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Award Review Article#

Recent Insights into the Biological Action of Heavy-Ion Radiation

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Heavy ions/Tumor radioresistance/Tumor sensitization/Normal tissue protection/Bystander effect.

Biological effectiveness varies with the linear energy transfer (LET) of ionizing radiation. During cancer therapy or long-term interplanetary manned explorations, humans are exposed to high-LET energetic heavy ions that inactivate cells more effectively than low-LET photons like X-rays and γ-rays. Recent biological studies have illustrated that heavy ions overcome tumor radioresistance caused by *Bcl-2* overexpression, *p53* mutations and intratumor hypoxia, and possess antiangiogenic and antimetastatic potential. Compared with heavy ions alone, the combination with chemical agents (a Bcl-2 inhibitor HA14-1, an anticancer drug docetaxel, and a halogenated pyrimidine analogue 5-iodo-2'-deoxyuridine) or hyperthermia further enhances tumor cell killing. Beer, its certain constituents, or melatonin ameliorate heavy ion-induced damage to normal cells. In addition to effects in cells directly targeted with heavy ions, there is mounting evidence for nontargeted biological effects in cells that have not themselves been directly irradiated. The bystander effect of heavy ions manifests itself as the loss of clonogenic potential, a transient apoptotic response, delayed p53 phosphorylation, alterations in gene expression profiles, and the elevated frequency of gene mutations, micronuclei and chromosome aberrations, which arise in nonirradiated cells having received signals from irradiated cells. Proposed mediating mechanisms involve gap junctional intercellular communication, reactive oxygen species and nitric oxide. This paper reviews briefly the current knowledge of the biological effects of heavy-ion irradiation with a focus on recent findings regarding its potential benefits for therapeutic use as well as on the bystander effect.

INTRODUCTION

For nearly a century, ionizing radiation has been indispensable to medical diagnosis and therapy. The goal of radiation therapy for cancer is to eradicate tumors without harming healthy tissues. It is well established that biological effectiveness of radiation differs with the linear energy transfer (LET), namely, the average amount of energy deposited per unit length (e.g., keV/μm). High-LET energetic heavy ions (charged particles heavier than helium ions) produce dense ionization along their trajectories, and cause complex and irreparable clustered DNA damage.[1)] Compared with low-LET photons and protons, heavy ions have higher relative biological effectiveness (RBE) with less cell-cycle and oxygen dependency of radiosensitivity,[2)] and possess greater potential to suppress angiogenesis, metastasis and arrhythmia.[3–5)] The biological evidence that the therapeutic ratio escalates with an increase in the dose per fraction[6)] has provided the basis for the short-course hypofractionated regimens.[7)] Unlike photons, heavy ions form the Bragg peak (a sharp rise in energy deposition at the end of their range) with a steep dose falloff downstream, thereby enabling dose escalation to the target tumor volume without much exacerbation of normal tissue complications. The same holds true for protons; however, the ratio of dose at the Bragg peak to that in the entrance region is higher for heavy ions.[8)] Such superb biological effectiveness and dose conformity represent a rationale for heavy-ion therapy. Ever since the first clinical experience in 1977–1992,[9)] the number of treated patients has been growing steadily and exceeded 5000 in total. Heavy-ion therapy is currently available at the National Institute of Radiological Sciences (NIRS, Chiba, Japan), the Gesellschaft für Schwerionenforschung (GSI, Darmstadt, Germany) and Hyogo Ion Beam Medical Center (Hyogo, Japan), and has thus far achieved good cancer controllability in short treatment times while sparing critical normal organs.[7,10–12)] Several other facilities including Gunma University (Gunma, Japan) are also becoming operational.

In addition to relevance to radiation therapy, the study of

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biological effects of heavy-ion radiation is also significant to radiation protection issues. Humans may be exposed to energetic heavy ions during prolonged missions in deep space, where health risk to astronauts is a matter of grave concern, necessitating the development of adequate radiation protection strategies for space travel.[13] A growing body of *in vivo* evidence suggests that heavy ions cause tumorigenesis more effectively than photons, particularly at low doses.[14–16] It should be stressed that in a cell population exposed to a lower dose of higher-LET heavy ions, less irradiated cells coexist with more nonirradiated counterparts.[17,18] Hence, to decipher the biological mechanism of heavy-ion action, not merely the effects occurring in irradiated cells but those in nonirradiated cells should also be elucidated if the effects could arise in nonirradiated cells. In this regard, since 1992,[19] significant evidence has accumulated demonstrating that radiation causes biological effects in nonirradiated bystander cells having received signals from either nucleus- or cytoplasm-irradiated cells.[20,21] Bystander cells manifest genetic and epigenetic changes, alterations in gene expression, activation of signal transduction pathways, and delayed effects in their descendants.[22–24] Proposed mediating mechanisms involve gap junctional intercellular communication (GJIC), reactive oxygen species (ROS), nitric oxide (NO), secreted soluble factors, lipid rafts and calcium fluxes.[25–27] Experimental systems include the use of microbeams that selectively target a preset fraction of cells each with a precise number of particle(s).[17,18] At present, heavy-ion microbeams are available for biological studies at three facilities: microbeams are collimated through microapertures at JAEA-Takasaki (Gunma, Japan),[28] and focused with magnetic lenses at GSI[29] and the Technische Universität München (Munich, Germany).[30]

This paper reviews briefly the current knowledge of the biological effects of heavy ions with emphasis on recent findings concerning their potential benefits for therapeutic use as well as their impact on propagation of bystander responses.

HEAVY IONS AND THERAPEUTIC GAIN

Overcoming tumor radioresistance

Genetic changes that accompany cancer development and progression endow tumor cells with a survival advantage over their normal counterparts, and often bring about a poor prognosis because of resistance to a multitude of therapeutic modalities. Heavy-ion therapy improves the responsiveness of photon-refractory tumors,[7] and recent biological studies have advanced our understanding of the underlying mechanisms.

The overexpression of the antiapoptotic oncoprotein Bcl-2 is found in almost half of human cancers and has been associated with radio- and chemoresistance.[31,32] Restoring susceptibility by nullifying the effects of Bcl-2 would hence be an attractive strategy to improve the therapeutic efficacy.[33] We reported that high-LET heavy ions (76–1610 keV/μm) overcome radioresistance caused by Bcl-2 overexpression in human cervical cancer HeLa cells *in vitro*, which may be potentially accounted for by the enhanced apoptotic response and prolonged G_2/M arrest.[34] Our preliminary data show that whilst exposure to neither carbon ions (108 keV/μm) nor γ-rays alters the amount of Bcl-2 proteins, the former augments Bcl-2 phosphorylation at serine 70 more effectively than the latter (unpublished data), encouraging further analysis to delineate the mediating molecular events.

In response to a range of environmental stimuli, the p53 tumor suppressor protein, often termed the guardian of the genome, becomes functionally active via posttranslational modifications like phosphorylation, leading to a transient cell cycle arrest, apoptosis or cellular senescence.[35] *p53* mutations arise in nearly half of human cancers and have been related to radio- and chemoresistance.[36,37] Regardless of *p53* status, high-LET carbon ions (70–100 keV/μm) effectively inactivate tumors and induce apoptosis but not necrosis *in vitro* in human non-small-cell lung cancer, glioblastoma and glioma cells.[38–40] Such *p53*-independent apoptosis appears to involve the activation of Caspase-3 via Caspase-9 in human gingival squamous cell carcinoma cells.[41] *In vivo* evidence also suggests that carbon ions are more effective than photons at inducing apoptosis in radioresistant human glioblastoma but not in radiosensitive human ependymoblastoma cells.[42]

Tumor oxygenation affects radiotherapy outcome. Hypoxia is a common feature of the tumor microenvironment and promotes radioresistance.[43] Evidence has been presented that in mice bearing mouse squamous cell carcinoma cells, high-LET carbon ions decrease radioresistance of intratumor quiescent cell populations,[44] which contain a higher fraction of hypoxic cells than proliferating cell populations in solid tumors.[45] The clinical evidence has further documented that there is little difference in the disease-free survival and local control rates between hypoxic and oxygenated uterine cervical cancers before and during carbon-ion treatments,[46] revealing that heavy-ion therapy reduces hypoxia-driven tumor radioresistance.

Altogether, these findings predict that heavy-ion therapy would be a promising modality for a wide variety of radioresistant tumors, and possibly for chemoresistant tumors as well.

Enhancing tumor cell killing

In spite of considerable interest in combined modalities (e.g., chemoradiotherapy) or in molecularly targeted approaches to radiosensitize tumors for conventional photon therapy, such information is very limited for heavy-ion therapy.

Several studies have proposed that preirradiation chemical treatment at clinically attainable, noncytotoxic concentrations potentiates tumor cell killing by heavy ions. HA14-1

is a nonpeptidic small-molecule ligand of a Bcl-2 surface pocket, which was recently identified from *in silico* screens.[47] HA14-1 selectively disturbs the interaction between Bcl-2 and Bax, and sensitizes tumors to photons.[48,49] We found that HA14-1 sensitizes HeLa cells and its Bcl-2 overexpressing counterparts, but not normal human fibroblasts, to carbon ions (108 keV/μm) and γ-rays (0.2 keV/μm) *in vitro*,[50] suggesting that Bcl-2 may be an attractive target. *In vitro* sensitization produced by the halogenated pyrimidine analogue, 5-iodo-2'-deoxyuridine, decreased with increasing LET, such that human kidney cells were sensitized to X-rays and three types of neon ions (38, 82 and 183 keV/μm), but not to lanthanum ions (1000 keV/μm).[51] An anticancer drug docetaxel[52] rendered human esophageal squamous cell carcinoma cells more vulnerable to two types of carbon ions (50 and 70 keV/μm) both *in vitro* and *in vivo*.[53]

Other combination regimens have also been proposed. One regimen involves the combination with gene therapy.[54] Adenovirus-mediated *p53* gene transfer resulted in the enhancement of carbon ion-induced cell killing in human esophageal squamous cell carcinoma, hepatocellular carcinoma, cervical cancer and mouse melanoma cells.[55–58] Of interest in this respect is that irradiation elevates adenovirus-mediated gene transfer and expression of exogenous genes.[59] An alternative regimen includes the combination with hyperthermia.[60] Carbon-ion irradiation and subsequent hyperthermia augmented killing of human glioblastoma and tongue squamous cell carcinoma cells *in vitro* supraadditively at ≤ 70 keV/μm and additively at ≥ 100 keV/μm in wild-type *p53* cells, but additively in *p53*-mutated cells irrespective of LET.[61,62] Such treatments synergistically suppressed growth of human head and neck squamous cell carcinoma cells *in vivo*.[63] Another regimen is the combination with high-energy X-rays, which acted additively and did not depend on the irradiation sequence *in vitro*.[64]

The above combined approaches may enhance the efficacy of heavy-ion therapy.

Protecting normal cells

The therapeutic ratio relies on the relative probability of tumor control and normal tissue complications. Normal tissue protection may thus offer an improved therapeutic outcome,[65] and is also critical for reducing health risks to astronauts. Incubation of primary human lymphocytes in beer, and its constituents β-pseudouridine and glycine betaine, but not ethanol, decreased the frequency of chromosome aberrations induced by carbon ions (50 keV/μm). Such treatments also reduced mouse mortality.[66–70] Melatonin,[71] which the epiphysis synthesizes and secretes, improved the survival and lowered the frequency of gene mutations induced by carbon ions (100 keV/μm) in Chinese hamster fibroblasts.[72] L-selenomethionine abolished the induction of nearly half of the genes whose expression changed in iron ion-irradiated human thyroid epithelial cells.[73] Such protectors may relieve heavy ion-induced damage to normal tissues, warranting further studies to assess its impact on tumor control.

BYSTANDER EFFECTS

In vitro experimental approaches used to study the bystander effect broadly fall into two categories. The first category involves the bystander effect in confluent cultures, where direct intercellular interactions (e.g., GJIC) between irradiated and bystander cells are operational. The second includes the bystander effect in the cells treated with conditioned medium from irradiated cells, whereby such direct interactions are inoperable and secreted soluble factor(s) would be a central player. Further details of culture and irradiation systems have been reviewed previously.[26]

Effects in normal cells

All data available hitherto for heavy ion-induced bystander effect in normal cells were obtained with normal human fibroblasts.

To examine the direct intercellular interaction-mediated bystander effect, we targeted 0.00026, 0.0013 or 0.0066% of cells in confluent cultures with microbeams of carbon (103 keV/μm) or two types of neon ions (294 and 375 keV/μm) in confluent cultures. Irrespective of ion species and the fraction of hit cells, similar bystander responses were observed.[74–76] First, bystander cells underwent a transient apoptotic response and delayed p53 phosphorylation in comparison with irradiated cells.[74,75] There is also the evidence for delayed foci formation of phosphorylated histone H2AX (γH2AX),[77] which forms discrete foci at the site of each nascent DNA double-strand break (DSB).[78,79] This thence suggests a temporally distinct response of irradiated and bystander cells. Secondly, gene expression was altered at a genome-wide level.[76] More than half of the genes whose expression changed in bystander cells were downregulated.[76] This was in contrast to the lack of downregulation among genes examined in bystander cells that were treated with conditioned medium from irradiated cells,[24] indicative of temporal differences in gene expression or dissimilar mechanism for direct intercellular interaction-mediated versus medium-mediated bystander effect. Expression profiles differed greatly between irradiated and bystander cells, such that most of the genes upregulated in irradiated cells were downregulated in bystander cells.[76] Pathway analysis revealed serial activation of NF-κB (nuclear factor κB) and $p21^{Waf1}$ pathways in irradiated cells, but G protein/PI-3 (phosphatidylinositide 3) kinase pathway in bystander cells.[76] Upregulated genes included interleukin genes in irradiated cells, but its receptor gene in bystander cells.[76] This implies that intercellular signaling between irradiated and bystander cells activate intracellular signaling, leading to the transcriptional response in bystander cells. Thirdly, the bystander effect was manifested as reduced survival.[74,75]

Loss of clonogenicity should reflect the summed response of a plated parental cell and its progeny. In this light, convincing evidence now exists that the progeny of bystander cells express delayed phenotypes, and that the neighbors of the progeny of surviving cells show bystander responses.[80,81] These findings are suggestive of the interrelation between the bystander effect and genomic instability, for which persistent oxidative stress may be a common mechanism.[25,82] This highlights that continual spatiotemporal propagation of signals initially transmitted from irradiated to bystander cells may perpetuate the radiation effects in their progeny over time. Accordingly, bystander-induced reductions in survival may be attributable to death of plated bystander cells and delayed death of their progeny. We recently found clonal morphotypic heterogeneity and delayed loss of clonogenicity in colonies arising from heavy ion-irradiated cells, both of which occur in a LET-dependent fashion.[83–86] This encourages further studies to test if this is also the case for colonies arising from bystander cells.

Other studies have also assessed the direct intercellular interaction-mediated bystander effect. ROS and GJIC mediated bystander-induced micronucleation regardless of ion species (100–1260 keV/μm).[87–89] GJIC contributed to carbon ion-induced bystander effect, which was expressed as increased gene mutation frequency and decreased survival.[90] Irrespective of ion species (11–15000 keV/μm), bystander cells went through a transient G_1 arrest along with concurrent accumulation of p53 and its downstream $p21^{Waf1}$ proteins.[91]

In terms of a medium-mediated bystander effect, exposure to iron ions (151 keV/μm) and X-rays similarly produced bystander-induced reductions in survival and formation of micronuclei and γH2AX foci, in which NO and ROS participated.[92,93] Irradiation with neon ions (437 keV/μm) gave rise to chromosome aberrations in bystander cells, which involved NO and may be partially reparable by DNA-PKcs (catalytic subunit of DNA-dependent protein kinase)-mediated DSB repair machinery, in particular nonhomologous end-joining.[94]

Effects in tumor cells

As regards the direct intercellular interaction-mediated bystander effect, clonogenic potential of bystander HeLa cells was inactivated irrespective of ion species (103–375 keV/μm) and Bcl-2 overexpression, but was less pronounced than in normal human fibroblasts.[75] This may be partially explained by a lack of GJIC (a key contributor to the direct intercellular interaction-mediated bystander response)[25] in HeLa cells.[95]

With respect to the medium-mediated bystander effect, exposure of human salivary gland tumor (HSG) cells to X-rays and two types of carbon ions (13 and 100 keV/μm) increased cell proliferation, the plating efficiency and micronucleus frequency in bystander HSG cells.[96–98] Irradiation of HSG cells with X-rays and carbon ions (100 keV/μm) decreased the survival and induced apoptosis and necrosis in bystander murine lymphoma cells.[99] Such bystander responses involved NO and varied with the radiation quality.[96–99]

LET dependency

There is currently limited evidence for heavy ion-induced bystander effects. It is nonetheless tempting to speculate on the LET dependency. Most data reported to date imply its potential LET independence for normal human fibroblasts; by comparison, its potential LET dependence has been demonstrated for several types of tumor cells.[96–99] Furthermore, with regard to the bystander effect of neon ions (437 keV/μm) and soft X-rays (5 keV/μm) for chromosome aberrations in normal human fibroblasts, there was a difference in the types of aberrations but little difference in total yields,[94] suggesting that the underlying causes differ with the radiation quality. More specifically, even with similar ion species at a similar LET, exposure to broadbeam[100] but not to microbeam[101] reduced the survival of bystander normal human fibroblasts. Collectively, the LET dependence of the bystander effect appears possibly to vary among experimental systems, and therefore must be more carefully confirmed or refuted under comparable experimental conditions (e.g., consistent endpoint, dose, irradiation system and cell type).

Significance

Observed measurable bystander responses may result from a cascade of feed-forward signal amplification events, such that signal(s) from irradiated cells are transduced into primary bystander cells, which in turn produce signals further transmissible to their secondary bystander cells one after another.[26] On one hand, the cytotoxic bystander effect arising in normal cells could be a defensive mechanism that would avert or minimize further expansion of aberrant cells, thus maintaining genome integrity and cellular homeostasis.[74] On the other, the bystander effect occurring in tumor cells, and the pertinent phenomenon of anti-tumor abscopal effects for cancers distant from an irradiated local area of the body,[102] may be beneficial to heavy-ion therapy in eradicating more tumor cells than targeted.[103,104] This is reminiscent of the case for the bystander effect of suicide gene therapy, which has been incorporated into clinical studies.[105] Potentiation of bystander responses by enhancing GJIC between tumors (e.g., via the introduction of *connexin* genes, or administration of chemicals such as retinoids, carotenoids and green tea components that upregulate GJIC) may allow heavy-ion irradiation of a smaller target volume with far less dose to the surroundings.[26] The expression of bystander effects *in vivo* and further characterization of the communication between irradiated and bystander cells as well as crosstalk between irradiated tumor and bystander cells await further investigation.

PERSPECTIVES

Despite a series of studies, the question as to how heavy ions enhance cell killing remains fully open. The LET dependence of DSB and clustered DNA damage induction is still a fascinating question.[1,106] Not just direct action but indirect action may play parts in heavy ion-induced cell inactivation.[107] Cell death modes[40,84,85,108–110] and the influence of cellular ultrastructure[111,112] may differ with LET following acute or chronic exposure even at an isosurvival level. Biological effectiveness may also vary among ion species (or track structure) even at a comparable LET.[113–116] To gain a deeper insight into the biological mechanism of heavy-ion action, dependence of the early- and late-arising effects upon LET and ion species therefore needs to be characterized more extensively. The nematode,[117–119] tardigrades,[120,121] silkworms[122,123] and chironomid[124] have emerged as *in vivo* model systems, in addition to rodents. Such basic but informative *in vitro* and *in vivo* studies would be of extreme importance and should be continued to reduce uncertainties in assessing health risks to astronauts, and to improve the therapeutic efficacy. For future heavy-ion therapy, in efforts to maximize the therapeutic ratio, identification of the potential agents or combination regimens that could enhance tumor control without aggravating or even with assuaging normal tissue complications warrants further extensive studies. Closer interdisciplinary interactions (e.g., between clinicians and biologists) could facilitate the bedside-to-bench interpretation as well as the bench-to-bedside translation.

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