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Molecular Signaling in Response to Charged Particle Exposures and its Importance in Particle Therapy

PD Dr Christine E. Hellweg[1]; Dr Arif Ali Chishti[1,2]; Sebastian Diegeler, MSc[1]; Dr Luis F. Spitta[1]; Bernd Henschenmacher, MSc[1]; and Prof Dr Christa Baumstark-Khan[1]

[1]German Aerospace Center (DLR), Institute of Aerospace Medicine, Radiation Biology Department, Köln, Germany
[2]The Karachi Institute of Biotechnology and Genetic Engineering, University of Karachi, Karachi, Pakistan

Abstract

Energetic, charged particles elicit an orchestrated DNA damage response (DDR) during their traversal through healthy tissues and tumors. Complex DNA damage formation, after exposure to high linear energy transfer (LET) charged particles, results in DNA repair foci formation, which begins within seconds. More protein modifications occur after high-LET, compared with low-LET, irradiation. Charged-particle exposure activates several transcription factors that are cytoprotective or cytodestructive, or that upregulate cytokine and chemokine expression, and are involved in bystander signaling. Molecular signaling for a survival or death decision in different tumor types and healthy tissues should be studied as prerequisite for shaping sensitizing and protective strategies. Long-term signaling and gene expression changes were found in various tissues of animals exposed to charged particles, and elucidation of their role in chronic and late effects of charged-particle therapy will help to develop effective preventive measures.

Keywords: DNA damage response; nuclear factor κB; DNA repair foci; linear energy transfer; bystander effect

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Corresponding Author:
PD Dr Christine E. Hellweg
German Aerospace Center (DLR)
Radiation Biology Department
Institute of Aerospace Medicine
Linder Höhe, D-51147, Köln, Germany
Phone: +49 (2203) 601-3243
christine.hellweg@dlr.de

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Introduction

In radiotherapy, energetic charged particles are used because of their favorable dose distribution with better sparing of healthy tissue [1] and greater biological efficiency in a defined linear energy transfer (LET) range. High-LET radiation has greater DNA-damaging capabilities than low-LET radiation has [2], resulting in more-effective cell killing or proliferation stops [3, 4].

A major difference between low-LET and high-LET radiation is the microscopic dose deposition. Charged particles deposit their energy along densely ionized tracks [5]. In chromosomes within those tracks, complex damage is produced, defined as 2 or more abasic sites, oxidized bases on opposing strands or the same strand, and strand breaks on opposite DNA strands within a few helical turns (**Figure 1**) [5–12]. That damage is difficult to repair and affects rejoining faithfulness [13–15]. DNA repair systems have an intrinsic weakness in processing complex damages [16]. Molecular signaling in response to charged-particle exposure is predominantly a DNA damage response (DDR), turning the switch toward cellular survival or death (**Figure 2**).

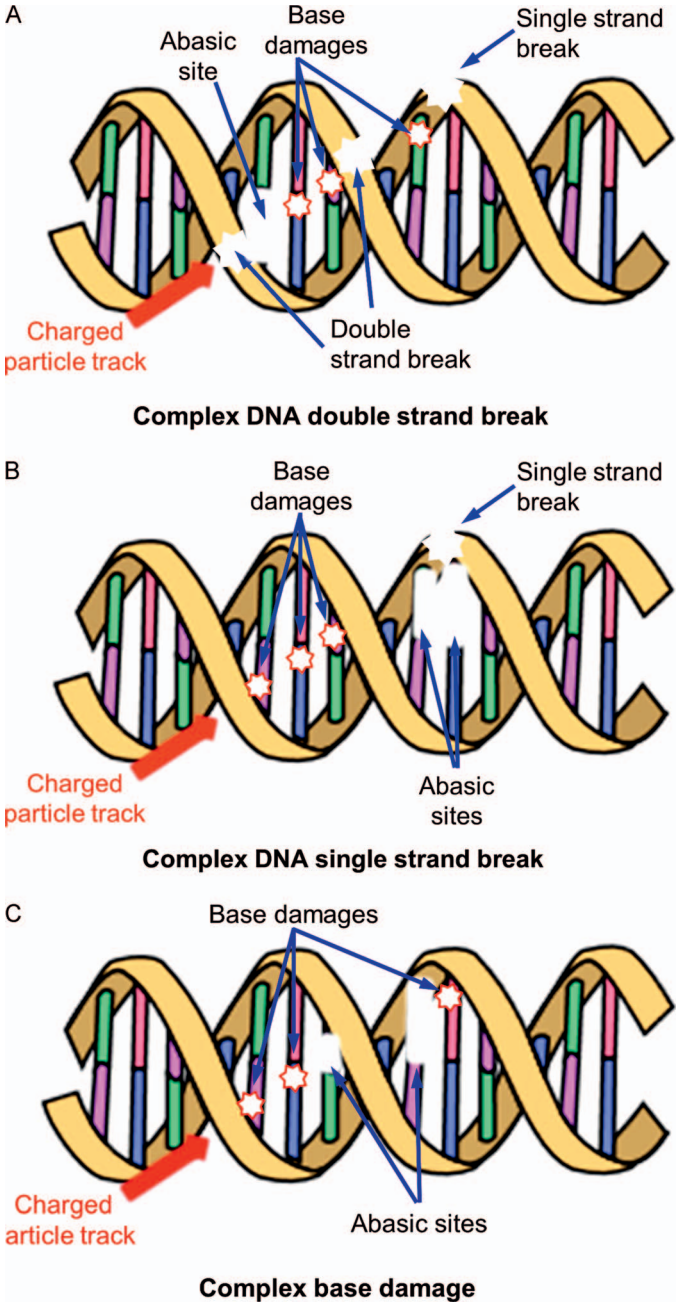

Figure 1. Examples of complex DNA damage. For details, see text. Adapted from Georgakilas [113], DNA structure from Wikimedia Commons.

However, complex DNA damage can be more efficient at killing tumor and healthy cells. In surviving cells, complex DNA damage might induce long-lasting signaling and gene expression changes, which might be tumor promoting and/or cause degenerative diseases.

Here, we summarize the current knowledge on molecular signaling after charged-particle exposure, and we highlight quantitative and qualitative differences in molecular signaling after charged-article exposure compared with low-LET irradiation.

Intracellular Molecular Signaling after Charged-Particle Exposure

Ionizing radiation induces multiple genotoxic, stress-induced signaling pathways that regulate cellular growth, proliferation, cell cycle progression, DNA replication, DNA repair, cell death, apoptosis, and cell-cell adhesion [17–19]. That very efficient DDR signaling network ensures the integrity of the genome by cell-cycle checkpoints and DNA repair. The DDR genes are also involved in transcriptional regulation and chromatin remodeling [20].

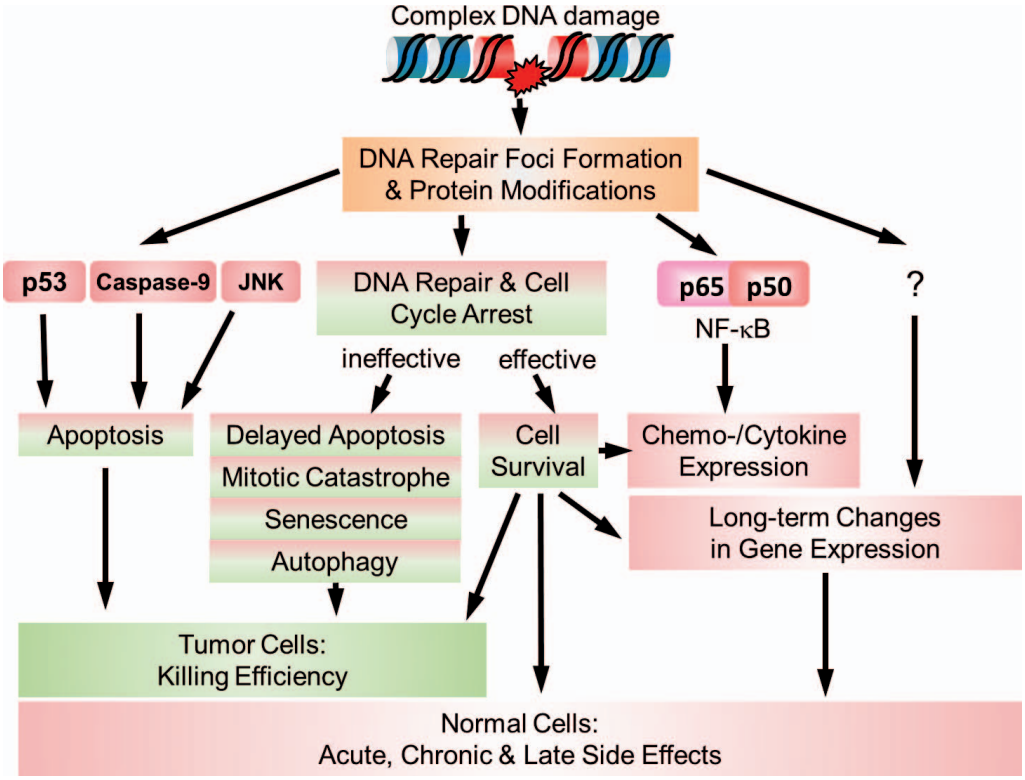

Figure 2. Molecular signaling and outcome after charged particle exposure. For details, see text

DNA Repair Foci Formation

Ionizing radiation activates phosphatidylinositol-3-kinase-related enzymes, including ataxia telangiectasia mutant (ATM), ataxia telangiectasia, Rad3-related protein (ATR), and DNA-dependent protein kinase (DNA-PK) [21]. The ATM and ATR are recruited to complex double-strand breaks (DSBs) (**Figure 3**) [22].

Mutations in ATM cause radiation hypersensitivity in patients with the autosomal recessive disorder ataxia-telangiectasia [16]. Mice with ATM haploinsufficiency develop cataracts earlier compared with wild-type animals, and the enhanced sensitivity was greater for high-LET heavy ions compared with low-LET x-rays [23].

There are 4 autophosphorylation sites in ATM: Ser-367, Ser-1893, Ser-1981, and Ser-2996. Ser-1981 phosphorylation is associated with ATM monomerization. In human fibroblasts, ATM phosphorylated at Ser-367 is recruited to DNA damage sites after exposure to xenon ions (LET 800 keV/μm) [24].

Very early events include phosphorylation of the histone variant H2AX on Ser-139 (γH2AX) by ATM [25]. That results in protein recruitment to the DNA lesions, forming foci in LET-dependent kinetics [26]. The fast-recruited proteins are responsible for damage recognition, and slower accumulating proteins are predominantly involved in subsequent repair events [27]. Meiotic recombination 11 homolog A (Mre11), Rad50 [28], p53-binding protein 1 (53BP1) [29], proliferating cell nuclear antigen (PCNA) [30], x-ray repair cross-complementing 1 (XRCC1) [31], aprataxin [32, 33], p21 [28], RNF8 [34], and BRCA1 [35] form foci at charged-particle–induced DNA lesions.

In addition to foci formation, α-particle irradiation (approximately 5.5 MeV) induces pannuclear phosphorylation of ATM and H2AX in human peripheral blood lymphocytes and fibroblasts, and that γH2AX formation is dependent on ATM [36]. Poly(adenosine diphosphate [ADP]-ribose) synthesis by poly([ADP]-ribose) polymerase-1 (PARP-1) colocalizes with γH2AX after proton exposure (3.2 MeV) in HeLa and V79 cells [37].

The Mre11/Rad50/NBS1 (MRN) complex has a central role as a DNA DSB sensor and is suggested to process a subclass of high-LET radiation-induced complex DNA damage [38]. Moreover, MRN binds directly to the DSB strand ends forming the inner focus [39] and supports efficient ATM activation and recruitment [40] as well as further H2AX phosphorylation. Mediators of DNA-damage checkpoint protein 1 (MDC/NFBD1) must be recruited to γH2AX before MRN can bind in the outer focus. In a final step, ATM binds to recruited MDC1 [39]. In U2OS cells, an acceleration of NBS and MDC1 foci formation was observed up to an LET of 3000 and 9000 keV/μm, respectively [39].

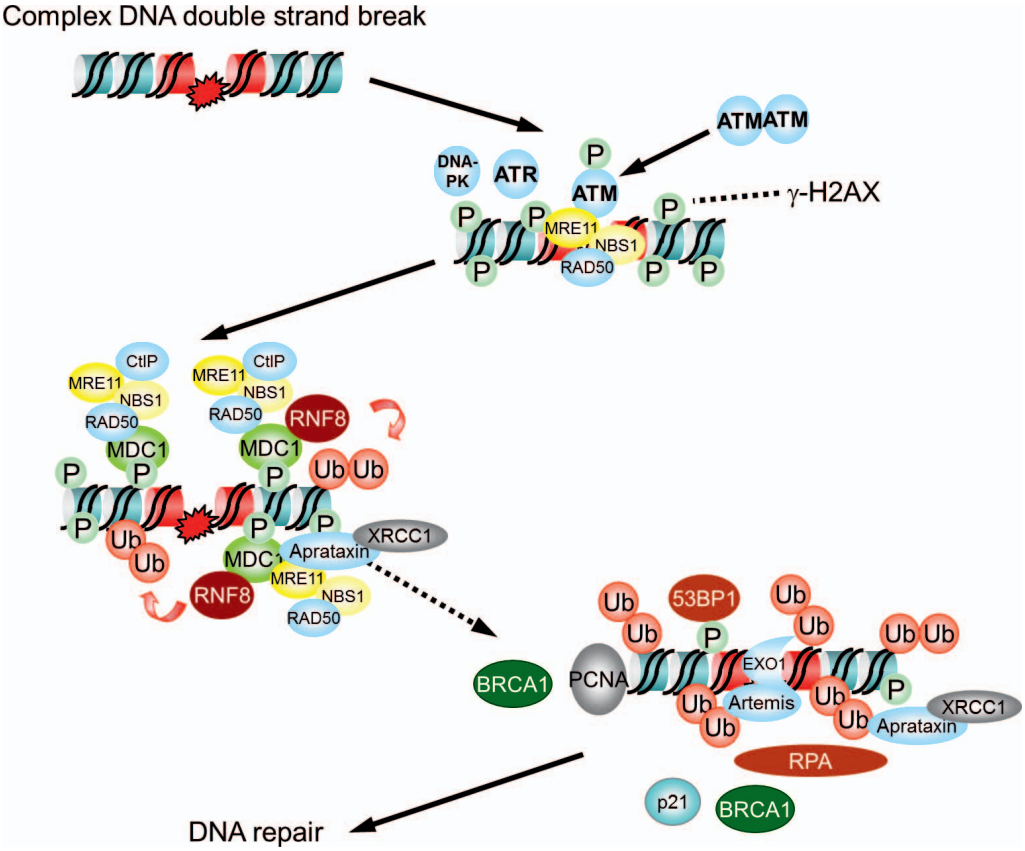

Figure 3. Formation of charged particle-induced foci at sites of complex DNA double strand breaks. For details, see text. Only proteins that were experimentally shown to accumulate at charged particle induced damage sites are shown. Adapted from Bekker-Jensen and Mailand [114] and references in the text. Not all proteins shown here might accumulate in every charged particle-induced focus; for example DNA-PK and Artemis are generally involved in nonhomologous end-joining, and BRCA1 and RPA in homologous recombination, indicating the branching into one of these DNA double-strand break repair pathways.

The ATM substrate Rad50 was phosphorylated at Ser-635 within 15 to 120 min after exposure of U2OS cells to ^{197}Au ions (LET 13 050 keV/μm) [41].

MRE11, CTBP-interacting protein (CtIP), and exonuclease 1 (EXO1) are suggested to drive resection of complex DSBs [22]. Furthermore, replication protein A (RPA) foci are formed at the sites of complex damages, indicating DSB resection after accelerated-ion exposure [22].

In A549 lung carcinoma cells, carbon-ion exposure (LET 290 keV/μm) induced large BRCA1 foci and more p-ATM/p-ATR foci per cell compared with γ-irradiation [35]. BRCA1 activates DNA-end resection and thereby promotes homologous recombination [42]. In a human bronchial epithelial cell line, γ-rays and heavy-ion exposure initiated a *BRCA1*-centric DDR involving *CDKN1A*, *RBBP8*, and *RAD51* [43].

In addition, 53BP1 forms a barrier that inhibits DNA-end resection (42). In human neonatal dermal fibroblasts, γH2AX and 53BP1 foci colocalize in particle tracks (^{11}B, ^{20}Ne, LET approximately 135 keV/μm) [29].

XRCC1, a scaffold DNA repair protein for single-strand breaks, also colocalizes with γH2AX, but the foci are smaller and disappear faster, and foci in heterochromatic regions are relocated to adjacent euchromatin [31]. Local heterochromatin decondensation at the sites of ion hits allows DSB repair [31].

Aprataxin, a nuclear protein involved in DNA strand break repair, base excision repair [44, 45], and mitochondrial function [46], accumulates at sites of iron or xenon-ion hits within seconds [32, 33]. It binds to MDC/NFBD1 in heavy-ion–exposed HeLa cells, indicating its involvement in the repair of very high-LET radiation-induced DNA DSB [47]. Aprataxin colocalizes with XRCC1 along tracks induced by uranium-ion (3.5 MeV/μm) exposure of HeLa cells [48].

Artemis has 5′ to 3′ exonuclease activity specific for single-stranded DNA, which can process damaged termini and is involved with that activity in the repair of complex DNA damage [49]. Artemis is also involved in processing lesions induced by 76-MeV protons in the spread-out Bragg peak [50].

In addition, p21 forms foci within 2 min after exposure to lead or chromium ions, whereas p21 is diffusely spread after x-irradiation [28].

The ubiquitin ligase RNF8, a key regulator of rapid DNA repair complex assembly, accumulates at DNA damage sites in α-particle (3 MeV)-irradiated HTB96 U2OS cells within 30 min [34].

Protein Modifications

Compared with low-LET radiation, high-LET radiation causes greater protein modifications via posttranslational and oxidative processes [51–55]. The ubiquitin/proteasome system might modulate the cellular radiation response by affecting protein turnover [56] and acts together with phosphorylation, methylation, and acetylation of, for example, H2AX [57] and p53 [58], ADP-ribosylation, and other ubiquitin-like modifiers [59].

In addition to fast phosphorylation (see DNA Repair Foci Formation), other protein modifications, with slower kinetics, such as ubiquitinylation, have been reported. A recent study with HeLa and oropharyngeal squamous cell carcinoma cells showed that the histone H2B is specifically ubiquitinylated at Lys-120 ($H2B_{ub}$) several hours after irradiation in response to a high dose (10 Gy) of high-LET α-particles (LET 121 keV/μm) and protons (LET 12 keV/μm) but not by low-LET protons (1 keV/μm) or x-rays/γ-radiation [60]. The ubiquitin ligases MSL2 and the RNF20/RNF40 complex control $H2B_{ub}$ and are essential for complex DNA-damage processing [60], and their knockdown results in reduced survival after proton exposure (LET 12 keV/μm).

Signaling Pathways and Gene Expression Changes

Carbon (LET 30/70 keV/μm) and iron-ion exposure (LET 180 keV/μm) kills lymphoblastoid cells, independent of p53 [61]. High-LET (>70-85 keV/μm) heavy-ion irradiation-induced, p53-independent apoptosis might be mediated by a mitochondria-associated apoptotic pathway involving caspase-9 [62–64].

The cytoplasmic mitogen-activated protein kinase (MAPK) pathways, extracellular signal-regulated kinase (ERK; cytoprotective), and c-Jun N-terminal kinase (JNK; proapoptotic), which feed into and are fed upon by DDR also have an equally important role in deciding the fate of the irradiated cell [35]. Moreover, ERK is phosphorylated in A549 cells after exposure to 1 Gy γ-rays, but not after carbon-ion irradiation (LET 290 keV/μm), whereas JNK is transiently phosphorylated only after carbon-ion exposure [35], suggesting proapoptotic signal predominance after carbon-ion exposure.

Heavy-ion beams suppressed serine/threonine kinase B (AKT) survival signaling and might enhance caspase activation for carbon-ion–induced autophagy and apoptosis [65].

The DDR results in activation of several transcription factors (reviewed in Hellweg et al [66]). Nuclear factor κB (NF-κB) is strongly activated in human cells by heavy ions, with an LET of 70 to 300 keV/μm [67, 68]. Its role in the radiation response as a link to the immune system was recently reviewed in Hellweg [69]. That strong NF-κB activation by heavy ions does not protect cells from heavy-ion–induced cell death, but it does induce stronger expression of several cytokines and chemokines compared with x-irradiation [70].

The role of microRNAs in the cellular response to charged-particle exposure and in cellular radiosensitivity is still unclear; for low-LET radiation, in addition to ATM, BRCA1, and transcription factors (p53, NF-κB, Myc, and E2F), ΔNp73 was suggested as a potential microRNA expression regulator in that response [71].

Radiation quality has been suggested to be the most significant source of variation in cellular signaling and overall gene expression [43, 70, 72–74].

Cardiovascular System

In the heart (and bone marrow) from ^{28}Si ion (LET 77 keV/μm)-irradiated mice, cleaved PARP-1, activated NF-κB, and interleukin (IL)-6 and IL-1β remained elevated for 1 week to 6 months [75]. In cardiomyocytes isolated from mice 28 days after their exposure, Fe ions (LET 155 keV/μm, 150 mGy) regulated a long-lived signaling mechanism for ERK1/2 and p38 MAPK signaling, with NFATc4, GATA4, STAT3, and NF-κB as regulators of the response [76].

Endothelial cells can be relevant to pathophysiologic manifestations of radiation toxicity in many organs, and their dysfunction was observed in response to γ-irradiation [77]. Both helium-ion (LET 76 keV/μm) and x-ray (250 kV) exposure (0.1-2 Gy) of human microvascular endothelial cells decreased TNF-α–induced leukocyte adhesion to endothelial cells under laminar conditions [78]. Baselet et al [79] compared differences in signaling after exposure of endothelial cells from human coronary arteries to x-rays and ^{56}Fe ions (LET 155 keV/μm). Endothelial inflammation and adhesiveness increased with x-rays (250 kV) but decreased after ^{56}Fe-ion exposure (155 keV/μm). Moreover, 2-Gy x-rays and iron ions both enhanced the expression of proteins involved in caveolar-mediated endocytosis signaling and cell-cell adhesion [79]. After x-irradiation, genes involved in cell-cycle control were upregulated, whereas cell-adhesion genes were downregulated. After ^{56}Fe-ion exposure, p53 and genes controlling apoptosis were upregulated [79]. In the human endothelial cell line EA.hy926, ^{58}Ni-ion (LET 183 keV/μm) exposure induced expression of genes involved in endothelial permeability and apoptosis signaling [80].

Iron ions induce proatherosclerotic processes in endothelial cells that are different in nature and kinetics than those induced by x-rays [81].

Lung

In a human bronchial epithelial cell line, the acute phase response pathway was more strongly activated by heavy ions (^{56}Fe, LET 150 keV/μm; ^{28}Si, LET 44 keV/μm) compared with γ-irradiation (LET 0.2 keV/μm) [43]. In general, gene expression patterns induced by different radiation species were related to distinct ionization densities but not to delivered dose [43]. Notch signaling, which is involved in regulation of cell fate and differentiation, proliferation, and migration during development, was specific to ^{56}Fe-ion (LET 150 keV/μm), and phospholipase C signaling was specific to ^{28}Si-ion (LET 150 keV/μm) irradiation, whereas genes involved in inhibition of angiogenesis, cell migration, and invasion; proapoptosis signaling; and mechanisms of viral exit from host-cells pathways responded only to γ-irradiation [43].

Gastrointestinal Tract

The intestinal epithelium undergoes continuous renewal with proliferation, differentiation, migration, and apoptosis. Deregulated WNT signaling with transcriptional coactivator β-catenin and ubiquitin-proteasome pathway has been implicated in colorectal carcinogenesis [2].

In Fe-ion (LET 148 keV/μm, 1.6 Gy)–induced intestinal tumors in mice, long-term accumulation of the transcription factor TCF4 and its coactivator β-catenin was found, which can upregulate the target genes c-Myc and cyclin D1. After exposure to 1.6 Gy ^{56}Fe ions, compared with 2 Gy γ-rays, a stronger decrease in expression of adenomatosis polyposis coli–independent retinoid X receptor α (RXR-α) was observed in tumors and in tumor-free areas of the intestine [2, 82].

Immune System

Lymphocytes depend on survival signals and are particularly prone to radiation-induced apoptosis. Leukocytes decrease after acute ^{56}Fe-ions exposure in mice, and lymphocyte populations in blood and spleen exhibit varying degrees of susceptibility (B > T > NK and T cytotoxic > T helper cells) [81].

In the p53 wild-type human lymphoblastoid cell line TK6, a large set of histone genes was downregulated 24 h after exposure to equitoxic doses of high-LET (1.67 Gy ^{56}Fe ions, LET 148 keV/μm) or low-LET (2.5 Gy γ-rays) radiation [83]. Both high- and low-LET radiation exposure negatively regulated histone gene expression in human lymphoblastoid cell lines independent of p53 status [83].

Nervous System

Neuronal cells as terminally differentiated cells with long dendrites and an axon represent special charged particles' targets. A recent modeling approach visualizes the microscopic energy deposition in hippocampal neurons [84]. In rats, persistent changes in the expression of NMDA receptor subunit genes were observed 3 months after exposure to 0.6 Gy iron ions (LET 150 keV/μm), affecting hippocampal glutamatergic transmission [85]. In hippocampal slices from proton-exposed mice, inhibitory GABAergic synaptic transmission was decreased 3 month later [86]. In female mice, 2 and 12 months after exposure to 1.6 Gy ^{56}Fe ions (LET 150 keV/μm), levels of reactive oxygen species (ROS) were persistently raised in cerebral cortical cells with concomitant lipid peroxidation. DNA repair proteins were decreased, whereas DDR marker proteins and expression of nestin and glial fibrillary acidic protein (GFAP), the major intermediate filament protein of mature astrocytes, were increased in the cerebral cortex [51].

Intercellular Molecular Signaling after Charged Particle Exposure

In bystander effects, unirradiated cells receive signals either from nearby irradiated cells [8, 87] via gap-junction and medium-mediated diffusion or are cultured in medium transferred from previously irradiated cell cultures [88, 89] (reviewed in Prise and O'Sullivan [90]). After tumor irradiation, rescue or sparing effects mediated by healthy tissue, as well as detrimental effects on bystander cells, can occur [91]. The DDR is a sensible starting point for bystander signaling after exposure to high-LET irradiation. Bystander effects elicited by exposure to carbon ions (LET 76 keV/μm) were diminished after inhibition of DNA-PKcs and ATM in irradiated cells [92]. After α-particle exposure, ATM initiates bystander signaling, which is mediated via NF-κB regulated cytokines (IL-6, IL8, IL-33, TNF, and TRAIL) and leads to activation of different pathways, such as JAK2-Stat3, MAPK, or NF-κB, in bystander cells [93]. NF-κB activation,

cyclooxygenase-2 (COX-2) upregulation, and DNA damage in bystander cells form a positive-feedback loop (LET 13-1130 keV/μm) [94–96]. Activation of ERK and p38 signaling pathways in bystander cells occurred after α-particle exposure (LET 120 keV/μm) as well as p53-independent ROS production after C-ion (LET 30/70 keV/μm) and Fe-ion (LET 180 keV/μm) exposure [61, 97]. Gap-junction inhibition diminished bystander effects after C-ion exposure (LET 76 keV/μm) [98].

Relevance of Molecular Signaling for Particle Therapy

Molecular signaling is important for the therapeutic outcome of proton and carbon-ion radiotherapy via (1) killing of tumor cells, (2) acute damage to healthy tissue, and (3) chronic and late effects.

Killing Tumor Cells

The DDR proteins represent excellent targets to augment radiotherapy. Clinical trials combining DDR inhibitors, radiation, and genotoxic chemotherapy are ongoing [16]. High-LET charged particles induce an intense DDR. The potential of DDR inhibitors should be evaluated for radiosensitization of tumors with defective or enhanced signaling. Patient-derived glioblastoma cell lines were more resistant to x-rays and carbon ions when ATM signaling is impaired [99]. Human non–small cell lung cancer models were sensitized to photon and carbon-ion (in the spread-out Bragg peak region) irradiation by ATM and DNA-PK inhibitors, whereby the sensitizing effect for carbon-ion exposure was stronger for the DNA-PK inhibitor compared with the ATM inhibitor [100].

An understanding of direct effects of charged-particle irradiation on the immune system [101] and indirect effects on immune cells via immunogenic death of tumor cells [101, 102] with release of damage-associated molecular patterns (DAMPs) [103, 104] is a prerequisite for developing effective immunotherapy in combination with charged-particle irradiation [105]. Currently, there is a lack of preclinical in vivo data combining proton therapy and immunotherapy, and the number of preclinical studies with carbon ions is very limited [101].

Acute Damage to Healthy Tissue

In spite of excellent healthy tissue sparing by the favorable dose distribution in charged-particle therapy, concerns about healthy tissue exposure in carbon-ion therapy exist because of higher relative biologic effectiveness [2]. Cell death-associated healthy-tissue complications might result. Therefore, DDR mitigation is suggested for radioprotection of healthy tissue [16]. Furthermore, the stronger cytokine and chemokine expression after heavy-ion exposure [70] might contribute to inflammatory reactions and represent a suitable target for reduction of acute side effects.

Chronic and Late Effects

Potential late radiation effects encompass secondary cancers, hereditary effects, and degenerative diseases. The major degenerative late effects [106] that can result from exposure to high-energy charged particles are: late damage to the central nervous system, cataract formation, cardiovascular diseases (vascular damage, accelerated atherosclerosis, myocardial fibrosis, and cardiac conduction and valve abnormalities), fibrosis, and other diseases related to accelerated senescence, including digestive and respiratory diseases and endocrine and immune system dysfunctions [107–111]. Recent investigations have shown strikingly long-lasting changes in molecular signaling. Small-molecule inhibitors targeting the involved pathways might interrupt the deleterious signaling changes.

Behind the tumor, a low-dose tail is produced by particles travelling beyond the Bragg peak [112]. After sublethal doses, the failure to eliminate mutated cells or cells with chromosomal aberrations can result in carcinogenesis and cataracts [112]: for example, after pelvic irradiation, a secondary cancer in the colorectal region might develop [56]. Recent studies have revealed the role of the ubiquitin-proteasome pathway for secondary tumor formation in the gastrointestinal tract after Fe-ions exposure [2].

Discussion

Charged particles induce complex DNA damage, resulting in immediate recruitment of DNA damage sensors and repair proteins and growth of DNA repair foci for up to 1 hour and in DDR initiation. Because repair of complex damage is slow and incomplete, a strong and sustained DDR might shape the cellular response and possible acute and late effects. Cellular signaling after radiation exposure varies among different cell types and also depends on time, dose, and radiation quality,

where in general, the effect of high-LET charged particles on signaling can be more pronounced and longer lasting compared with low-LET radiation because of complex DNA damage [79]. Signaling pathways elicited by densely ionizing radiation can be quite different to those activated by sparsely ionizing radiation [106]. Many studies were performed with HeLa, U2OS or 293/ human embryonic kidney cells, or human fibroblasts, and fragmented knowledge for some relevant organ systems exists. In tumors, cell-specific abnormalities in the DDR machinery might exist [1].

The initial repair foci formation after charged particle exposure is already described in detail, but many of those studies were performed with very high-LET heavy ions (>1000 keV/μm), which do not represent the therapeutically relevant LET range of approximately 30 to 70 keV/μm carbon ions. In addition, many studies were performed with heavy ions relevant for the chronic cosmic ray exposure during spaceflight, such as 1 GeV/n Fe ions, which have a higher relative biologic effectiveness for various endpoints than do therapy relevant carbon ions. Knowledge about pathway activation by charged particles, in addition to the central foci formation, DNA repair induction, and cell cycle arrest, is rather fragmented, with a focus on the role of p53, LET-dependent activation of NF-κB, and MAPK regulation. The criteria for selection of a particular cell-death pathway, considering the full concert of signaling pathways, warrant further investigation after charged-particle exposure, in tumor cells as well as healthy cells. There is also a huge knowledge gap between these signaling events occurring within the first hours up to 1 day after exposure and the sustained signaling changes observed in irradiated animals weeks or months later, when complex damage should be repaired. The underlying mechanisms for those long-term changes are still under investigation; for example, mitochondrial damages and sustained ROS production are suggested. With hypofractionation (1-3 fractions with a very high dose [up to 25-30 Gy]), more or other pathways might be modulated compared with lower doses per fraction. Therefore, molecular profiling studies with relevant tumors in the high dose range are required to personalize radiotherapy in cases of therapy resistance.

More research on the healthy tissue response to charged particle irradiation is required to understand the role of signaling in low-dose tissue effects at tumor margins because gene expression might affect the risk of radiation-induced, secondary cancer.

ADDITIONAL INFORMATION AND DECLARATIONS

Conflicts of Interest: The authors have no conflicts to disclose.

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