mL doxycycline (BD Biosciences, San Jose, CA) on days 3 with Dulbecco’s Modification of Eagle’s medium with 4.5g/L glucose, without L-glutamine and sodium pyruvate (CellGro, Manassas, VA) supplemented with 5% Tet system Approved fetal bovine serum (BD Biosciences, San Jose, CA), 200 \(\mu\)g/mL Hygromycin B (Invitrogen, Carlsbad, CA), and 100 \(\mu\)g/mL Geneticin (GIBCO, Carlsbad, CA). The cells were subsequently reseeded on days 4. Experiments were started 24 h after last feeding. The cells were maintained in a 37°C humidified incubator in an atmosphere of 5% CO\(_2\) in air. Control cells were sham-treated and handled in parallel with the test cells.
Irradiation Condition

Cells were exposed to γ rays (LET ~0.9 keV/μm from $^{137}$Cs source at 1.3 Gy/min) (J.L. Shepherd Mark I, San Fernando, CA). For α particle-irradiation, cells were exposed at 37°C to a 0.0002 Ci $^{241}$Am-collimated source housed a helium-filled plexiglass box at a dose rate of 2 cGy/min. Irradiation was carried out from below, through the mylar base, with α particles with an average energy of 3.2 MeV (LET ~122 keV/μm). The source was fitted with a photographic shutter to allow accurate delivery 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 down to the 2500 μm$^3$ level. The fraction of cells whose nucleus was actually traversed by an α particle was derived from Poisson statistics and estimate involving cell geometry, α particles fluence and energy loss. For energetic protons and HZE particles, cells were exposed to graded doses from 1 GeV protons or 1 GeV/u iron ions at the NASA Space Radiation Laboratory (NSRL, Brookhaven National Laboratory, Upton NY). The Physics Dosimetry group at NSRL carried out the dosimetry and provided the information about the ions beam characteristics (http://www.bnl.gov/medical/nasa/LTSF.asp). Samples were placed in the plateau region of the Bragg curve and irradiated at room temperature. The exposure times for proton-or iron ion-irradiation varied from 1 to 10 min depending on the dose. The culture flasks were positioned perpendicular to the beam such as 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 measured to be ~151 keV/μm for 1 GeV/u iron ion-irradiation and ~0.2 keV/μm for proton-irradiation. The flasks were filled to capacity with growth medium that was pH-and temperature-equilibrated, 3 to 6 h before the radiation exposure. 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 associated with a prolonged change from the normal horizontal culture position to the vertical position of vessels containing a small volume of medium.\textsuperscript{21} In all cases, control cells were handled in parallel with cells destined for irradiation but were sham-irradiated.

The absorbed dose received by a single traversal of α particle or iron ion through the cell nucleus and the percentage of cells traversed by the α particle or iron ion can be calculated. The absorbed dose received as a result of a single particle traversal through a radius of 10 μm of the cell nucleus of HeLa cell\textsuperscript{22}, and the percentage of cells traversed in an exposed culture, may be calculated using the terminology and methods given by Charlton and Sephton.\textsuperscript{23} Briefly, the dose per traversal to the thin disk-shaped cell nucleus of the HeLa cell is

\[ d = \frac{0.16 \times \text{LET}}{A}, \]

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 protons, γ rays, α particles or iron ions are ~0.2, 0.9, 122 and 151 keV/μm, respectively, and the mean nuclear area of an HeLa cell is 314 μm\(^2\),\textsuperscript{22} the absorbed dose from a proton, γ ray, α particle or an iron ion traversal would be ~0.01, 0.04, 6.21 and 7.69 cGy, respectively.

The fraction of cells \( f \) receiving exactly \( i \) traversals was calculated according to the equation

\[ f = \frac{(D/d)^i \exp(-D/d/i!)}{i!} \]

where \( D \) is the mean dose to the cell population and \( d \) is the dose to an HeLa cell from a proton, γ ray, α particle or an iron ion traversal.\textsuperscript{23} Thus, in an HeLa confluent culture exposed to mean doses of 0.05, 0.1, 0.25, 0.5, 0.8 or 1.0 Gy from α particles, ~55, 80, 98, 100, 100 and 100% of the cells, respectively or in HeLa cultures exposed to mean doses of 0.1, 0.25, 0.5, 1.0 or 2.0 Gy from iron ions, ~73, 96, 100, 100 and 100% would be traversed through the nucleus by an average of one or more particle tracks. In contrast, in confluent cultures exposed to
any doses from protons or $\gamma$ rays, every cell in the population is traversed by an increasing number of tracks, respectively that effect a rather uniform irradiation of the population.

Cell Survival Analysis

Cell survival curves were generated in HeLa cells exposed to energetic protons, $\gamma$ rays, $\alpha$ particles or iron ions by a standard colony formation assay. Confluent (G$_1$ phase) HeLa cells with expression of Cx26 or Cx32 were trypsinized within 5-10 min after exposure at 37°C in 5% CO$_2$ in air atmosphere, and the cells were suspended in 5% Tet system Approved fetal bovine serum and 20% conditioned medium. The cells were counted, diluted, and seeded in 60-mm dishes at numbers estimated to give about 80 to 100 clonogenic cells per dish. Three or four replicates were done for each experiment point, and the experiments were repeated at least twice. After an incubation of 8 to 10 days, the plates were rinsed with phosphate buffered saline (PBS), fixed in ethanol, and stained with crystal violet, and macroscopic clonoies were counted. Survival values were corrected for the plating efficiency, which ranged from 50 to 70%.

Micronucleus Assay

The frequency of micronucleus formation was measured by the cytokinesis block technique. After treatments, cell populations were subcultured and approximately $\sim 3 \times 10^4$ cells were seeded in chamber flaskettes (Nalgene Nunc, Rochester, NY) in the presence of 1.5 $\mu$g/ml cytochalasin B (Sigma, St. Louis, MO) and incubated at 37°C. After 48 h, the cells were rinsed in PBS, fixed in ethanol, stained with Hoechst 33342 solution (1 $\mu$g/ml PBS, Molecular Probes, Eugene, OR), and viewed under a fluorescence microscope. At least 1000 cells/experiment were examined, and only micronuclei in binucleate cells were considered for analysis. At the concentration used, cytochalasin B was not toxic to HeLa cells.
Metabolic Activity (MTT) Assay

Briefly, ~5×10^4 cells/mL were incubated in Dulbecco's Modification of Eagle's medium after exposed to either protons of γ rays with a dose of 0 and 4 Gy, α particles with a dose of 0 and 80 cGy or iron ions with a dose of 0 and 1.5 Gy in standard 24-well plates. Then, 2 mM of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT (Sigma, St. Louis, MO) was added into cells at 72 hours and the cells were further incubated for 4 hours in a 95% humidified incubator at 37°C with 5% CO₂. The MTT reduction is catalyzed by succinate dehydrogenase, a component of complex II of electron transport chain in the inner membrane of mitochondria. The blue crystal product, formazan reflects the energetic stage of mitochondria or refers to viability of cell.²⁵-²⁶ Four hours after cells incubation with MTT, they were harvested and rinsed once with PBS. Then formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The optical density of formazan at 560 nm was determined and represented number of viable cells.

Statistical Analysis

The statistical significance in measurements of the fraction of micronucleated cells was determined using Chi-square analysis. Statistical analyses of clonogenic survival and MTT-reduction activity measurements were carried out using Student's t test. A p value of less than 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.
3. Results

Effect of Selective Permeability of Gap Junction Channels on Radiation Sensitivity: Cell Killing, Induction of DNA Damage and Metabolically Viable Cells

The objective of this study was to investigate whether the involvement of gap junction communication through specific connexin channels modulates the intracellular propagation of toxic or protective effects in human cells exposed to different types of ionizing radiation. To test this hypothesis, HeLa cells expressing Cx26 or Cx32 and their respective controls were exposed to protons, γ rays, α particles and iron ions at isosurvival dose levels and trypsinized within 5-10 min after irradiation and assaying for clonogenic survival, micronucleus formation and metabolically viable cells.

Figure 1 shows the clonogenic survival of HeLa cells expressing Cx26 or Cx32 and their respective control were exposed to protons, γ rays, α particles or iron ions, varies with LET from ~0.2 to 151 keV/μm at isosurvival dose levels. As expected, HeLa cells that express Cx26 was more effective in cell killing than cells expressing Cx32 in all case. In addition to the above, these results indicate the relative biological effectiveness (RBE) was calculated from isoeffective dose levels for a reduction of the surviving fraction to 10% or D$_{10}$ of γ rays. We found that the RBE based on D$_{10}$ of HeLa cells that express Cx26 were approximately 1.1, 4.8 and 3.1 for protons, α particles and iron ions, respectively. In contrast with HeLa cells expressing Cx32 were approximately 0.8, 4.6 and 2.7 for protons, α particles and iron ions, respectively. Interestingly, the relative fractions of survival cells for all cell line types that exposed to α particles exhibited the expected higher RBE than iron ions.

We also investigated the chromosomal damage as determined by micronucleus formation in HeLa cells expressing Cx26 or Cx32 and their respective control were exposed to protons, γ rays, α particles or iron ions. Results presented in Figure 2
indicate that HeLa cells that express Cx26 had a higher percentage of binucleated cells with micronuclei at isosurvival dose levels in all types of IR, compared to HeLa cells expressing Cx32 and their respective control. Together, our results indicated that an increase of DNA damage (Fig. 2) is consistent with the decrease in survival (Fig. 1) observed in all of IR and cell line types we used. These data strongly suggest that the persistence of DNA damage had a high rate of micronucleus formation, reflecting their inability to repair DNA double-strand breaks (DSBs) and consistent with the results from colony formation.

We next asked whether the change of metabolic activity via MTT assay in HeLa cells that express different of connexins would exhibit cellular sensitivity to low- or high-LET radiation. Thus HeLa cells expressing Cx26 or Cx32 and their respective control were irradiated to different types of IR at doses that result in isosurvival levels and MTT assay was performed after 72 h. Unirradiated cells were taken as respective control. We found that either low- or high-LET radiation, HeLa cells that express Cx32 junctional channels had a higher percentage of cell viability, compared with cells expressing Cx26 proteins (Figs. 4A-D). As expected, HeLa cells that express functional Cx26 junctional channels show a significantly \( p < 0.0001 \) enhanced sensitivity than HeLa cells expressing Cx32. They confirm that the selective permeability of gap junction plays a prominent role in the modulation of radiation sensitivity in human cells exposed to low- or high-LET radiations.

4. Discussion

It has been reported that direct cell-to-cell communication through connexin channels mediated IR-induced damaging effect in irradiated cells and between irradiated and non-irradiated bystander cells cultures exposed to low- or high-LET
radiations.\textsuperscript{1-7, 15-17} This outcome is in line with our recent study, showing strongly support that the toxic molecules in human cells exposed to $\alpha$ particles may spread through Cx43 to modulate the cellular response.\textsuperscript{15-16} However, very little known about the biological effects of other types of connexin in cells exposed to different types of IR. In this present study, we demonstrate the presence of selective permeability of different connexin channels in HeLa cells exposed to low- or high-LET radiations and we investigate the role of Cx26 or Cx32 and its channels in the propagation of stressful or protective effects from low- to high-LET radiation ($\sim$0.2 to 151 keV/\(\mu\)m) with various biological end points.

Here, we show that HeLa cells that express Cx26 were more toxic than HeLa cells expressing Cx32 both for low- or high-LET radiation (Fig. 1). Interestingly, the RBE values at 10% survival of $\alpha$ particles of HeLa cells expressing Cx26 or Cx32 had a greater of RBE for cell killing than iron ions. Whereas a dose of 80 cGy from $\alpha$ particles, each cells is traversed on average by $\sim$13 particle tracks. In contrast, in cultures cell exposed to iron ions (1.5 Gy), each, cells are traversed by $\sim$19 particle tracks. However, the biological effects of iron ions are due to the combination of a cyclindrical "core" produced by the ion track and the energetic secondary electrons ($\delta$ rays) surrounding the track structures, and the biological effects of those $\delta$ rays is the same as that of the reference radiation (e.g., $\gamma$ rays). Whereas the radiation track of $\alpha$ particles consist only of core the ion track itself, resulting in the induction of multiple of DNA DSBs and DNA complex lesion with in $\alpha$ particles track.\textsuperscript{27-28} These data illustrate the severity of the damaging effects of the dense ionizations and excitations produced along the high LET $\alpha$ particle tracks than the track of energetic iron particles.

Similar finding are also consistent with results obtained from induction of DNA damage by micronucleus formation. We found that LET-dependent increase in
micronucleus formation in HeLa cells expressing Cx26 or Cx32, with a greater effect produced by α particles than iron ions. It also appears that the induction of DNA damage through Cx32 is somehow limited by protective mechanisms compared with cells that express Cx26. In addition, these results (Figs. 1 and 2) indicated the different of toxic or protective molecules spread through cells that express Cx26 or Cx32 exposed to low- or high-LET radiations, which may be exploited in cancer therapy. However, this may be prevented by gap junction open or closure, and therefore an understanding of these mechanisms is an important.

Taken with the MTT-reduction assay is as alternative to the colony formation assay, to confirm our finding about the cellular response of HeLa cells expressing Cx26 or Cx32 and their respective control. These results was similar to that observed with data of clonogenic survival, we presented that HeLa cells that express Cx32 had a higher significantly ($p < 0.0001$) percentages of cell viability than HeLa cells expressing Cx26 both for low- and high-LET radiations at isosurvival dose levels (Fig. 1). Taken together, a good correlation was obtained between cell survival and MTT-reduction assay in HeLa cells expressing Cx26 or Cx32 exposure to low- or high-LET radiation confirm that high-LET radiations were more effective at cell killing than that of low-LET radiations and cells that express Cx26 propagate toxic effects than cells expressing Cx32 channels. These findings support current study of the role of specific connexins channels on radiation sensitivity (Figs. 1-3). However, little is known about the possible reasons for these differences. This may due to differences in the processing of cellular response of IR or the involvement of the permeability of connexin channels are more dependent on radiation quality.

In conclusion, this represents the first study to demonstrate the role of selective permeability of different connexin proteins in human cells were exposed to protons, γ
rays, α particles or iron ions. We showed that selective permeability of gap junction channels has a role in modulating radiation sensitivity and/or cytotoxicity. Whereas Cx32 channels propagate pro-survival effects, Cx26 and Cx43 channels likely propagate toxic effects. Characterizing the nature and amount of communicated molecules among irradiated cells and between irradiated and non-irradiated bystander cells through gap junction channels would have translational implication in radiation therapy and the formation of countermeasure against the toxic effect of low- or high-LET radiations.

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Figure 1: Clonogenic survival of HeLa cells expressing connexin26 or connexin32 and their respective control were exposed to different types of ionizing radiation at isosurvival dose levels within 5-10 min after irradiation. Panel A: protons. Panel B: γ rays. Panel C: α particles. Panel D: iron ions (*p < 0.08; **p < 0.005; ***p < 0.005).
Figure 2: Fraction of micronucleated cells in HeLa cells expressing connexin26 or connexin32 and their respective control were exposed to different types of ionizing radiation within 5-10 min after irradiation. Panel A: protons. Panel B: γ rays. Panel C: α particles. Panel D: iron ions (*p < 0.05; **p < 0.005; ***p < 0.0008).
Figure 3: Cell viability of HeLa cells expressing connexin26 or connexin32 and their respective control were exposed to different types of ionizing radiation and assay for MTT-reduction within 5-10 min after irradiation. Panel A: protons. Panel B: γ rays. Panel C: α particles. Panel D: iron ions (*p < 0.05; **p < 0.005; ***p < 0.008; ****p < 0.0001).
References


V. Discussion

V.1. The role of gap junction intercellular communication in human cells exposed to high LET radiation

The results of our studies presented in Chapters II–III clearly show that confluent, density-inhibited AG1522 human fibroblasts exposed to high LET radiation (α particles or iron ions) were more prone to cell killing and the induction of DNA damage than when exposed to low LET radiation (energetic protons or γ rays). When the cells were assayed within 5-10 min after irradiation, and considering the 10% survival levels, the RBE of protons (4 Gy), α particles (0.8 Gy), iron ions (1.5 Gy) compared to the reference γ rays (4 Gy) were ~1, 5 and 2.7, respectively, (Fig. 1). Holding AG1522 cells in the confluent state for 3 h, we found that PLDR occurred in proton- and γ-irradiated cells, while, a decrease in survival was detected in cells exposed to α particles or iron ions. These results therefore show that during incubation period in the confluent state, high LET radiation-induced toxic effects were enhanced rather than attenuated.

Relative to γ rays, the RBE of protons, α particles and iron ions when cells were assayed for clonogenic survival 3 h after irradiation (Fig. 1) were ~ 1.3, 12.5 and 4.5, respectively (a ~30, 150 or 67% increase over the RBE calculated in cells assayed shortly (within 5-10 min) after proton-, α particle- and iron ion-irradiation, respectively. These results indicated that the damage caused by high LET radiation as being poorly repaired or irreparable (LITTLE, 1973; ELKIND 1984). The explanation of this event that cells traversal by an α particles or iron ions results in the deposition of a large amount of energy along the ionizing radiation track, with the potential of producing
clustered DNA damage and damage to other macromolecule(s) such as proteins or lipids (SPITZ et al., 2004).

When AG1522 cell cultures are exposed to 0.8 Gy of α particles, ~1.6, 4.8, 9.5 and 14% of the cells would be traversed on average by 1, 2, 3 or 4 particle tracks, respectively (CHARLTON and SEPHTON, 1991). In contrast, at dose of 4 Gy from protons or γ rays, all of cells would be traversed on average by multiple particle tracks. However, cellular response to protons or γ rays generates sparse ionizations that results mostly in non-clustered DNA damage and can be repaired correctly (ELKIND and SUTTON, 1960; LITTLE, 1973; ELKIND 1984). These results show that the RBE for the induction of cell killing depends on with the expression of PLDR and highlights the importance of the radiation track structure.

![Figure 1](image)

**Figure 1.** The RBE at the 10% survival level as a function of LET obtained in confluent AG1522 cells exposed to protons (LET ~0.2 keV/μm), α particles (LET ~122 keV/μm) and iron ions (LET ~151 keV/μm), relative to γ rays and assay for clonogenic survival within 5-10 min or held in confluent state for 3 h after irradiation.
To better understand the mechanisms underlying the enhanced cell killing effect during confluent holding, it was provocative to determine whether direct cell-cell communication via GJIC is involved in the propagation of stressful effect among human cell exposed to high LET radiation. Studies using gap junction inhibitor or the use of connexin knockout cells have explored their involvement in the propagation of various radiation-induced bystander effects (AZZAM et al., 1998). The data in Figure 2 show clonogenic survival in low (sparse) or high (confluent) density AG1522 cell cultures exposed to γ rays or α particles. We found that at isosurvival dose, the survival fraction in confluent AG1522 cells exposed to γ rays was significantly increased ($p < 0.0001$) compared with sparse AG1522 cells. In contrast, a significant decrease ($p < 0.0001$) in the survival fraction was observed in confluent AG1522 cells exposed to α particles, but at sparse AG1522 cells, there was little effect. Therefore, these data suggest a mode of cell killing that is mediated by GJIC. Overall, these data described here indicated that GJIC play an important role in modulation killing effects and PLDR in cells exposed to high LET radiations. Our finding are consistent with those of Jensen and Glazer (JENSEN and GLAZER, 2004) that showed greater cell killing by cisplatin in high density cell cultures consisting of gap junction proficient cells.

To test the role of GJIC in the propagation of stressful among high LET-irradiated cells, we used several methods to manipulate gap junction expression and function, including chemical inhibitor and RNA interference. We investigated whether intercellular communication among irradiated cells is involved in the propagation of toxic effects in the presence or absence of gap junction inhibitor AGA. The data in Figure 3 (Chapter II and III) show that treatment with AGA, did not significantly affect survival of proton- or γ-irradiated cells during the post-irradiation incubation time;
however, it prevented the decrease in survival that is observed in α particle- or iron ion-irradiated cells.

![Graph](image)

**Figure 2.** Clonogenic survival of confluent-or sparse-AG1522 cells exposed to low LET γ rays or high LET α particles within 5-10 min or incubation for 3 h after irradiation. (*p < 0.05; **p < 0.01)

Consistent with the above finding, the fraction of micronucleated cells was decreased in α particle- or iron ion-irradiated cells. In contrast, AGA did not alter micronucleus formation in proton- or γ-irradiated cells. This study and other (SUZUKI and TSURUOKA, 2004) indicated that gap junction inhibitor reduced the toxic effects in cells exposed to high LET radiation. All together, the data in Figures 3 and 4 (Chapter II and III) support the involvement of GJIC in the propagation of protective or stressful effects. They suggest that molecule(s) with different effects, or different amounts of the same molecules(s), may be propagated via gap junction among cells exposed to low- or high-LET radiations.
Figure 3. Role of gap junction intercellular communication in the propagation of stressful effects among irradiated confluent cells: Effects of gap junction inhibitor AGA. I: Clonogenic survival of AG1522 cells exposed to different types of ionizing radiation at isosurvival dose levels in the presence or absence of AGA. (Panel A: protons, 4 Gy); (Panel B: γ rays, 4 Gy); (Panel C: α particles, 0.8 Gy); (Panel D: iron ions, 1.5 Gy).
Figure 4. Role of gap junction intercellular communication in the propagation of stressful effects among irradiated confluent cells: Effects of gap junction inhibitor AGA. II: Fraction of micronucleated cells in control-and irradiated-AG1522 cells exposed to different types of ionizing radiation at isosurvival dose levels in the presence or absence of AGA. (Panel A: protons, 4 Gy); (Panel B: γ rays, 4 Gy); (Panel C: α particles, 0.8 Gy); (Panel D: iron ions, 1.5 Gy). (*: p < 0.05; **: p < 0.01; ***: p < 0.0001)
We further confirm the role of GJIC in the propagation of toxic effect among α-irradiated cells by a more direct approach, confluent AG1522 cells in which the levels of connexin43 expression was decreased by about ~85% by siRNA approach. When AG1522 cells transfected with Cx43-siRNA were exposed to 0.8 Gy lethal dose of α particles and held in confluent state for 3 h, clonogenic survival was increased when compared with scrambled siRNA-transfected cells. It was also associated with a decrease in micronucleus formation. These results were consistent with the reduced up-regulation of the stress-responsive protein p21\textsuperscript{wafl}. Collectively, these data strongly support the involvement of connexin43 in the modulation of killing effect and PLD repair in human cells exposed to high LET radiation. Additional investigation is required to elucidate how knockdown regulations of connexin43 proteins regulate the expression of other connexin proteins in confluent AG1522 cells.

Our data provide new evidence that the direct cell-to-cell communication via gap junction channels play an important role in the propagation of stressful effects in cells exposed to high LET radiation. They show that GJIC modulates potentially lethal damage repair and induction of DNA damage. The enhancement of killing effect in cells exposed to high-LET radiation that would otherwise survive the toxic effect of radiation during the holding in the confluent state after irradiation promoted decreased survival and increased chromosomal damage. Inhibiting gap junction communication with chemical inhibitor or by a knockdown of connexin43, protected againsts the toxic effects from high LET radiation during confluent holding. Overall, these finding indicate that the stressful effects in cell exposed to high but not low LET radiation are amplified by GJIC. Therefore, enhancement of GJIC by chemotherapeutic agents in tumor cells, coupled with high LET radiation and the associated transmission of toxic
molecules between cells in the irradiated tumor, would have important implications to radiotherapy.

V.2. The role of intercellular communication and oxidative metabolism in the propagation of stressful effects in human cell exposed to α particles

High LET radiation has been previously shown to initiate the biological production of reactive oxygen species (ROS) such as superoxide anions (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) in human cells (HALLIWELL and GUTTERIDGE, 1999; MEESUNGNOEN et al, 2002). Though a burst of excess ROS is initially produced around these ionizing radiation tracks at the time of irradiation and is believed to persist for only microseconds or less (MUROYA et al., 2006), radiation-induced oxidative stress on cells may be prolonged due to persistent long-term effects on oxidative metabolism. High LET α particles-induced ROS are known to participate in damage to various cellular components and produce DNA double-strand breaks in addition to base damage and DNA single-strand breaks (AZZAM et al, 2000, 2002, 2003, 2004).

We investigated whether the increase in oxidative stress detected 3 h after irradiation (Chapter II) contributes to the observed increase in cell killing and induction of DNA damage. To this end, we measured clonogenic survival and micronucleus formation in α particle-irradiated cells in which the antioxidant GPX was ectopically over-expressed and in their respective controls. The GPX enzyme converts H$_2$O$_2$, a product of dismutation of O$_2^-$ by the superoxide dismutases, to water (HALLIWELL and GUTTERIDGE, 1999). In parallel experiment, we also investigated lipid peroxidation and protein oxidation in control or α particle-irradiated cells; we harvested cells for analyses following 3 h incubation after irradiation, a time during
which propagation of toxic molecules through gap junction channels among irradiated
cells occurs, and compared the results with effects measured within 5-10 min after
irradiation. Similar to the enhanced toxicity described in Chapter II, holding empty
vector-transduced cells in the confluent state for 3 h after exposure to a mean dose of
0.8 Gy resulted in significant decrease in survival. Ectopic overexpression of GPX
significantly attenuated cell killing measured shortly after irradiation indicating that
oxidative stress contributes to cell killing in α particle-irradiated cells. In this context,
it is of interest to note that the yield of H₂O₂ in irradiated cells is thought to increase
with increasing LET (MUROYA et al., 2006; AUTSAVAPROMPORN et al., 2007).
Thus, by more efficiently scavenging H₂O₂ in α particle-irradiated cells, ectopic
overexpression of GPX would protect against chemical changes to cellular
macromolecules that would be caused by H₂O₂ or by 'OH and O₂·− that result from its
dissociation by the Haber-Weiss reaction (HALLIWELL and GUTTERIDGE, 1999).
Interestingly however, holding GPX-transduced cells for 3 h after α particle irradiation
did not increase survival or decrease micronucleus formation over what was observed
when cells were assayed shortly after irradiation. The latter results suggest that death-
inducing or clastogenic factors other than, or in addition to, oxidizing species may be
directly communicated through gap junctions to enhance cell killing of irradiated cells
that would otherwise survive.

Furthermore, we investigated the role of gap junction communication and
oxidative metabolism in α particle-irradiated cells that were incubated for 3 h after
exposure, in the presence or absence of gap junction inhibitor AGA. As expected,
Figure 5 shows holding irradiated cells that overexpress GPX in the confluent state for
3 h that resulted in significant increase (p < 0.05) in cell survival compared with empty
vector-transduced cells. Interestingly, inhibition of gap junction communication by
AGA in empty vector-transduced cells resulted in substantially increased survival. In contrast, a decrease of survival cells overexpressing GPX was observed.

The micronucleus formation data in Figure 6, show that holding AG1522 cells that overexpress GPX in the confluent state for 3 h after exposure to α particles resulted in a significant decrease ($p < 0.001$) of micronucleus formation. In contrast, inhibition of gap junction channels by AGA in cells overexpressing GPX cells that were held for 3 h in the confluent state after irradiation resulted in an increase of micronucleus formation. It is likely that the propagation of toxic molecules through gap junctions among the irradiated cells may have countered any repair activities in the antioxidant over-expressing cells. Whereas inhibition of gap junction by AGA in GPX-overexpressing and irradiated cells may inhibit the DNA repair activities and perturb oxidative metabolism, which can result in the persistent oxidative stress and increased lethality. These results further indicate that GJIC play a critical role in maintaining the redox mechanisms, which typically protect against damage from high levels of oxidative stress.
**Figure 5.** The role of gap junction communication and oxidative metabolism in the propagation of α particle-induced stressful effects. AG1522 cells were transduced with glutathione peroxidase (GPX) or empty adenovirus vector in the presence or absence of gap junction inhibitor AGA, exposed to 80 cGy from α particles that followed 3 h incubation at 37°C. Clonogenic survival of confluent AG1522 cells exposed to high LET α particles holding in confluent for 3 h after irradiation. (*p < 0.05; **p < 0.01, n=2).
Figure 6. The role of gap junction communication and oxidative metabolism in the propagation of α particle-induced stressful effects. AG1522 cells were transduced with glutathione peroxidase (GPX) or empty adenovirus vector in the presence or absence of gap junction inhibitor AGA, exposed to 0 or 80 cGy from α particles that followed 3 h incubation at 37°C. Micronucleus formation of confluent AG1522 cells exposed to high LET α particles holding in confluent for 3 h after irradiation. (**p < 0.001, n=2)
Consistent with the enhanced toxicity expressed during the 3 h incubation period after α particle irradiation, the increase in lipid peroxidation [4-Hydroxynoneal (HNE)], and protein carbonylation observed in our studies during confluent holding of α particle-irradiated cells (Chapter II) may be caused by excess ROS generated from an effect of the irradiation on oxidative metabolism, ROS generated at the time of the irradiation may have contributed to these effect. Whereas ~60 ROS per nanogram of tissue were estimated to be generated from a hit caused by γ rays (FEINENDEGEN, 2002) (i.e., ~10.4 ROS per cell nucleus, using a nuclear mass of ~173 pg, thus corresponding to a yield of about 1 ROS/100 eV), we can estimate that over 2000 ROS are generated from an α particle traversal, corresponding to a ROS concentration in the nucleus of ~19 nM. Such a ROS concentration can obviously cause oxidative DNA damage (HALLIWELL and ARUOMA, 1991).

Recently, it has been found that the formation of 4-HNE-inhibited DNA repair activities such as inhibit nucleotide excision repair through its direct interaction with proteins involved in DNA repair (FENG et al., 2004) or involved in the regulation and function of p53 (FORD et al., 1995). These finding point that increased protein carbonyl formation in α particle-irradiated cells was associated with the formation of 4-HNE, which plays an important role of oxidative stress in human cells exposed to α particles. These findings raise the possibility that the increased ROS generation overwhelm the antioxidant enzymes resulting in oxidative stress and damage to macromolecules such as DNA, proteins and lipids. These damages are less-readily repaired and their level depend on the time of assay after irradiation (ELKIND, 1984; GOODHEAD; 1993). Understanding the mechanism underlying the repair of damage induced by various types of ionizing radiation is important in estimates of the risks to human health.
V.3. The role of selective permeability of gap junction composed of different
of connexins in human cells in the nature of biological effect propagated
among cells exposed to low- or high-LET radiations

Our studies (Chapter II) indicated that the toxic molecules in confluent AG1522
cells exposed to α particles may spread through Cx43 to modulate the killing effect
and induction of DNA damage as well as the persistence of the level of oxidative
stress. However, little is known about the biological effect of other connexin channels
in confluent AG1522 cells exposed to low- or high-LET radiations. In this regard, we
investigated various biological endpoints in HeLa cells that express Cx26 or Cx32
following exposure to low LET γ rays or protons and high LET α particles or iron ions.
We found that HeLa cells that express Cx26 were more sensitive to DNA damage and
killing effect than HeLa cells that express Cx32 in all case. Interestingly, the RBE
values at 10% survival (D_{10}) of α particles of HeLa cells expressing Cx26 or Cx32 was
greater than the RBE for cell killing after iron ion irradiation (Fig. 7). Whereas at
isosurvival dose levels of 80 cGy from α particles and 150 cGy from iron ions, each
cells nucleus is traversed on average ~13 and 19 particle tracks, respectively. In
contrast, in cultures cell exposed to 4 Gy from protons or γ rays, each cells are
traversed by ~39200 and 8711 particle tracks, respectively. These data illustrate the
severity of the damaging effects of the dense ionizations and excitations produced
along the tracks of high LET radiations. The bursts of ROS in and around these
radiation tracks as well as in the intercellular matrix alter the cellular redox
environment, modify signaling cascades and normal biochemical reactions, and
generate damage to cellular molecules and organelles that is far more extensive than
the damage produced along low LET radiation such as protons or γ rays (SPITZ et al.,
2004; MUROYA et al., 2006).
Figure 7. The RBE at the 10% survival level as a function of LET obtained in HeLa cells that express Cx26 and Cx32 and their respective control exposed to protons (LET ~0.2 keV/μm), α particles (LET ~122 keV/μm) and iron ions (LET ~151 keV/μm), relative to γ rays and assay for clonogenic survival within 5-10 min after irradiation.

Our cell survival data were consistent with the results obtained from micronucleus formation and enzymatic activities by MTT-reduction method. We found that HeLa cells expressing Cx26 mediated the propagation of stressful effects among irradiated cells; HeLa cells that express Cx32 conferred the propagation of protective effects. These finding supports the role of specific permeability of connexin channels in the propagation of stressful or protective effects in culture cells exposed to low- or high-LET radiations. There is an increasing number of studies indicate that the direct cell-to-cell communication via gap junction channels are implicated in the regulation of cell survival and/or cell death that may communicate pro-apoptotic or cell-protective
signalling events to neighbouring bystander cells, but the role of connexin channels in the regulation of low- or high-LET radiation-induced apoptosis is currently not fully established (NORBURY and ZHIVOTOVSKY, 2004; TAKAHASHI et al., 2004). Our results indicated that HeLa cells that express Cx26 showed an increased of apoptosis when exposed to high LET α particles than cell expressing Cx32, but that low LET γ rays with very little effect (Fig. 8). Therefore, the induction of apoptosis varies in function of specific connexin channel permeability and radiation quality. High LET radiation has enhanced effect at inducing apoptosis than low LET radiation and the spread of damaging effect through Cx32 is somehow limited by the protective mechanism. The difference in toxic or protective molecules spread through Cx26 or Cx32 channels between low- and high-LET radiations may be exploited in cancer therapy. One might expect that bystander cell killing could be used in clinical cancer therapy studies. The enhancement of connexin expression and gap junction communication in tumor cells could increase the effectiveness of cancer therapies, and therefore an understanding of these mechanisms is important.
Figure 8. Induction of apoptosis in HeLa cells expressing connexin26 or connexin32 and their respective control were irradiated with a dose of 4 Gy from γ rays or a dose of 80cGy from α particles and incubated for 15 h after irradiation at 37°C. They were then stained with Annexin V-FITC/PI and observed under flow cytometry (n=2). Panel A: γ rays. Panel B: α particles.
Our studies are first to demonstrate the role of selective permeability of different connexin proteins in human cells exposed to protons, γ rays, α particles or iron ions. We showed that selective permeability of gap junction channels has a role in modulating radiation sensitivity and/or cytotoxicity. Whereas Cx32 channels propagate pro-survival effects, Cx26 and Cx43 channels likely propagate toxic effects. Their expansion to investigate the nature and amount of the communicated molecules through Cx26, Cx32 and Cx43 in irradiated cells and between irradiated and bystander cells should increase our knowledge of the biological effects of low- or high-LET radiation and would have translational implications in radiotherapy and the formulation of countermeasures against the toxic effects of ionizing radiation.

V.4. Future directions

In parallel with my Ph.D. studies, I had the great opportunity to participate in a joint project between New Jersey Medical School Cancer Center (USA), Columbia University (USA) and National Institute of Radiological Sciences (NIRS, JAPAN), under the supervision of Drs. Masao Suzuki, Edouard I. Azzam, Tom K. Hei and Yukio Uchihori (November 29, 2010-March 29, 2011). This work extends and complements my current Ph.D. research on the role of GJIC and oxidative metabolism in modulation of human cellular response to protons (LET ~0.2 keV/μm), γ rays (LET ~0.9 keV/μm), α particles (LET ~122 keV/μm) and iron ions (LET ~151 keV/μm). Particular focus of my Ph.D. study is to investigate the communication of signalling events between irradiated cells. We concluded that GJIC participates in the propagation of stressful effects in human cells exposure to high LET radiations and results in enhanced oxidative stress (Chapter II and III). These findings are consistent with the concept that LET of the radiation is an important factor in determining the propagation of stressful or protective effects. However, the biochemical and molecular events
underlying the role of GJIC dependence on radiation quality or LET among irradiated cells and between irradiated and bystander cells remain largely unclear.

The objective of the collaborative project in Japan is to investigate mechanism(s) underlying the role of GJIC in mediating communication of low- or high-LET radiation-induced targeted and bystander effects. Particular focus is to investigate the communication of signalling events among irradiated cells and between irradiated and bystander cells. This work is based on studies using various biological endpoints such as clonogenic survival, micronucleus formation, premature chromosome aberration, mutation assay and change in gene expression. As an application related to cancer therapy, we will investigate the role of intercellular communication in the propagation of stressful effects among human cells exposed to low- or high-LET carbon ions that are currently used to treat patients. The studies are also relevant to radiation protection. We hypothesize that the role of GJIC determines in a LET dependent manner, the magnitude and nature of biological effects propagated among irradiated cells and between irradiated and bystander cells.

Using an in vitro approach the goals of the project will be addressed in the following 3 specific aims:

1) To investigate the role of GJIC in the propagation of stressful effects in human cells exposed to low- or high-LET carbon ions.

We found that GJIC participates in the propagation of high LET radiation-induced stress and enhances toxic effects in α particle- or iron ion-irradiated cells. We also found that PLDR is LET dependent (Chapter II and III). To address this important issue, confluent human cells were exposed to low LET carbon ions (LET~13 keV/μm) or high LET carbon ions (LET~76 keV/μm) at isosurvival dose levels in the presence or absence of gap junction inhibitor (Heavy-ion Medical Accelerator, NIRS). 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, micronucleus formation and γ-H2AX formation.

(2) To investigate the role of GJIC in cell killing and induction of DNA damage in bystander cells exposed to low- or high-LET radiations.

We have recently shown that high LET radiation induce more persistent DNA damage and cell killing in confluent human cells than low LET radiation (Chapter II-IV). To further confirm this finding, we will use microbeam or broad beams (X rays, protons, carbon ions, neon ions and argon ions that vary in LET from ~6 to 1060 keV/µm) at National Institute of Radiological Sciences (NIRS), Japan Atomic Energy Agency (JAEA) and High Energy Accelerator Research Organization (KEK) to demonstrate the role of GJIC-mediated bystander effects induced by low- or high-LET radiation. To further understand the mechanism underlying the role of GJIC in the modulation of the cell killing and DNA damage, we will use gap junction inhibitor to investigate whether the involvement of GJIC in bystander cells using several different endpoints.

(3) To identify that high LET radiation may induce a different gene and/or protein expression profiles than that induced by low LET radiations in bystander cells.

We proposed to verify that high LET radiation would generate a different and/or a unique radiation response in bystander cells as compared to low LET radiation in conjunction with the capabilities of NIRS such as western blot analysis and cDNA microarray. Toward this end, a more comprehensive understanding of the molecular mechanisms of DNA damage, repair, signalling and change in global gene expression patterns by low- or high-LET radiation will identify biomarkers of radiation damage in
bystander cells, as well as enhancing our understanding of the mechanism by which low- or high-LET radiations may lead to the development of cancer and other long term effects.

Preliminary results

We present below some of our preliminary results on the effects of functional gap junction communication in the response of targeted and bystander cells to different types of IR (Aims 1 and 2).

1) Role of GJIC in propagation of stressful effects among carbon-irradiated cells and its correlation with potentially lethal damage repair

Confluent human fibroblast NB1RGB cells were exposed to low- or high-LET carbon ions and held in confluence at 37°C for 5-10 min, 3 h or 24 h prior to subculture and assaying for clonogenic survival. As expected, the data in Figure 9A indicate PLDR in cell exposed to low LET carbon ions. While a minor PLDR occurred in cell exposed to high LET carbon ions (Fig. 9B). These results indicate that PLDR is LET dependent. Therefore we hypothesized that GJIC may contribute to the propagation of PLDR and/or stressful effects among the irradiated cells during confluent holding. To test this hypothesis and further elucidate the role of LET in expression of PLDR, confluent cells were exposed, in the presence of gap junction inhibitor AGA. Figure 9B also show that incubation with AGA prevented the decrease in survival that occurred in cells exposed to high LET carbon ions. In contrast, there was a small effect of GJIC on cell survival after low LET carbon ions (Fig. 9A). These data support the studies in Aim 1.
Figure 9. Clonogenic survival of confluent NB1RGB normal human fibroblasts exposed to low- or high-LET carbon ions at isosurvival dose levels and subcultured for assay within 5-10 min after irradiation or following 3 and 24 h at 37°C in the presence or absence of gap junction inhibitor. Panel A: low LET carbon ions. Panel B: high LET carbon ions (* p<0.05, ** p<0.008, *** p<0.005, n=2).

2) Radiation-induced bystander effects depend on radiation quality and dose and correlate with the role of intercellular communication

Using microbeam irradiation, we investigated the role of intercellular communication dependence of radiation-induced stressful effects in the bystander cells on radiation quality and dose. We evaluated the micronucleus formation of bystander NB1RGB cells under the condition in which only 0.2% of cell population was targeted with 5.35 keV X rays (LET~ 6 keV/μm) or 460 MeV/u argon ions (LET ~1060 keV/μm) in the presence or absence of gap junction inhibitor AGA following 4 h incubation at 37°C. Figures 10 and 11 show the dependence of micronucleus in bystander cells as a function of dose. Interestingly, these results also show that incubation with AGA eliminated the bystander effects following exposure to high, but not low LET radiation. Thus, the bystander effects appeared to be mediated by communication via gap junctions in as much as it was inhibited by treatment of cells.
with a gap junction inhibitor. However, when there is no cell to cell contact and cells are distance apart, secretion of a factor in the medium is involved in the transduction of the stressful bystander signal. Here, we found that cells irradiated with 5.35 keV X rays (LET~ 6 keV/um) released certain cytotoxic factors into the culture medium that killed the non-irradiated cells (data not shown). This evidence suggest that cell-cell communications through gap junction and/or secretion of a cytotoxic factor into the culture medium may be involved in the bystander response.

Taken together, our results show that the propagation of clastogenic effects from irradiated to bystander cells depend on the quality and dose of the radiation and that intercellular communication promote the propagation of stressful bystander effects in high LET-irradiated cell cultures. These data strongly support the rationale and feasibility of the studies in Aim 2.

Conclusion

1) Potentially lethal damage repair is LET dependent.

2) Gap junction intercellular communication participates in critical response to low- or high-LET radiation. It modulates potentially lethal damage repair and induction of DNA damage.

3) The propagation of stressful effects from irradiated to bystander cells depends on the LET and dose. Gap junction communication and secreted factor may be involved.

4) Signalling events leading to bystander effects are more likely to be expressed in cells exposed to high LET radiations.

In studies related to this project, we are focusing at determining the nature of the molecules communicated by gap junction channels among irradiated cells and between irradiated and bystander cells. This may help formulate countermeasures to
attenuate ionizing radiation-induced harmful effect and would have translational in cancer therapy.

**Figure 10.** Fraction of micronucleated cells in bystander cells exposed to X rays microbeam as a function of dose in the presence or absence of gap junction inhibitor (n=1).
**Figure 11.** Fraction of micronucleated cells in bystander cells exposed to argon ions microbeam as a function of dose in the presence or absence of gap junction inhibitor (n=1).
VI. Conclusion

These studies provide evidence that gap junction intercellular communication and oxidative stress modulate the killing effect, induction of DNA damage and protein oxidation and lipid peroxidation in an LET dependent manner. This observation could be a reflection that GJIC plays different role(s) in signaling DNA damage and its repair in cells exposed to low- or high-LET radiations. Therefore it should be pointed out that the GJIC participates in the propagation of high LET stressful effects and results in enhanced oxidative stress. In contrast, holding low LET-irradiated cells in the confluent state for several hours after irradiation promoted increased survival and decreased chromosome damage.

Up-regulation of antioxidant defense, by ectopic overexpression of GPX, protected against toxic effects expressed shortly after α particle-irradiation. However, holding GPX-tranduced cells for 3 h after α particle irradiation did not increase cell survival or decrease induction of DNA damage. These results suggest that the damaging effect of high LET α particles results in oxidative stress, and the toxic effects in the hours after irradiation are amplified by GJIC, but the communicated molecules(s) is unlikely to be a substrate of GPX.

In addition, we found that in the presence of gap junction inhibitor AGA in GPX-tranduced cells, the damaging effects of α particle were enhanced. These results clearly support the role of GJIC and oxidative metabolism in mediating the propagation of toxic effect between α particle-irradiated human cells.

We suggest that our findings are consistent with the concept that inhibition of gap junction intercellular communication adversely affects cellular repair following exposure to high LET radiation. This may represent a novel approach to modify radiation sensitivity. Moreover, the outcome of this work would have important
implications in cancer therapy by chemotherapeutic agents in tumor cells coupled with radiotherapy using α particles; the transmission of toxic compounds between cells in the irradiated tumor would offer a therapeutic gain.

Using human adenocarcinoma (HeLa) cells in which specific connexins can be expressed in the absence of endogenous connexins, we found that permeability aspects of gap junctions affect the response to radiation. Whereas connexin26 and connexin43 channels mediated the propagation of toxic effects among irradiated cells, connexin32 channels conferred protective effects. Characterization of the molecules that are communicated through specific connexin channels from tumor to normal cells would help formulate countermeasures to protect normal tissues during radiotherapy. Future in vivo research would contribute to validating these concepts.
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