

review

Carcinogenesis induced by low-dose radiation

Igor Piotrowski¹, Katarzyna Kulcenty^{1,2}, Wiktoria Maria Suchorska^{1,2}, Agnieszka Skrobata^{2,3}, Małgorzata Skórska³, Marta Kruszyna-Mochalska^{2,3}, Anna Kowalik³, Weronika Jackowiak⁴, Julian Malicki^{2,3}

¹ Radiobiology Laboratory, Department of Medical Physics, Greater Poland Cancer Centre, Poznań, Poland

² Department of Electroradiology, University of Medical Sciences, Poznań, Poland

³ Department of Medical Physics, Greater Poland Cancer Centre, Poznań, Poland

⁴ Radiotherapy Ward I, Greater Poland Cancer Centre, Poznań, Poland

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Correspondence to: Dr. Katarzyna Kulcenty, Radiobiology Laboratory, Department of Medical Physics, Greater Poland Cancer Centre, Garbary 15 Street, Poznań, Poland. E-mail: katarzyna.kulcenty@wco.pl

Igor Piotrowski and Katarzyna Kulcenty contributed equally to this work.

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Background. Although the effects of high dose radiation on human cells and tissues are relatively well defined, there is no consensus regarding the effects of low and very low radiation doses on the organism. Ionizing radiation has been shown to induce gene mutations and chromosome aberrations which are known to be involved in the process of carcinogenesis. The induction of secondary cancers is a challenging long-term side effect in oncologic patients treated with radiation. Medical sources of radiation like intensity modulated radiotherapy used in cancer treatment and computed tomography used in diagnostics, deliver very low doses of radiation to large volumes of healthy tissue, which might contribute to increased cancer rates in long surviving patients and in the general population. Research shows that because of the phenomena characteristic for low dose radiation the risk of cancer induction from exposure of healthy tissues to low dose radiation can be greater than the risk calculated from linear no-threshold model. Epidemiological data collected from radiation workers and atomic bomb survivors confirms that exposure to low dose radiation can contribute to increased cancer risk and also that the risk might correlate with the age at exposure.

Conclusions. Understanding the molecular mechanisms of response to low dose radiation is crucial for the proper evaluation of risks and benefits that stem from these exposures and should be considered in the radiotherapy treatment planning and in determining the allowed occupational exposures.

Key words: low-dose radiation; carcinogenesis; DNA; radiation induced bystander effect

Introduction

Ionizing radiation is widely used in medical procedures, including cancer radiotherapy and the use in diagnosis.¹ For over a century the biological effects of medium- and high-dose radiation (over 0.5 Gy) on human health have been investigated. Induction of cancer is one of the most severe long-term effects of radiotherapy. While the relationship between radiation effects and dose is well defined at higher doses, the effects of doses below 0.5 Gy are still unclear. Even though the use of radiotherapy resulted in a significant increase in cancer survivors², it is important to investigate the potential

negative long-term effects of radiation, considering that further advancements in therapy result in increased length of patients life after therapy. Table 1 presents the classification of radiation doses according to Kadhim *et al.*³ paired with examples of exposures.

The damage to DNA is induced by radiation through two mechanisms: the direct and indirect effect.⁹ Directly induced damage results from the deposition of radiation energy in the DNA molecule creating a break. Damage induced indirectly is a result of an attack of the reactive species, which might be produced from ionization of water, on DNA molecule. These processes induced by radia-

TABLE 1. Dose ranges and sources of radiation exposure

	Dose range	Examples of exposure
Very low doses	< 0.05 Gy	Mammography ⁴ , chest X-ray ⁴
Low doses	0.05–0.5 Gy	Cardiac CT angiogram ⁴
Medium doses	0.5–5 Gy	One fraction dose in standard fractionated radiotherapy ⁵ , dose absorbed by workers during Fukushima accident ⁶
High doses	5–15 Gy	Intraoperative radiotherapy (as boost) ⁷
Very high doses	> 15 Gy	Intraoperative radiotherapy ⁸ , cumulative dose delivered during fractionated radiotherapy ⁵

tion result in creation of single-strand breaks (SSB) and double-strand breaks (DSB) in DNA molecule.¹⁰ DSBs are prime lesions induced by ionizing radiation, and are responsible for its deleterious effect. Densely ionizing radiation has the ability to induce more damage per cell, making it possible for two or more lesions to be induced in proximity. These groups of breaks are called clustered DNA lesions, and are harder to repair than single DSBs and SSBs.¹¹ Density of radiation correlates with reparability of DNA lesions, with densely ionizing radiation being harder to repair. Radiation stimulates DNA damage repair through non-homologous end-joining (NHEJ)¹², an error prone pathway that can lead to induction of chromosome aberrations, which in turn may cause genomic instability.¹³ Genomic instability has been connected to induction of most of the human cancers, however currently the exact mechanism of radiation-induced carcinogenesis is not clear.

The dose-rate of radiation plays a major role in how much damage an exposed cell receives. High

dose-rate irradiation, like radiation created by atomic bomb, results in deployment of energy in very short time to every irradiated cell. However if radiation is protracted over long periods of time, like the dose-rates considered in calculations of occupational and environmental radiation risks, the cell turnover influences how many 'hits' a cell will receive. Low-dose rates also influence how cell reacts regarding the repair of DNA damage, since low-dose rates allow more time for damage to be repaired which makes it more favorable for cells than high-dose rate radiation.¹⁴

This review contains a description of biological consequences of low-dose radiation and possible induction of cancer (Figure 1).

DNA damage

Damage to DNA is one of the most important factors in radiation induced cancer transformation. Even though ionizing radiation induces one DSB per 20 SSBs, research indicates that DSBs are much more impactful effect of irradiation (IR).¹⁵ This difference might be caused by the fact that SSBs are repaired by error-free mechanisms and are not sustained, while DSBs induced by radiation are repaired by mechanisms prone to mis-repair and repair failure, making DSBs a main cause of radiation induced cell death.¹⁶ Recently many authors pointed towards important differences between DNA damage response exerted by high and low doses of radiation.^{17,18} Research of low dose effect on normal tissues is especially significant considering side effects of radiotherapy treatment.

Induction of DSBs is considered to be one of the main mechanisms by which radiation exerts its deleterious effect. One of the earliest events appearing in response to DSB induction is phosphorylation of histone H2AX by protein kinases: DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia telangiectasia and Rad3-related (ATR) and ataxia telangiectasia mutated (ATM).¹⁹ Phosphorylated H2AX (γ H2AX) foci appears around the DNA break as a part of DSB repair mechanism²⁰ and it corresponds directly with the number of DSBs.²¹ The correlation between the radiation dose and the initial number of γ H2AX foci it induces has already been thoroughly described.^{21,22} Rothkamm *et al.*²³ have shown that this linear correlation holds true also for the X-ray doses as low as 1 mGy (at dose rate of 6–60 mGy/min) for nondividing human fibroblasts cultured *in vitro*. Interestingly, authors came to the conclu-

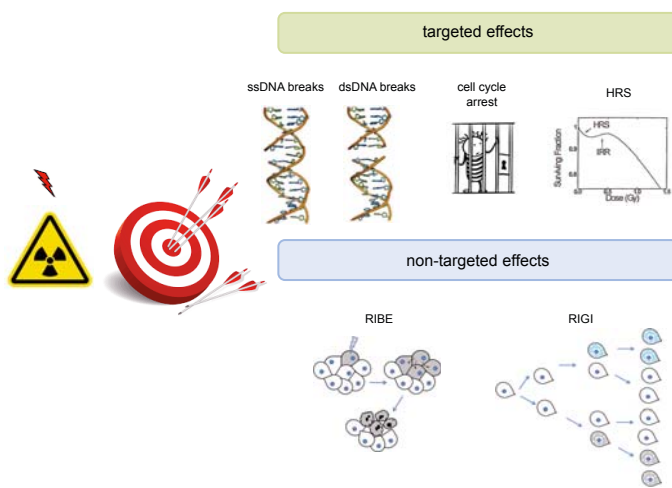


FIGURE 1. Diagram illustrating targeted and non-targeted effect induced by radiation (all described in the main text).

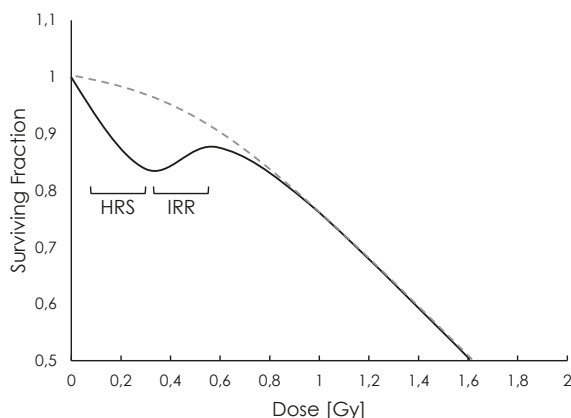


FIGURE 2. Schematic diagram illustrating cell survival curve for cells exhibiting low dose hyper radiosensitivity (HRS). Solid line shows survival curve with evidence of HRS and increased radioresistance (IRR). Dashed line shows survival curve with no evidence of HRS and IRR. Image adapted from.²⁹

sion that the damage induced by these very low doses remains unrepaired and evidence suggests that the cells with unrepaired DSBs are eliminated. Similar results were observed recently by Osipov *et al.* in research conducted on human MSCs isolated from oral mucosa.²⁴ Authors measured kinetics of γ H2AX and pATM foci formation after irradiation with X-rays at doses ranging from 20 mGy to 250 mGy and a dose rate of 40 mGy/min. Linear correlation between the number of γ H2AX foci and radiation dose was observed at all time-points after irradiation. For the intermediate doses (160 and 250 mGy) the highest number of foci was observed at 60 min followed by a significant decrease, which is typical for γ H2AX kinetics observed at higher doses. However for the lower doses (20, 40 and 80 mGy) after initial rise in γ H2AX foci number no decrease was observed. Considering the fact that pATM foci did not co-localize with γ H2AX foci at low doses and the fact that low dose radiation stimulates the MSC proliferation²⁵, authors propose that the persistence of γ H2AX foci after irradiation with low dose X-rays might be a consequence of new DSBs appearing as a result of replication stress rather than resulting from inefficient repair of DSBs. It is important to note that doses used in these experiments are in range of doses used in diagnostic procedures like CT scan.²⁶ However high linear energy transfer (LET) radiation has also been shown to induce ATM-dependent responses at low doses.²⁷ Fibroblasts isolated from patients with ataxia telangiectasia and healthy persons were irradiated with carbon ion beams (LET = 70 keV/ μ m) or with X-rays at doses between 0.1 to 2 Gy. Results

have shown that mutation frequency and cell survival after low dose carbon ion irradiation were dependent on ATM status. Authors also compared the number of γ H2AX foci in cells with different activity of ATM irradiated with 0.2 Gy of carbon ion radiation. Results indicate that ATM plays an important role in early DSB recognition and can be responsible for inefficient DSB repair, which is in line with conclusions reached by Osipov *et al.*²⁴ Authors also observed that doses higher than 0.5 Gy of carbon ion radiation caused an early G2/M arrest dependent on ATM. Some authors suggest that occurrence of this arrest might support DNA DSB repair, while a failure to induce early G2 arrest can be one of the factors responsible for low dose hyper-radiosensitivity (HRS).²⁸

Low-dose hyper radiosensitivity (HRS)

HRS is described as an increase in radiosensitivity in cells exposed to low-dose radiation, usually below 0.2–0.3 Gy for low LET radiation. When cells are exposed to doses higher than 0.3 Gy an increase in radioresistance is observed and the transition towards it on dose response curve is described as increased radioresistance (IRR).²⁹ Figure 2 presents the dose-response curves with and without the evidence of HRS and IRR. Occurrence of HRS and IRR in cells was first described using an *in vitro* culture of V79 Chinese hamster cells.³⁰ Cells were exposed to X-ray radiation doses ranging between 0.01 and 10 Gy. Using clonogenic assay authors have shown increased effectiveness of X-ray doses below 0.2 Gy. Cells exposed to doses higher than 0.4 Gy experienced a rise in radioresistance. Authors hypothesized that only X-ray doses above certain threshold can induce enough damage to activate DNA damage repair mechanisms. The dependence of HRS occurrence on cell cycle phase was also firstly noted in this research. Since then, research of HRS on different cell lines reported that HRS is increased in G2-phase cells.^{31,32} To prove this concept Short *et al.*³¹ used two glioma cell lines – T98G exhibiting HRS and U373 that did not exhibit HRS. Using cell counting authors have shown that G2 population of T98G cells irradiated with low dose X-rays demonstrated more pronounced HRS than cells in asynchronous population. Interestingly the G2 population of U373 cells also exhibited HRS indicating that in the asynchronous population the response of G1 and S-phase cells dominates. These results show that actively proliferating cells might

be more sensitive to low doses of radiation. It is important to note, that HRS can potentially be used to therapeutic advantage. Using ultra-fractionation with doses in HRS range it is possible to achieve more cytotoxic effects than after administration of same total dose at once.³³

Cell cycle arrest

In order to repair the damaged DNA, irradiated cells utilize cell cycle arrest to allow sufficient time for the DNA repair. The most important checkpoint pathways that initiate the radiation-induced arrest inhibit the cell cycle progression from G2 phase to mitosis. Two checkpoints are utilized for the repair of DNA damage induced by radiation. The classic G2/M checkpoint is activated after high doses of radiation and its role is to arrest the cells damaged in S or G1 cell cycle phase.³⁴ The early G2/M checkpoint is activated shortly after exposure to radiation and it exclusively protects cells in G2 phase of cell cycle from radiation effects.³⁵ This checkpoint is especially relevant to assessment of low-dose radiation risks because induction of this checkpoint occurs after irradiation with doses up to 10 Gy of low LET radiation³⁵ and a threshold for its activation is observed at radiation doses of 0.3 Gy.³⁶ This threshold dose coincides with radiation doses at which a transition between HRS and IRR is observed, suggesting a correlation between the two phenomenon, which was first suggested by Marples *et al.*³⁶ The activation of early G2/M checkpoint is mediated by radiation-induced, DSB-dependent phosphorylation of ATM.³⁷ It has been hypothesized that ATM acts by an interplay with Chk1 and Chk2.³² Inhibition of these factors in fibroblast cells results in an enhanced HRS response, however it also causes an increase in IRR. It is important to note that the response varied depending on the HRS status of the cell lines used.

While even very low doses of radiation are capable of inducing DSBs, there is evidence suggesting a threshold number of 10–20 DSBs for the triggering of ATM dependent G2/M checkpoint.³⁸ This can mean that low doses of radiation inducing few DSBs may not trigger G2/M arrest in irradiated cells, making it possible for cells with unrepaired DSBs to enter mitosis, which in turn might result in loss of genetic material.³⁹ Deckbar *et al.*⁴⁰ investigated induction of G2 arrest by X-ray radiation on immortalized fibroblasts and on primary human cells. γ H2AX foci count confirms, that cells irradiated with X-ray doses inducing fewer than ~20

DSBs (below 0.6 Gy for immortalized fibroblasts and below 0.2 Gy for primary human cells) did not enter G2 arrest. In cells irradiated with doses above the threshold dose, cells that exited the G2 checkpoint still had ~20 unrepaired DSBs and 1–2 chromosome breaks. These results indicate that a certain threshold of DSBs and chromosome aberrations is necessary for both activating and maintaining the G2 checkpoint arrest. The threshold dose at which G2 arrest is induced can play a role in low-dose radiosensitivity of a cell. Different cell lines show variability in this threshold dose. Although this threshold usually varies between 0.2 Gy and 0.6 Gy, in some cell lines the cell cycle arrest is induced without threshold.⁴¹ Currently the mechanism behind this difference in threshold dose is not known, however a hypothesis has been proposed stating that cell lines which show early G2 checkpoint after exposure to very low doses might have higher base level of DSBs, making it much easier to reach the number of DSBs needed for G2 arrest induction.⁴¹

Mutation in ATM gene observed in Ataxia telangiectasia, has been connected with increased chromosomal radiosensitivity and increased susceptibility to cancer.⁴² Since both DNA damage repair and G2 checkpoint induction depend on ATM kinase, Ataxia telangiectasia (AT) cells are often used to investigate the mechanisms of these processes. Using AT cells researchers were able to show for the first time that G2 checkpoint facilitates not only the repair of DSBs, but also the repair of chromosomal breaks, after irradiation with 1 Gy ionizing radiation.⁴³ Failure to activate the G2 checkpoint might be one of the reasons for increased cancer rates in patients with Ataxia telangiectasia, since unrepaired damage to DNA might alter cells genome leading to malignant transformation.⁴²

Non-targeted effects

Radiation induced genomic instability (RIGI)

Radiation induced DNA damage, specifically DSBs, are very important in occurrence of chromosomal instability, which may involve aneuploidy, deletions, and aberrations, which in turn might contribute to carcinogenesis.⁴⁴ The non-targeted effects, including bystander effects and genomic instability, contribute to the induction of cancer in a less clear way.⁴⁵ The progeny of exposed cell might exhibit phenotypes such as chromosomal aberrations and rearrangements, gene mutations and en-

hanced cell death rate.⁴⁶ Acquisition of alterations in the genome of progeny of irradiated cells is described as genomic instability (GI) and it has been accepted as the hallmark of cancer cells and one of the most important factors involved in the development of some cancers.⁴⁴ RIGI was firstly observed in one-cell mouse embryos irradiated with X-rays and with neutrons.⁴⁷ The results suggested that chromosomal aberrations might appear de novo two or three mitoses after initial exposure and also that radiation of a different quality (different LET) induces aberrations at different frequencies.

In the context of RIGI the effect on genome is mediated via the accumulation of genetic changes in the progeny of surviving irradiated cells through many generations.⁴⁸ The capacity to induce genomic instability depends on both dose and quality of radiation, with high LET radiation generally inducing chromosome- and chromatid-type aberrations more effectively.⁴⁹ This effect was observed in murine bone marrow cells irradiated with 0.25 Gy, 0.5 Gy and 1 Gy of densely ionizing α -radiation.⁵⁰ Colonies arising from irradiated cells exhibited high frequency of non-clonally induced chromatid aberrations consistent with them arising de novo and not directly from irradiation. Early studies using human lymphocytes irradiated *in vitro* show the ability of low dose X-rays to induce chromosomal aberrations.⁵¹ Linearity of chromosomal aberration yield was observed above doses of 20 mGy while doses below 20 mGy resulted in yields lower than background. Many researchers have tried to elucidate the mechanisms behind RIGI, especially induced by low dose radiation. Maxwell *et al.* observed that in HMEC cells irradiated with X-ray doses ranging from 10 to 200 cGy frequency of chromosomal aberrations increased with dose.⁵² In cells irradiated with 50 cGy the frequency of chromosome aberrations increased with time after irradiation up to 72 h. Interestingly, administration of TGF- β , a growth factor whose activity is induced by radiation, resulted in elimination of cells with chromosome aberrations. The elimination was mediated by induction of p53-dependent apoptosis in genomically unstable cells. Authors also concluded that chromosomal aberrations induced in this experiment are a targeted effect, as induction of chromosomal aberrations above 10 cGy was proportional to dose.

TGF- β signaling can be induced in nontransformed cells irradiated with very low doses of radiation. It has been shown that TGF- β signaling selectively induces reactive oxygen species (ROS) production in transformed cells leading to their

apoptosis.⁵³ This signaling pathway could be a part of surveillance network protecting cells from malignant transformation. It is important to note, that radiation induced EMT phenotype can be mediated by TGF- β , making it a "two-edged sword" in need of further investigation.⁵⁴ Authors suggest that while TGF- β might play a role in elimination of irradiated, genomically unstable cells, the chronic exposition to radiation and TGF- β signaling can induce EMT in remaining cells.⁵³

Dicentric chromosomes are an established marker used to assess IR induced chromosomal aberrations.⁵⁵ Using the measurement of dicentric frequency in human fibroblasts after irradiation, researchers were able to investigate the role of dose-rate effect in induction of chromosome aberrations.¹³¹ In cells irradiated with a single X-ray dose the frequency of dicentrics correlated exponentially with doses higher than 0.2 Gy. Irradiation of cells with a single 1 Gy dose of X-rays resulted in similar rise of frequency of dicentrics as irradiation with fractionated 1 Gy dose (fractionation: 10 x 0.1 Gy; 5 x 0.2 Gy; 2 x 0.5 Gy) given with intervals of 1 min between fractions. When intervals between fractions were increased above 5 min, the frequency of dicentric chromosomes was significantly lower, which suggests that the maximum of DNA damage repair, in relation to chromosome dicentrics, is reached after 5 min. These results show that high dose rate irradiation is more harmful to the genome than low dose rate. The measurement of frequency of dicentrics in patients lymphocytes could be utilized in examination of radiosensitivity. Linear dose response of dicentric frequency was observed in human lymphocytes after irradiation with X-ray doses above 20 mGy.⁵¹ Frequency of dicentrics was also used to evaluate human lymphocytes response to low LET γ -rays. While for doses above 20 mGy a linear increase in dicentric frequency was observed, authors were not able to detect statistically significant changes in dicentric frequencies below 20 mGy doses, despite counting over 5000 metaphases.⁵⁶ This means that dicentric frequency count might not be a precise enough method for assessment of response to very low doses.

Aneuploidy is a phenotype very often observed in tumors and it arises from incorrect chromosome segregation during mitosis.⁵⁷ Delayed appearance of aneuploid cells induced by low-dose radiation has been observed *in vitro* in human fibroblasts.⁵⁸ Fibroblasts were irradiated with X-ray doses of 20, 50 or 100 cGy and cultured for five passages. As an end point authors picked assessment of mi-

micronuclei (MN) formation with special probes for analysis of structural and numerical aberrations⁵⁹, with chromosomes 1 and 4 picked for aneuploidy analysis. Micronuclei are generated in the progeny of irradiated cells as a result of *de novo* generation of chromosome aberrations.⁴⁹ After X-ray irradiation with doses of 50 and 100 cGy frequencies of centromere-positive and negative MNs increased significantly. Frequency of aneuploidy increased with dose in a dose-dependent manner and after irradiation with 50 and 100 cGy the increase in aneuploidy with time was observed. The data show that after irradiation with low doses, chromosomal aberrations appear *de novo* up to five population doublings, which as authors suggest, might be connected with variability in the number of centrosomes.⁶⁰

One of the radiation induced phenotypes used to investigate genetic effects of low-dose radiation is loss of heterozygosity (LOH). LOH is conventionally associated with cancer as a mechanism inactivating tumor suppressor genes, however it is also found in regions of genome responsible for cancer induction.⁶¹ For research on induction of mutations *in vitro*, TK6 cells are often used, as this cell line contains heterozygosity at the thymidine kinase (TK) locus. In one of the experiments irradiation of TK6 cells with 10 cGy X-rays caused a significant increase in hemizygous LOH.⁶¹ Hemizygous type of LOH is a result of homologous end joining, a repair pathway observed after irradiation. Umabayashi *et al.* analyzed induction of LOH in TK6 cells after irradiation with low-dose, low dose-rate γ -irradiation.⁶² Even irradiation with the dose as low as 30 mGy increased the frequency of early mutations in TK gene almost 2-fold. Authors claim that detection of LOH is an efficient system for estimation of genetic effects induced by low-dose radiation.

Radiation induced bystander effect

Another non-targeted effect playing a role in carcinogenesis is radiation induced bystander effect (RIBE). This effect together with other non-targeted effects, is often described as most relevant to low-dose radiation⁶³ and is mediated through two mechanisms: secretion of soluble factors by irradiated cells⁶⁴ and also by signaling through cell-to-cell junctions.⁶⁵ One of the early works in which this effect was described used Chinese hamster ovary (CHO) cells irradiated with low doses of α -radiation.⁶⁶ The authors chose very low radiation dose (0.31 mGy), so that less than 1% of cells

were traversed through by the radiation. After irradiation close to 30% cells exhibited presence of sister chromatid exchanges which suggests, that the genetic damage was induced even in the cells whose nuclei was not traversed through by radiation. Authors speculated that this effect might have been mediated through the production of ROS by the cells which were irradiated directly. The occurrence of RIBE was also confirmed for very low doses of X-ray radiation in human fibroblasts.⁶⁷ After irradiation with doses ranging from 1.2 to 200 mGy the induction of DSBs, measured by ATM phosphorylation, followed a supralinear relationship. While cells were treated with an inhibitor of gap junction intercellular communication, the number of DSBs induced by radiation was smaller than in untreated cells. The largest differences between treated and untreated cells were observed at doses between 1.2 to 5 mGy, meaning that RIBE has the most influence on DSB induction at doses up to 10 mGy. RIBE has also been shown to induce mutations in bystander cells and the mechanism of these mutations is different than in the cells irradiated directly.⁶⁸ In bystander CHO cells, the frequency of deletions was higher after using 10 cGy α -radiation and after using 0.5 cGy α -radiation the frequency of point mutations was higher. Very low doses of α -radiation also have the ability to induce ROS production in irradiated cells⁶⁹ and ROS has been shown to induce point mutations⁷⁰ making it the likely mechanism for induction of mutations in bystander cells. However it is not clear whether the effect is mediated through ROS produced by irradiated cells and transferred to unirradiated cells, or the irradiated cells produce factors inducing ROS production in unirradiated cells.

The role of factors secreted by irradiated cells has also been confirmed for low-dose induced RIBE. Seymour and colleagues⁷¹ irradiated human keratinocytes with doses ranging from 0.01 to 0.5 Gy of γ -radiation. Then the medium from irradiated cells was collected and transferred to unirradiated cell culture. The results of clonogenic assays measuring clonogenic death of cells show that RIBE mediated by the secreted substances is most predominant in doses below 0.5 Gy. RIBE has also been confirmed to occur *in vivo* and cause chromosomal instabilities. To first demonstrate this effect *in vivo*, Watson *et al.*⁷² transplanted a mixture of irradiated (0.5 Gy, neutrons) and unirradiated bone marrow cells into unirradiated recipient. After transplantation some of the progeny of the unirradiated donor cells showed chromosomal aberrations induced through the bystander effect.

Evidence has also been presented for oncogenic effect of RIBE in *in vivo* mouse model, however the effect was observed only after irradiation with 3 Gy of X-rays.⁷³ Currently no research was able to confirm with confidence the oncogenic effect of low-dose RIBE, and even the exact mechanism of RIBE *in vivo* is not clear.⁷⁴ Presented results suggest that bystander effect plays an important role in induction of mutations after low dose irradiation, making it a potential oncogenic factor.

Conclusions

Radiation to which humans are exposed comprises mainly of low-dose and low-dose rate radiation from both natural and man-made sources. In recent years the biological effects of low-dose radiation became a point of interest due to the increase in popularity of radiation therapy and diagnostic radiology. Even though many studies point toward a link between carcinogenesis and exposure to radiation, the exact mechanism is still not clear. Induction of genomic instability is suspected to play a major role in malignant transformation after high-dose irradiation, and it might be responsible for carcinogenesis after exposure to lower doses. Latest research suggests that phenomena characteristic for low-dose exposures like HRS and RIBE might be the factors contributing to induction of genomic instability after exposure. Better understanding of these processes is crucial for the proper estimation of low-dose exposure risks for radiation workers, patients and people exposed to high background radiation.

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