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Fractionated exposure to low doses of ionizing radiation results in accumulation of DNA damage in mouse spleen tissue and activation of apoptosis in a p53/Atm-independent manner

Igor Koturbash^{*,†}, Matt Merrifield^{*}, Olga Kovalchuk

Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, Canada

Abstract

Purpose: While the effects of high doses of ionizing radiation (IR) are relatively well characterized, the molecular mechanisms underlying cellular responses to prolonged exposure to low doses of radiation remain largely under-investigated.

Materials and methods: Here, we addressed the DNA damage and apoptotic response in the spleen tissue of C57BL/6 male mice after fractionated exposure to X-rays within the 0.1–0.5Gy dose range.

Results: The response to initial exposure to 0.1 Gy of IR was characterized by increased DNA damage and elevated levels of apoptosis. Subsequent exposures (cumulative doses of 0.2 and 0.3 Gy) resulted in adaptive response-like changes, represented as increased proliferation and apoptotic response. Cumulative doses of 0.4 and 0.5 Gy were characterized by accumulation of DNA damage and reactivation of apoptosis and apoptosis-related proteins. Additionally, spleen cells with irreversible damage caused by radiation can undergo apoptosis via activation of p38, which does not necessarily involve the Atm/p53 pathway.

Conclusions: Fractionated exposure to low doses of X-rays resulted in accumulation of DNA damage in the murine spleen and induction of apoptotic response in p53/Atm-independent manner. Further studies are needed to understand the outcomes and molecular mechanisms underlying cellular responses and early induction of p38 in response to prolonged exposure to IR.

Keywords

Apoptosis; DNA damage; fractionated irradiation; low dose radiation; p53

Introduction

While the effects of high doses of ionizing radiation (IR) have been relatively well studied, the molecular mechanisms underlying cellular responses to prolonged exposure to low doses

CONTACT Dr Olga Kovalchuk olga.kovalchuk@uleth.ca Department of Biological Sciences, University of Lethbridge, 4401 University Drive, Lethbridge, Alberta, Canada T1K 3M4.

^{*}These authors contributed equally to the research, their names are listed in alphabetical order.

[†]Dr Igor Koturbash is currently at: Department of Environmental and Occupational Health, Fay W. Boozman College of Public Health, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA.

Disclosure statement

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of radiation remain largely under-investigated (Merrifield and Kovalchuk 2013; Calabrese and O'Connor 2014; Majer et al. 2014; Kamiya et al. 2015; Langie et al. 2015) Owing to the marked increase in accessibility of IR-based diagnostics and therapeutics and the introduction of innovative technologies that utilize IR, the number of people who receive chronic exposure to low doses of radiation via occupational, diagnostic or treatment-related modalities is progressively rising (Merrifield and Kovalchuk 2013). Even though recent advances in diagnostics and treatments utilizing IR have led to increases in cancer patient survival rates, the increased risk of radiation-induced health effects cannot be overlooked.

The biological consequences associated with low doses of IR effects constitute one of the high-priority issues facing modern cancer research; however, the data that has been obtained from studies of high doses of radiation can unfortunately not be directly applied to the effects of low doses of radiation exposure (Barcellos-Hoff et al. 2011). The linear correlation (increased dose leading to increased damage) that characterizes exposures to over 1 Gy of radiation is not relevant to lower doses.

Ionizing radiation is a DNA-damaging agent that can lead to the disturbance of the regular DNA structure, resulting from base damage, and/or single- or double-strand breaks (Jeggo and Löbrich 2006; Kovalchuk and Baulch 2008). This DNA damage can be repaired by cellular and molecular repair machinery or, if the damage is irreversible, the cell will necessarily undergo programmed death or apoptosis (Jeggo and Löbrich 2006). Apoptosis is an evolutionary conserved defensive mechanism adopted by cells to protect the living organismal system from development of potentially dangerous clonal genetic alterations (Elmore 2007). Apoptosis is also directly correlated to cell cycle delay. This cell cycle delay can be managed by two different mechanisms: the long-term response, which requires hours and the involvement of p53 (Pearce and Humphrey 2001; Mikhailov et al. 2005), or a more rapid mechanism that occurs within minutes after introduction of a stressor and is often characterized by involvement of mitogen-activated protein kinase 14, or p38 (Mikhailov et al. 2005). This short-term mechanism does not necessarily require p53. Our previous studies on radiation-induced molecular changes in the spleen tissue of C57/BL6 mice have shown that p53 activation after exposure to 1 Gy of ionizing radiation requires more than 6 h to manifest and can be observed up to approximately 96 h after treatment (Koturbash et al. 2008b). We have also previously reported that there is a rapid increase in the levels of p38 shortly after administration of 1 Gy of ionizing radiation in both directly and indirectly exposed murine spleen tissue (Koturbash et al. 2008c).

In previous studies, we have also demonstrated that fractionated exposure to low doses of ionizing radiation (cumulative dose of 0.5 Gy) leads to accumulation of DNA damage, as well as distortion of several epigenetic parameters in the hematopoietic tissue (Pogribny et al. 2005). However, this previous research evaluated only the final outcomes of fractionated radiation exposure; the intermediate events that could occur between the treatments remain to be revealed. To fill this gap, we have conducted the following study in order to further understand the time- and dose-dependent dynamics of the molecular mechanisms underlying response of radiation-target organ – murine spleen, during fractionated exposure to low doses of ionizing radiation (cumulative dose range of 0.1–0.5 Gy). For congruency, we have utilized the C57/BL6 strain of mice, the same strain that was used in our previous studies

(Pogribny et al. 2005; Koturbash et al. 2008b, 2008c) and focused on the same target organspleen.

Materials and methods

Animal model, irradiation of animals

In this study, we examined the molecular changes in the spleen of C57BL/6 male mice following in vivo whole-body irradiation exposure. Handling and care of the animals were in strict accordance with the recommendations of the Canadian Council for Animal Care and Use. Animals were kept in a virus-free facility at the University of Lethbridge in a temperature-controlled room with a 12-h light/dark cycle, housed three per cage, and given food and water *ad libitum*. The procedures were approved by the University of Lethbridge Animal Welfare Committee.

The 60-day-old animals were randomly assigned to control ($n = 8$) and treatment groups ($n = 60$) (Figure 1). At Day 1 of the experiment, 60 animals received a whole-body exposure to 0.1 Gy of X rays (5 cGy/s, 90 kV, 5 mA). Six hours after exposure to ionizing radiation, six animals were randomly chosen and humanely sacrificed. Twenty-four hours after administration of treatment (Day 2 of the experiment), another six mice were randomly chosen and humanly sacrificed. At the same time, the remaining 48 animals were exposed to a subsequent dose of 0.1 Gy of X rays (cumulative dose of 0.2 Gy). Previously described procedures were repeated on Days 3, 4, 5 and 6 of the experiment. The control mice were sham-treated. Half of the control animals ($n = 4$) were sacrificed on Day 1 of the experiment. The remaining animals ($n = 4$) were sacrificed on the last day, Day 6, of the experiment. In each dose group, animals were sacrificed 6 h after exposure to see the early effects, and 24 h after exposure to IR to study delayed effects. Spleen tissue was sampled upon sacrifice; half of the tissue was fixed in 4% neutral formalin for immunohistochemical analysis; the other half was snap-frozen in liquid nitrogen and stored at -80°C for further molecular analysis.

DNA damage analysis

Total DNA was prepared from spleen tissues using the Qiagen DNAeasy™ Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A modification of the random oligonucleotide-primed synthesis assay (ROPS) was utilized to identify the presence of DNA strand breaks in high molecular weight DNA, as previously described (Basnakian and James 1996; Koturbash et al. 2008a).

Detection of apoptosis

To evaluate programmed cell death events in the murine spleen after fractionated exposure to low doses of IR, we used a terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling (TUNEL) assay. Slide sections were scored by determining the percentage of positive cells per field of view as a multiple of the stain intensity (0–3 scale based on darkness) in at least 20 high power fields, in a double blind manner. The score ($[\text{percent of positive cells per FOV}] \times [\text{intensity}]$) relative to those of control animals is shown as the mean $\pm$ SE; * $p < .05$.

Immunohistochemical analysis

Paraffin embedding and sectioning was conducted at Pantomics, Inc. (Richmond, CA). In brief, upon citrate buffer epitope retrieval, slides were rinsed and subjected to peroxidase blocking, followed by serum blocking to prevent the non-specific binding of immunoglobulins. Sections were then incubated overnight with either Atm, p53, p38, Bcl2, Ki67 or PCNA, and rinsed. The slides were then incubated with the secondary biotinylated antibody for at least 1 h, subjected to HRP-Streptavidin detection and counterstained with hematoxylin. Cells positive for Atm, p53, p38, Bcl2, Ki67 and PCNA presented as purple/brown, while negative cells stained blue. Tonsil tissue served as a positive control. Atm, p53, p38, Bcl2 and Ki67 indexes were quantified by enumerating Atm-, p53-, p38-, Bcl2- and Ki67-positive cells in at 12–20 high power fields in a double-blind manner. The numbers of Atm-, p53-, p38-, Bcl2- and Ki67-positive cells relative to those of control animals are shown as the mean $\pm$ SE; * $p < .01$. PCNA-stained slides were scored by determining the percentage of positive cells per field of view as a multiple of the stain intensity (0–3 scale based on darkness) in 12–20 high power fields in a double-blind manner. The score ([percent of positive cells per FOV] $\times$ [intensity]) relative to those of control animals is shown as the mean $\pm$ SE; * $p < .01$.

Immunofluorescence

Upon organ extraction, tissues were fixed in 4% PFA (pH 7.4) for 48 h and subsequently rinsed $\times 2$ in 1 $\times$ PBS (pH 7.4). Tissues were then stored in 70% ethanol for shipping. Paraffin embedding and assembly of 4.5 mm core tissue microarrays (TMA) was conducted by Pantomics, Inc. (Richmond, CA). Sectioning (5 μ m thick) of TMA was conducted at the Histology Department (Chinook Regional Hospital). In brief, upon citrate buffer epitope retrieval, slides were rinsed and subjected to serum blocking to prevent the non-specific binding of immunoglobulins. Sections were then incubated overnight with p38 MAPK rabbit antibody (Cell Signalling #9212). Slides were then washed 3 $\times$ 10 min in 1 $\times$ PBS (pH 7.4), incubated with Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen A11034) for 1.5 h and subsequently washed 3 $\times$ 10 min in 1 $\times$ PBS (pH 7.4) before dehydration and counterstaining with 4',6'-diamidino-2-phenyl-indole, dihydrochloride (DAPI) (Prolong Gold anti-fade reagent with DAPI Invitrogen).

Statistical analysis

Statistical analyses (Student's t -test with Bonferroni correction for multiple comparisons) were performed using the MS Excel 2007 software package. Each exposure group was compared to the corresponding control group. To counteract the problem of multiple comparisons we used Bonferroni correction, whereby the Bonferroni correction tests each individual hypothesis at a significance level of α/m , whereby m = number of comparisons. Hence, comparing five doses to control (testing five hypotheses) with a desired $\alpha = 0.05$, then the Bonferroni correction would test each individual hypothesis at $\alpha = 0.05/5 = 0.01$ (<http://mathworld.wolfram.com/BonferroniCorrection.html>). The difference between the groups was considered significant if the p -value was less than .01.

Results

Analysis of DNA damage in the mouse spleen after fractionated exposure to low doses of ionizing radiation

Ionizing radiation causes DNA damage. One of the most dangerous types of damage is the formation of double-strand breaks (DSB) that can result in genomic instability. Additionally, radiation exposure causes single strand breaks (SSB) and non-DSB bi-stranded oxidatively generated clustered lesions (Georgakilas 2011). For this reason, we initially assessed the levels of DNA damage in the murine spleen tissue after fractionated exposure to low doses of ionizing radiation. We utilized a modification of the well-established random oligonucleotide prime synthesis assay (ROPS), which detects DNA damage represented as single- and double-strand breaks (Koturbash et al. 2008a). We have found that initial whole-body exposure to 0.1 Gy of ionizing radiation leads to a significant, 1.3-fold (p -value $< .05$) increase in the level of DNA damage in the mouse spleen 6 h after the treatment (Figure 2). However, 24 h post treatment, levels of DNA damage were comparable with the pre-exposure background levels (Figure 2). Interestingly, the next exposure to 0.1 Gy of radiation (cumulative dose of 0.2) did not result in any significant change, possibly suggesting a radioadaptive mechanism. Conversely, starting at Day 3 of the experiment (cumulative dose of 0.3 Gy), a significant increase in the levels of DNA damage was detected 6 h after exposure, and was also observed over the next two exposures, reaching up to 1.45-fold increase at cumulative doses of 0.4 and 0.5 Gy. In addition, a significant increase in the level of DNA damage was also observed 24 h after these last two exposures, suggesting the persistent nature of this radiation-induced DNA damage.

Levels of cell death in the murine spleen after fractionated exposure to low doses of ionizing radiation

Exposure to ionizing radiation leads to DNA damage (Iliakis et al. 2003). The cell can repair this damage by activation of repair mechanisms/pathways or, in the case of damage irreversibility, the cell will necessarily undergo apoptosis. Apoptosis is a protective mechanism that prevents a cell with irreparable damage from giving rise to a genetically unstable population (clone). Our initial analysis of DNA damage revealed not only a significant increase in the DNA damage after initial exposure to ionizing radiation but also significant accumulation, suggesting the presence of irreversible damage. That being said, here we used ROPS method that depends on the preservation of the 3-OH groups and their availability for the polymerase extension. Sometimes, radiation exposure might affect the efficiency of strand break end trimming and 3'-OH processing. Therefore, in the future, it would be important to use other methods for DNA damage analysis, such as γ H2AX foci analysis or Comet assay (Tice and Strauss 1995; Redon et al. 2009; Pouliliou and Koukourakis 2014).

Having seen radiation-induced DNA damage, as a next step of our study, we addressed the apoptotic response to whole-body fractionated exposure to ionizing radiation, using a TdT-mediated dUTP nick-end labelling (TUNEL) assay. TUNEL is one of the most frequently used methods for detection of apoptotic cells and is a practical assay for determining radiation-induced cell death (Laposa et al. 2007; Vyas et al. 2007; Saito et al. 2008).

Apoptotic events were significantly induced after the first initial exposure to 0.1 Gy of ionizing radiation and after the fourth and fifth subsequent exposures (cumulative doses of 0.4 and 0.5 Gy, respectively) (Figure 3). These findings display a similar trend to those observed in the DNA damage assay, suggesting a causal relationship.

Molecular response to fractionated exposure to ionizing radiation

To analyze further the nature of this apoptotic response to low doses of fractionated IR, an immunohistochemical approach was employed. Using the HRP method, we evaluated the contribution of key players involved in radiation-induced cellular molecular responses: Atm, p53 and p38.

Atm is a serine/threonine protein kinase that is activated shortly after administration of a DNA-damaging agent (particularly IR). Atm serves as one of the cell cycle checkpoint regulators and targets a number of tumor suppressor genes, including p53. Whole-body exposure to 0.1 Gy of ionizing radiation led to a 1.87-fold ($p < .05$) increase in Atm levels in splenocytes of experimental mice. Moreover, elevated levels of Atm were observed not only 6 h after treatment, but also 24 h after (2.24-fold, $p < .05$), as well as 6 h after the next exposure to 0.1 Gy of ionizing radiation (cumulative dose of 0.2 Gy); however, this difference was not as significant (1.67-fold, $p = .0532$) (Figure 4(A)). Interestingly, Atm resembled a 'mute' behavior after the following exposures (cumulative doses 0.3–0.5 Gy), where the cellular levels of Atm were fluctuating within the background levels, independently of cumulative dose and time after treatment.

One of the main downstream targets of Atm is p53, the so called 'guardian of the genome'. A prime method by which p53 exercises a tumor suppressor role is via the initiation of cell cycle arrest in the presence of DNA damage, which facilitates elimination of irreversibly damaged cells. Taking this into consideration, we performed immunohistochemical analysis of p53 levels in murine spleen after fractionated exposure to low doses of ionizing radiation. Quite surprisingly, we did not find the expected significant elevation in levels of p53 in the spleen tissue after treatment at any of the evaluated time-points (Figure 4(B)). Moreover, we found a significant decrease in p53 expression at the 24-h time-points in comparison with the 6-h time-points after cumulative doses of 0.2, 0.4 and 0.5 Gy ($p < .05$). These findings suggest the possibility of an Atm/p53-independent pathway of apoptotic regulation, in response to fractionated delivery of low doses of radiation.

With these findings in mind, we assessed levels of p38 mitogen-activated protein kinase. p38, also known as MAPK14, is a stress-activated protein kinase that has been shown to be involved in the G2/M checkpoint independently of Atm (Bulavin et al. 2002; Mikhailov et al. 2005). There is also strong evidence that p38 is involved in response to ionizing radiation (Koturbash et al. 2008c). In contrast to p53, a 1.5-fold induction of p38 was observed after the first exposure ($p < .05$) and up to 1.9-fold after the last two exposures (cumulative doses of 0.4 and 0.5 Gy, respectively, $p < .05$). It is noteworthy that, in the case of cumulative doses of 0.4 and 0.5 Gy, elevated levels of p38 were statistically significant, compared not only to the levels in control splenocytes but also to the tissues obtained from other groups of treatment throughout Day 1–3 of the experiment. Together with the previous findings, this strongly suggests an Atm/p53-independent, p38-mediated early mechanism of apoptosis

regulation after fractionated whole-body exposure to low doses of ionizing radiation (Figure 5).

Radiation-induced cellular proliferation in murine spleen tissue

Lower levels of apoptotic response and DNA damage after cumulative doses of 0.2 and 0.3 Gy attracted our attention. One of the known effects of exposure to ionizing radiation is post-treatment cellular proliferation. We therefore evaluated levels of two known proliferation markers, PCNA and Ki-67, and an anti-apoptotic protein, Bcl-2, using an immunohistochemical approach.

Bcl2, or *B cell lymphoma 2*, is a member of the Bcl2 family of genes that regulate the process of cell death. It also has been shown to be implicated in a number of cancers. Levels of Bcl-2 were increased throughout the time of the experiment (Figure 6). Intriguingly, the highest induction (up to 1.97-fold, $p < .05$) was observed after cumulative doses of 0.2 and 0.3 Gy, which appears to clearly correlate to the absence of a significant apoptotic response at these time-points (Figure 6).

In addition, we have assessed levels of two markers of cellular proliferation, Ki67 and PCNA. While Ki67 is present in the cell during all stages of the cell cycle except G0, PCNA is predominantly expressed during the S-phase. Surprisingly, levels of the proliferation marker Ki67 were significantly increased, by 1.43-fold and 1.60-fold, after the cumulative dose of 0.2 Gy 6 and 24 h after exposure, respectively (Figure 7(A)). Furthermore, analysis of PCNA showed that the highest levels were also observed after the second exposure (2.1-fold, $p < .05$), and these high levels remained throughout the treatment (Figure 7(B)).

Discussion

Even though the biology of low doses of ionizing radiation has been well studied for decades, there are still many gaps in knowledge to be filled and much controversial data that needs to be addressed. The aim of this study was to determine the dynamics of spleen tissue responses to ionizing radiation exposure. To our knowledge, this study is the first of its kind to evaluate time- and dose-dependent aspects underlying cellular survival and apoptotic responses arising from protracted exposure to low doses of ionizing radiation.

DNA damage is the hallmark of exposure to ionizing radiation. To maintain genomic stability, this damage has to be removed, either by activation of repair mechanisms or by induction of programmed cell death when damage is irreparable. Initial exposure to 0.1 Gy of ionizing radiation led to significant elevation of DNA damage in the murine spleen tissue after 6 h. This was paralleled by induction of Atm, a marker of radiation-induced DNA damage, and increased levels of a positive regulator of apoptosis (stress-activated kinase p38) and, subsequently, apoptosis. Remarkably, one of the downstream targets of Atm, p53, a positive regulator of apoptosis, was not induced as part of this response. No significant differences were detected in the levels of DNA damage, p38 or apoptosis 24 h after exposure. In summary, the data obtained from spleen tissue treated with 0.1 Gy of ionizing radiation clearly suggests that 'priming exposure' mostly leads to formation of DNA damage, which can be removed within the next 24 h. This may also be indicative of low-

dose hypersensitivity (Martin et al. 2013). Low-dose hypersensitivity was reported to be associated with ATM levels (Xue et al. 2009, 2015). Future studies are needed to dissect the molecular underpinning of low dose hypersensitivity and priming in context of whole-body fractionated low dose irradiation.

An additional exposure to 0.1 Gy of ionizing radiation (cumulative doses of 0.2 Gy) did not result in a significant increase in the levels of DNA damage or apoptosis at either time-point. After the next exposure (cumulative dose 0.3 Gy), there was a significant induction of DNA damage at 6 h, as well as apoptosis at the 24-h time-point. Moreover, the cellular levels of a protein from the Bcl-anti-apoptotic protein family (Bcl2) were significantly up-regulated, which was paralleled by considerable induction of both proliferation markers, Ki67 and PCNA. These findings could possibly be linked to radiation-induced cellular proliferation. For instance, such induction of proliferation has been observed in hematopoietic progenitor cells after exposure to 75 mGy of X rays (Li et al. 2004). On the other hand, increased levels of these proteins can also suggest radiation damage-induced cell cycle block – G1 to G2 in the case of Ki67 or S-phase in the case of PCNA (Koturbash et al. 2008a). Previous studies indicated that PCNA can be involved in DNA repair, not only as a processivity factor for DNA polymerase δ but also in complex with Rad 6 and Rad 18 in the repair of double-strand breaks (Moertl et al. 2008). This may in part explain the absence of detectable DNA damage after cumulative doses of 0.2 Gy at 6 and 24 h and 0.3 Gy at 6 h (experimental Days 2 and 3, time-points where the highest levels of PCNA were detected). Overall, these findings depict the response to cumulative doses of 0.2 and 0.3 Gy as an 'adaptive-like' response. This kind of response has previously been described in many cells and tissues, including spleen, characterized by enhancement of cellular proliferation after introduction of a challenging radiation treatment in the pre-exposed cells (Yoshida et al. 1993; Hyun et al. 1997; Li et al. 2004).

The last two treatments with ionizing radiation (cumulative doses of 0.4 and 0.5 Gy) resulted in significant elevation of DNA strand breaks, paralleled by up-regulation of programmed cell death events and induction of p38. Notably, at these time-points, the levels of strand breaks and p38 were the highest throughout the experiment. Remarkably, after the previous exposures to cumulative doses of 0.2 and 0.3 Gy of IR, double-strand breaks were resolved and levels of p38 returned to the background reading; however, whole-body fractionated exposure to total doses of 0.4 and 0.5 Gy led to persistence of DNA strand breaks and up-regulated levels of p38. These findings clearly point to accumulation of DNA damage, which tends to indicate a 'cumulative threshold level', where the cell is not able to resolve the overwhelming damage caused by repeated exposure to ionizing radiation.

One can speculate on the absence of p53 response to exposure to low doses of radiation. Indeed, p53 plays an imperative role as guardian of the genome, and as such is a participant in numerous cellular response pathways and mechanisms, which makes it difficult to define pathways that are clearly distinct from p53. The absence of p53 response may be explained by the fact that the p53-dependent response pathway is relatively slow and requires time; therefore, induction of p53 within a short period of time after exposure could not be detected, at least by the methods of immunohistochemistry. This data is also in good agreement with our previous studies of delayed p53-response to ionizing radiation exposure,

where no significant induction of p53 could be detected in the spleen tissue of the same mouse strain within 6 h of administration of the treatment (Koturbash et al. 2008b, 2008c).

In summary, we have shown the experimental evidence demonstrating a few of the time- and dose-dependent molecular mechanisms underlying murine spleen response during fractionated exposure to low doses of ionizing radiation (cumulative dose range of 0.1–0.5Gy). Interestingly, reports by other groups have shown accumulation of residual DNA damage during fractionated radiation exposure, albeit to much higher doses of radiation, 2 Gy per fraction (Klokov et al. 2006). In the future, it would be important to conduct an in-depth analysis of DNA damage induction and persistence upon irradiation using several methods for DNA damage analysis, such as ROPS assay used in this study, as well as γ H2AX foci analysis or Comet assay (Tice and Strauss 1995; Redon et al. 2009; Pouliliou and Koukourakis 2014). This may lead to a more complete and comprehensive understanding of DNA damage responses to fractional irradiation. Moreover, during low dose exposure not every cell in the tissue is directly traversed by radiation (Seymour and Mothersill 2000; Suchowerska et al. 2005). Now it is well-accepted that radiation effects can also be observed in naïve cells that were in contact with directly irradiated cells or cells that received certain irradiation ‘distress’ signals from directly exposed cells. Such communication is termed the ‘bystander effect’, and is now accepted as a ubiquitous consequence of radiation exposure (Mothersill and Seymour 2004; Suchowerska et al. 2005). Bystander effects manifest as DNA damage, altered gene expression, cellular proliferation, and apoptosis (Morgan 2003a, 2003b, 2003c; Kovalchuk and Baulch 2008; Ilnytskyy and Kovalchuk 2011). In the future, it would be important to determine the contribution of direct and indirect bystander effects to the observed changes.

In sum, studies are much needed to fully appreciate the outcomes and molecular mechanisms underlying cellular responses and early induction of p38 in response to prolonged exposure to ionizing radiation. Although the present study offers experimental evidence only, its future potential for clinical relevance will warrant further attention.

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Figure 1.

Experimental set-up. At Day 1 of the experiment, 60 animals received a whole body exposure to 0.1 Gy of X rays. Six hours after exposure, six animals were randomly chosen and sacrificed. Twenty-four hours after the initial exposure (Day 2), another group of six randomly chosen animals was sacrificed. The rest of the animals (48) received a subsequent exposure to 0.1 Gy of X rays (cumulative dose of 0.2 Gy). The above-mentioned procedures were repeated until the cumulative dose of 0.5 Gy of X rays was reached. Acute – animals that were sacrificed 6 h after exposure to IR. Delayed – animals that were sacrificed 24 h after exposure to IR. 0.1, 0.2, 0.3, 0.4 and 0.5 Gy – cumulative dose of exposure to IR at the time of sacrifice.

Figure 2.

Level of DNA damage in spleen tissue after fractionated exposure to IR. Values represent % control (disintegrations per min). Bars represent $\pm$ SE. * $p < .01$; # $p < .05$.

Figure 3.

TUNEL apoptotic assay of spleen tissue after fractionated exposure to IR. Bars represent $\pm$ SE. * $p < .01$; # $p < .05$.

Figure 4. Levels of Atm (A) and p53 (B) in murine splenocytes after fractionated IR exposure. Values represent average number of positive cells per field of view. Bars represent $\pm$ SE. * $p < .01$; # $p < .05$.

Figure 5.

Level of p38 in murine splenocytes after fractionated IR exposure. Values represent average number of positive cells per field of view. Bars represent $\pm$ SE. * $p < .01$; # $p < .05$.

Figure 6.

Level of Bcl2 in murine splenocytes after fractionated IR exposure. Values represent average number of positive cells per field of view. Bars represent $\pm$ SE. * $p < .01$; # $p < .05$.

Figure 7.

Level of Ki67 (A) and PCNA (B) in murine splenocytes after fractionated IR exposure.

Values represent average number of positive cells per field of view. Bars represent $\pm$ SE. * $p < .01$; # $p < .05$.