

ORIGINAL ARTICLE

Irradiation induces DNA damage and modulates epigenetic effectors in distant bystander tissue *in vivo*I Koturbash¹, RE Rugo², CA Hendricks², J Loree¹, B Thibault¹, K Kutanzi¹, I Pogribny³, JC Yanch⁴, BP Engelward² and O Kovalchuk¹¹Department of Biological Sciences, University of Lethbridge, Alberta, Canada; ²Division of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA; ³Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR, USA and ⁴Department of Nuclear Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

Irradiated cells induce chromosomal instability in unirradiated bystander cells *in vitro*. Although bystander effects are thought to be linked to radiation-induced secondary cancers, almost no studies have evaluated bystander effects *in vivo*. Furthermore, it has been proposed that epigenetic changes mediate bystander effects, but few studies have evaluated epigenetic factors in bystander tissues *in vivo*. Here, we describe studies in which mice were unilaterally exposed to X-irradiation and the levels of DNA damage, DNA methylation and protein expression were evaluated in irradiated and bystander cutaneous tissue. The data show that X-ray exposure to one side of the animal body induces DNA strand breaks and causes an increase in the levels of Rad51 in unexposed bystander tissue. In terms of epigenetic changes, unilateral radiation suppresses global methylation in directly irradiated tissue, but not in bystander tissue at given time-points studied. Intriguingly, however, we observed a significant reduction in the levels of the *de novo* DNA methyltransferases DNMT3a and 3b and a concurrent increase in the levels of the maintenance DNA methyltransferase DNMT1 in bystander tissues. Furthermore, the levels of two methyl-binding proteins known to be involved in transcriptional silencing, MeCP2 and MBD2, were also increased in bystander tissue. Together, these results show that irradiation induces DNA damage in bystander tissue more than a centimeter away from directly irradiated tissues, and suggests that epigenetic transcriptional regulation may be involved in the etiology of radiation-induced bystander effects.

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Introduction

Until recently, it has been broadly accepted that biological consequences following ionizing radiation (IR) exposure are attributable to the direct effects of DNA damage. However, a wealth of evidence now challenges this classical paradigm (Ward, 2002; Hall, 2003; Mothersill *et al.*, 2004; Mothersill and Seymour, 2004). In particular, it has been shown that irradiated cells can elicit increased levels of mutations and chromosome aberrations in neighboring cells that have never been exposed to radiation. These ‘bystander effects’ can lead to persistent genome destabilization and ultimately may contribute to carcinogenesis (Zhou *et al.*, 2000, 2002; Sawant *et al.*, 2001; Ward, 2002; Hall, 2003; Mothersill and Seymour, 2004; Mothersill *et al.*, 2004). Bystander effects encompass a wide range of genetic alterations, including gross genome rearrangements, chromosome aberrations, sister chromatid exchanges (SCEs), deletions, duplications and gene mutations and amplifications (Little, 1998, 1999; Morgan, 2003a, b).

As such, bystander effects are a negative complication in radiation oncology and are thought to contribute to secondary radiation carcinogenesis (Goldberg and Lehnert, 2002; Goldberg, 2003; Hall, 2003; Huang *et al.*, 2003; Sigurdson and Jones, 2003; Mothersill and Seymour, 2004). Despite its potential biological importance, most of what we currently know about radiation-induced bystander effects stems from studies of cultured cells, rather than from animal models (Goldberg and Lehnert, 2002; Goldberg, 2003; Hall, 2003). Furthermore, despite the clear link between radiation-induced bystander effects and radiation-induced genome instability (Morgan *et al.*, 2002, 2003a, b; Goldberg, 2003; Huang *et al.*, 2003; Sigurdson and Jones, 2003), very few studies have explored the potential impact of radiation exposure on distant organs and tissues (Xue *et al.*, 2002).

Radiation-induced genome instability has recently been suggested to be epigenetic in nature (Nagar *et al.*, 2003). Epigenetic changes are mitotically stable alterations that include DNA methylation and histone modifications (Robertson, 2002; Jaenisch and Bird,

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2003). Aberrant cytosine DNA methylation is well documented in cancer development and is linked to genomic instability and increased rates of genome rearrangements (Robertson and Wolffe 2000; Ehrlich, 2002; Gaudet *et al.*, 2003; Feinberg and Tycko, 2004). A variety of DNA damaging agents including IR are known to affect genome DNA methylation patterns and this may contribute to their genome destabilizing effects (Kalinich *et al.*, 1989; Tawa *et al.*, 1998; Minamoto *et al.*, 1999; Pogribny *et al.*, 2004; Raiche *et al.*, 2004; Koturbash *et al.*, 2005). To our knowledge, no studies have explored the possible roles of epigenetic mechanisms on somatic *in vivo* bystander effect.

To investigate the possibility that X-irradiation induces DNA damage in bystander tissue *in vivo*, we monitored the induction and repair of DNA strand breaks in cutaneous tissue. In addition, we also explored the possibility that epigenetic mechanisms (i.e. DNA methylation and alterations in DNA methyltransferases and methyl-binding proteins) are involved in the generation and/or maintenance of a radiation-induced bystander effect. Here, we report that radiation exposure to one-half of the body leads to elevated levels of DNA strand breaks, and alters the levels of key proteins known to modulate methylation patterns and silencing in the bystander half of the body at least 0.7 cm from the irradiated tissue. These are some of the first data to clearly demonstrate that bystander effects occur *in vivo* in distant tissue.

Results

Accumulation of DNA damage in bystander skin tissue in vivo

We studied bystander effects *in vivo* by placing lead shielding, one edge aligned along the spine, over half the body of a mouse, before exposure to 1 Gy of X-rays (Figure 1). Animals were immobilized during exposure and bystander ventral skin was taken from the area adjacent to the thigh at least 0.7 cm from the exposed half of the mouse. The lead shielding used for these studies is the same as that which is used for patients exposed to radiation in the clinic, and complete protection of shielded bystander tissue from radiation exposure was verified by careful dosimetry (see Materials and methods).

DNA damage in mammalian bystander cells has been studied *in vitro* by directly measuring double-strand breaks (DSBs) (Sokolov *et al.*, 2005), and by measuring sequence rearrangements that are thought to be caused by DSBs, such as chromosomal aberrations, SCEs, and homologous recombination (HR) between direct repeat (Nagasawa and Little, 1992; Huo *et al.*, 2001; Ponnaiya *et al.*, 2004; Rugo *et al.*, 2005). As single-strand breaks can become DSBs if encountered by the replication fork (McGlynn and Lloyd, 2002; Helleday, 2003), we measured single and DSBs in bystander tissues *in vivo* using a modified version of the random oligonucleotide-primed synthesis (ROPS) assay (Basnakan and James,

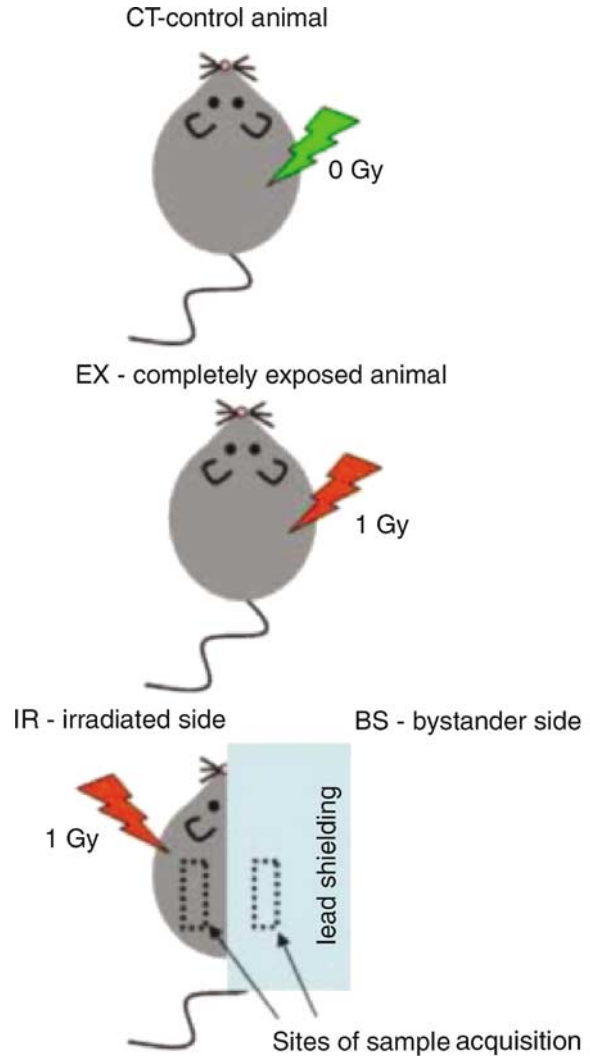


Figure 1 Induction of *in vivo* bystander effect. Animals had lead shielding covering half their bodies during exposure to 1 Gy of X-rays. Animals in the unirradiated control cohort were sham treated. Animals in the fully irradiated cohort received 1 Gy of whole-body X-ray exposure. CT – unirradiated skin; IR – skin from fully irradiated animals; IR $\frac{1}{2}$ – skin from the irradiated side of hemishielded animals; BS $\frac{1}{2}$ – skin from the shielded, bystander side of hemishielded animals. Note that ventral skin was taken from the area adjacent to the thigh, thus bystander samples were taken at least 0.7 cm from the edge of irradiated side.

1996). This assay is based on the ability of Klenow polymerase to initiate ROPS from the reannealed 3'-OH ends of single-stranded DNA. DNA strand breaks were quantified in skin from unirradiated control mice, fully irradiated mice, and from irradiated and bystander sides of mice (Figure 2a). DNA strand breaks were quantified at 6 h and at 4 days (precisely 96 h) after exposure in order to monitor the persistence and/or repair of irradiation-induced strand breaks over time. As expected, cells isolated from the skin of fully irradiated animals as well as the skin from the irradiated side of hemishielded animals showed significantly increased levels of strand breaks 6 h postirradiation compared to

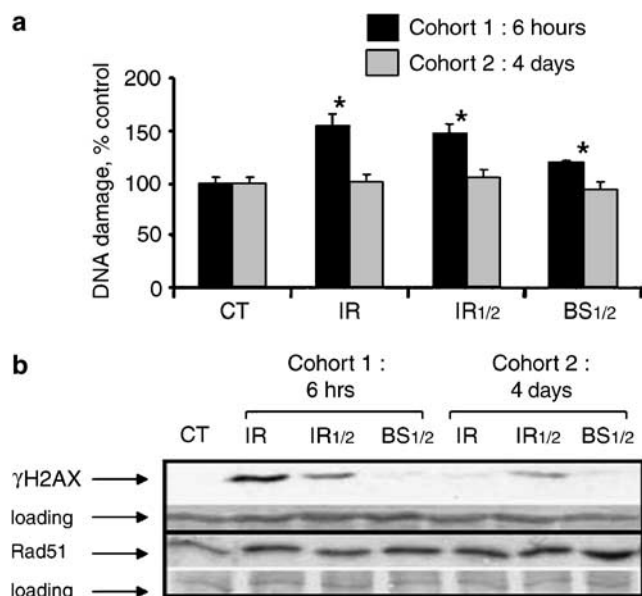


Figure 2 DNA damage in irradiated and bystander skin. (a) Levels of DNA strand breaks were revealed by a modified ROPS assay (see Materials and methods). Separate cohorts were analysed 6 h and 4 days after exposure. Levels of DNA strand breaks are presented as mean values \pm s.e.m., $n=10$; *denotes statistically significant difference compared to controls ($P<0.05$), Student's t -test. (b) Lysates from cutaneous tissue were immunoblotted using antibodies against RAD51 and γ H2AX. Representative blots from three independent experiments; each experiment included pooled lysates from five animals for each exposure condition, with equal representation of each animal. CT, IR, IR $\frac{1}{2}$ and BS $\frac{1}{2}$ are as described in Figure 1.

controls (50 and 48%, respectively; $P<0.05$; Figure 2a). Surprisingly, we also found significantly elevated levels of breaks in bystander skin from hemishielded mice 6 h post-treatment (20%; $P<0.05$), indicating that damage is induced in shielded tissue *in vivo*. At 4 days after exposure, the number of strand breaks under all conditions had returned to control levels (Figure 2a, gray bars), indicating that strand breaks induced directly (in the exposed tissue) and indirectly (in the shielded bystander tissue) had been repaired.

As an alternative approach for assessing the occurrence of DSBs, we assayed for the presence of γ H2AX phosphorylation. H2AX is a member of the H2A histone family which becomes phosphorylated at S139 (γ H2AX) as one of the earliest cellular responses to DSBs (Rogakou *et al.*, 1998). γ H2AX accumulates in the nucleus at DSBs forming the γ H2AX foci and a direct correlation has been found between the H2AX phosphorylation and the number of DSBs resulting from radiation. Although the majority of γ H2AX foci appear within minutes of radiation exposure, previous studies have shown that a certain subset of breaks and H2AX phosphorylation persists for many hours (Rothkamm and Lobrich, 2002). At 6 h after exposure, γ H2AX was undetectable by Western blot in skin from unirradiated control mice, but was significantly increased in skin from fully irradiated mice and in skin from the irradiated sides of hemishielded mice (~ 2.2 - and

~ 1.6 -fold over control, respectively; Figure 2b). In addition, there was a slight increase in γ H2AX in the skin from the bystander sides of hemishielded mice (although not readily apparent by eye, densitometry analysis shows that there is a statistically significant ~ 1.3 -fold increase in γ H2AX levels in bystander tissue compared to control cohorts; $P<0.05$, Student's t -test), which is consistent with DSBs in the bystander tissues 6 h after irradiation. By 4 days postirradiation, γ H2AX was still slightly elevated in cutaneous tissue from fully irradiated mice and from the irradiated side of hemishielded mice, but not in bystander skin. These data are consistent with the ROPS assay 6 h after exposure, indicating that X-irradiation resulted in the induction of DSBs in shielded bystander tissue *in vivo*.

Mammalian cells employ HR and nonhomologous end joining (NHEJ) as important pathways to repair DSBs (reviewed in Hoeijmakers, 2001; McGlynn and Lloyd, 2002; Helleday, 2003; West, 2003). Homologous recombination allows cells to use the undamaged sister chromatid or the homologous chromosome as a template for repair and thus is considered error-free (Hoeijmakers, 2001; McGlynn and Lloyd, 2002; Helleday, 2003; West, 2003). Nonhomologous end joining is a fast, yet error-prone process of linking broken DNA ends together without reference to the accurate base pairing (Hoeijmakers, 2001).

As we had found evidence of DSBs in bystander tissues, we next asked if HR or NHEJ related proteins were induced in bystander tissues. Rad51 is a key protein essential for repair of DSBs via HR in mammals (Sonoda *et al.*, 1998; Lundin *et al.*, 2003). Rad51 binds to single-stranded DNA and forms a nucleoprotein filament that catalyses homology searching, strand pairing, and strand exchange (Baumann *et al.*, 1996; Baumann and West, 1998). Ku70 is a key participant in the NHEJ pathway to repair DSBs (Hoeijmakers, 2001).

Western blots were performed on lysates of skin from control mice, fully irradiated mice, and the irradiated and bystander sides of hemishielded mice 6 h and 4 days post-treatment. No significant changes were found in the levels of Ku70 in the exposed and bystander mouse skin (data not shown).

Unexpectedly, we found that Rad51 expression is similarly elevated in tissues from fully exposed and the exposed and bystander sides of hemishielded mice at 6 h and 4 days after exposure (Figure 2c). Rad51 activity is generally thought to be controlled by subnuclear localization. However, the increased levels of Rad51 described here are nevertheless consistent with an earlier report of a transcription-dependent increase in Rad51 found in cultured human cells after DNA damage and measured by immunofluorescence (Haaf *et al.*, 1995).

Epigenetic changes in exposed and bystander tissue

Bystander effects are thought to arise via epigenetic mechanism(s). DNA methylation is an important epigenetic mechanism for regulating gene expression/silencing and there is increasing evidence that methylation also serves to help safeguard genome stability. For

example, decreased DNA methylation has been linked to elevated levels of transposon activation, SCEs and other gross genome rearrangements (Gonzalzo and Jones, 1997; Esteller and Herman, 2002; Robertson, 2002).

To test if changes in methylation were observed in bystander tissue, global cytosine methylation was measured in skin from unirradiated, irradiated, and the irradiated and bystander sides of hemishielded mice 6 h and 4 days after irradiation. To monitor changes in DNA methylation, we employed the sensitive *HpaII*-based cytosine extension assay, which measures the proportion of unmethylated CCGG sites in genomic DNA (Kovalchuk *et al.*, 2004; Pogribny *et al.*, 2004; Raiche *et al.*, 2004; Koturbash *et al.*, 2005). Using this approach, we found that exposure to 1 Gy of X-rays led to a significant decrease in DNA methylation in exposed skin 6 h post-treatment (decreased methylation appears as an increase in ³H-dCTP incorporation; Figure 3a). Methylation levels in the exposed tissue returned to normal in a separate cohort analysed 4 days post-treatment (gray bars; Figure 3a). DNA methylation changes were not significant in bystander tissue, although a slight decrease was observed (Figure 3a). Overall, unilateral irradiation suppressed global DNA methylation in directly irradiated, but not in bystander tissue at the time-points studied.

In mammals, three DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are primarily responsible for establishing and maintaining DNA methylation patterns at CpG sites (Robertson, 2001; Rountree *et al.*, 2001; Raiche *et al.*, 2004). In particular, DNMT3a and DNMT3b catalyse *de novo* methylation, while DNMT1 maintains existing methylation patterns. Deregulation of any or all of these proteins may result in perturbations of DNA methylation. To further investigate the basis of methylation loss in irradiated tissue, we monitored expression of DNMT1, DNMT3a and DNMT3b methyltransferase in skin from unirradiated, fully irradiated, and the irradiated and bystander sides of hemishielded mice at 6 h and 4 days post-irradiation. Interestingly, DNMT1 expression was significantly up-regulated in bystander tissue both 6 h and 4 days posttreatment (~1.4- and 1.8-fold respectively; Figure 3b), whereas expression remained unchanged in irradiated skin. Although these are somewhat subtle changes in the levels of DNMT1, it is striking that within the same animals, DNMT1 was consistently higher in bystander tissue than in directly irradiated tissue. Furthermore, each lane is a combined sample from five separate animals, and each Western blot was repeated three times, such that the fold induction reflects cohorts of at least 15 animals. In contrast to DNMT expression of the *de novo* methyltransferases DNMT3a and DNMT3b was slightly downregulated both in irradiated skin as well as in bystander tissue 6 h post-irradiation (Figure 3b). Suppression of DNMT3b was reduced at 4 days post-irradiation and levels of DNMTa had normalized by 96 h post-irradiation, which coincides with recovery of normal methylation levels by this time (Figure 3b) (densitometry analysis shows that the

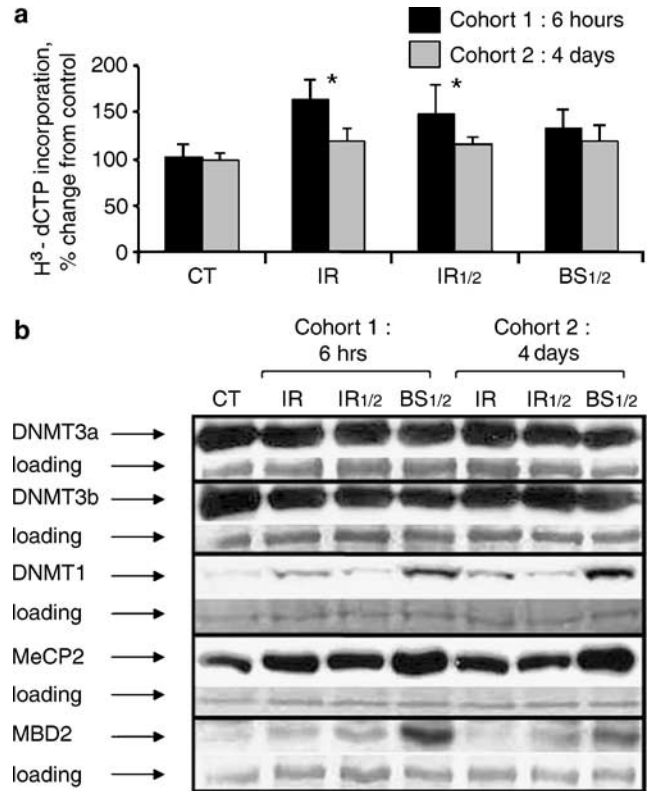


Figure 3 (a) Epigenetic effects of radiation exposure. Relative levels of global DNA methylation where an increase in ³H-dCTP indicates a decrease in global methylation in cutaneous tissues of unirradiated control, fully irradiated, and the irradiated and bystander sides of animals 6 h and 4 days after exposure. (b) Western analysis of DNMT1, DNMT3a, DNMT3b, MeCP2, MBD2. Protein levels relative to loading controls were compared and the statistically significant fold changes relative to untreated sham controls are shown. Representative blots from three independent experiments; each experiment included cohorts of five animals for each exposure condition, with equal representation of each animal. Each lane represents pooled lysates from five animals; experiments were repeated three times independently. CT, IR, IR $\frac{1}{2}$ and BS $\frac{1}{2}$ are as described in Figure 1.

decrease is statistically significant; $P < 0.05$, Student's *t*-test.)

In mammals, the association of DNA methylation with transcriptional repression is thought to be mediated by the MBD (methyl CpG-binding domain) family of proteins. Methyl CpG-binding domain proteins, including MeCP2, MBD1, MBD2, and MBD3, selectively interact with methylated DNA and play pivotal roles in methylation-mediated chromatin remodeling and gene silencing (Robertson and Wolffe, 2000; Wade, 2001; Robertson, 2002; Hendrich and Tweedie, 2003; Jaenisch and Bird, 2003; Bowen *et al.*, 2004). To test if MBD expression is altered in bystander tissue, we monitored expression of MeCP2 and MBD2 in skin from unirradiated, fully irradiated, and in skin from the irradiated and bystander sides of mice at 6 and 96 h post-irradiation. Both MeCP2 and MBD were significantly upregulated (~2-fold) in bystander skin (Figure 3b). As was observed with DNMT1, this

induction persisted 4 days post-treatment. In addition, a slight but statistically significant increase in MeCP2 expression (~1.25-fold) was also evident in irradiated skin at 6 h post-irradiation. Upregulation of the methyl-binding proteins MeCP2 and MBD2 in bystander skin is consistent with the observed induction of DNMT1 methyltransferase in this tissue.

Bystander effects are not the result of insufficient shielding or radiation scattering

As an independent measure of effective shielding, fully shielded mice were exposed to 1 Gy X-rays and ventral skin was analysed by Western blot for expression of Rad51, DNMT1, MeCP2, and MBD2 expression. As can be seen in Figure 4a, there was no induction. Furthermore, levels of the γ H2AX protein, an indicator of DNA damage, also remained unchanged from the unirradiated control (data not shown). As X-rays penetrate tissue, they can react with biological matter resulting in the deflection of their trajectories. This is referred to as scatter radiation. Low doses, close to the scatter-dose range levels were shown to induce bystander effects (Mothersill and Seymour, 2002; Kashino *et al.*, 2004; Maguire *et al.*, 2005; Mothersill *et al.*, 2005). Thus, to measure if scatter contributed to the effects seen in bystander tissue, mice were irradiated with a dose approximating the expected scatter dose (1.3 mGy, which was calculated as described in Materials and methods) and ventral skin was analysed as was performed for the fully shielded samples above. There was no protein induction seen (Figure 4b) indicating that the observed changes in expression were not likely to be caused by scatter radiation.

Exploration of possible effects of physiological asymmetry

It is well known that the mammalian body is not absolutely symmetrical and multiple organs do not have a pair. In our initial experiments we protected the right part of the animal body, exposing the left side. Thus, the spleen, an important hematopoietic organ was within the exposure field. On the other hand, irradiation of right side of the animal body exposes more of the liver. It is possible that differential exposure of organs has a role in the induction of bystander effects. To address this question, experiments were set up in which the right or the left half of the body was shielded while animals were exposed to X-rays. In a third experimental group, the heads were exposed to X-rays while the body was shielded by lead. Sham-treated animals served as experimental controls. Animals were killed 6 h and 4 days after exposure and tissue lysates analysed by Western blot. Consistent with the experiments shown in Figure 3, we found that DNMT1 expression was upregulated in bystander tissues but not on irradiated skin; this was the case regardless of which side of the body was irradiated (Figure 5). In addition, Rad51 was upregulated in all irradiated and bystander tissues, regardless of which half of the body was exposed (Figure 5). Interestingly, induced expression of MBD2

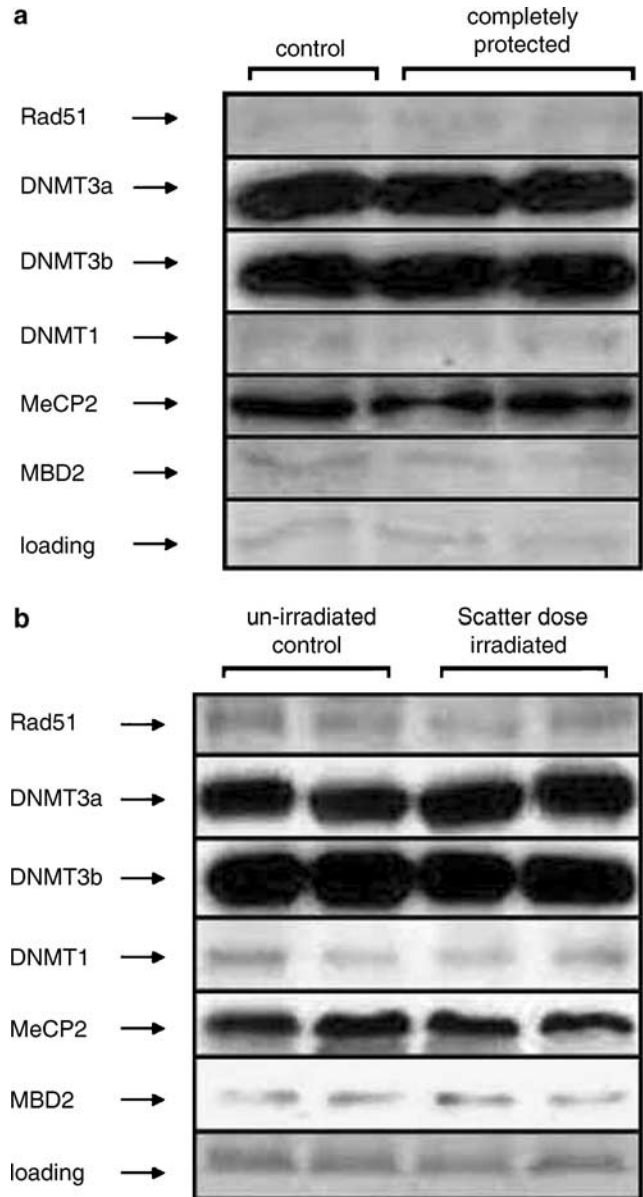


Figure 4 Bystander effects are not due to insufficient shielding or radiation scattering. (a) Western analysis of fully shielded animals. Animals in the control cohort were sham treated. Animals of the shielded cohort were exposed to 1 Gy of X-rays while their bodies were completely shielded with lead. Representative blots from three independent technical repeats are shown; each experiment included cohorts of five animals for each exposure condition, with equal representation of each animal. (b) Western analysis of animals exposed to the approximate dose expected from scatter. Animals in the control cohort were sham treated. Animals of the scatter-dose exposed cohort received an approximate 1.3 mGy dose (see Materials and methods). Representative blots from among three independent technical repeats are shown; each experiment included pooled lysates from five animals for each exposure condition, with equal representation of each animal.

and MeCP2 was noted in bystander skin only when the left side of the body was irradiated, suggesting the possibility that internal organs affect upregulation of MBD2 and MeCP2 in bystander tissue (Figure 5). Further studies are underway to elucidate the

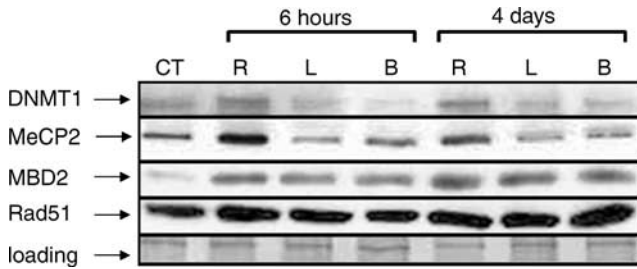


Figure 5 Role of internal organ exposure in the generation of bystander effects. Animals were irradiated as described in the text, three animals per treatment group. Lysates from the control and shielded/bystander cutaneous tissue were immunoblotted using antibodies against DNA repair and DNA methylation-related proteins. CT – lysate from control animal skin; R – lysate from the right body side shielded skin (left side was irradiated); L – lysate from the left body side shielded skin (right side was irradiated); B – lysate from the lower animal body shielded skin (head was irradiated). Protein levels relative to loading controls were compared. Representative blots from among three independent technical repeats are shown; each experiment included pooled lysates from three animals for each exposure condition, with equal representation of each animal.

molecular underpinnings of the regulation of MBD2 and MECP2.

As an independent approach for studying potential bystander effects, we also adjusted the exposure procedure so that for one cohort of animals, their heads were irradiated and unexposed cutaneous tissue from their backs was analysed. We found similar expression patterns for DNMT1 and Rad51 in bystander skin taken from the dorsal sides (at least 0.7 cm away from the irradiated tissue) of animals whose heads had been irradiated, which is consistent with the data from the unilateral exposure.

Discussion

Most reported bystander effects have been observed *in vitro* and the biological significance of bystander effects *in vivo* are unclear (Goldberg and Lehnert, 2002; Goldberg, 2003). Ideally, research on bystander effects would be performed in clinical trials. However, in the meantime, experiments involving animals could be useful for delineating the possible effects of irradiation on unexposed tissues and for dissecting the molecular basis for such effects (Balmain and Harris, 2000; Goldberg and Lehnert, 2002; Goldberg, 2003). We set out to explore DNA damage and repair in bystander tissues in a mouse model. The main findings of the present study are: (i) radiation exposure leads to the induction of DNA damage in distant (>0.7 cm from irradiated tissues), lead-shielded, bystander tissues *in vivo*; (ii) DNA damage in bystander tissues was present several hours after radiation; (iii) bystander tissues exhibited changes in expression of genes involved in methylation, indicating gene silencing by methylation may be involved in bystander effects; (iv) expression changes in bystander tissue persisted through 4 days

after irradiation; (v) expression changes in bystander tissue may be, in part, linked to internal organ exposure, as the more pronounced response was observed when the left side of the body was irradiated.

DNA damage and repair

We measured DNA damage in skin from unirradiated, irradiated and the irradiated and bystander sides of hemishielded mice. We found significant increases in strand breaks in irradiated and bystander samples 6 h after exposure but not after 4 days, which is consistent with the damage being repaired in irradiated and bystander tissues. In addition, we monitored γ H2AX as a specific marker of DSBs. As the appearance of γ H2AX occurs rapidly after DSBs are formed, it is often examined soon after damage is induced, for example, after 30 min. The fact that we observed an increase in γ H2AX in irradiated and bystander tissues 6 h after exposure is surprising as it indicates there may be unrepaired DSBs persisting after irradiation (Rothkamm and Lobrich, 2003). Alternatively, it may reflect an increase in the number of dividing cells in tissues following irradiation (Ichijima *et al.*, 2005).

The induction of Rad51 in irradiated tissue was somewhat unexpected. It is thought that Rad51 activity is regulated by subnuclear localization (West, 2003); however, there are earlier reports that are consistent with the induction of Rad51 expression by IR (Haaf *et al.*, 1995). Interestingly, we found Rad51 was similarly induced in both irradiated and bystander tissues even 4 days after irradiation, suggesting a persistent upregulation of DSB repair capacity. The cellular repercussions of this finding will have to be further elucidated, since it was recently shown that overexpression of Rad51 can lead to chromosome rearrangements and genome instability (Richardson *et al.*, 2004).

DNA methylation

Although it is hypothesized that bystander effects result from epigenetic changes to cells, the precise mechanism by which bystander effects are induced and maintained is not known. In this study, we found a decrease in global methylation in irradiated skin but not in bystander skin. The observed decrease in global DNA methylation in irradiated skin 6 h after irradiation correlated ($r^2 > 0.9$) with the accumulation of strand breaks, as monitored by the ROPS assay, as well as with the increase in recombination activity ($r^2 > 0.9$). Thus, the loss of methylation in cells from directly irradiated tissue may be related to the repair status of these cells. Indeed, it is known that DNA polymerases involved in repair and recombination incorporate cytosine but not methylcytosine during repair synthesis (Pogribny *et al.*, 2005). Thus, the induction of DNA lesions and subsequent activation of DNA repair and recombination mechanisms may result in DNA hypomethylation (Pogribny *et al.*, 2004, 2005; Koturbash *et al.*, 2005). We have previously shown that suppression of DNA methylation caused by exposure to gamma-irradiation is

linked to activation of DNA repair (Pogribny *et al.*, 2004). Another possible mechanism by which irradiation could reduce the levels of DNA methylation could be that DNA damage interferes with the ability of DNA methyltransferases to methylate DNA (Turk *et al.*, 1995; Panayiotidis *et al.*, 2004). However, it seems unlikely that the number of DNA lesions induced under these conditions would yield detectable changes in global methylation.

De novo methyltransferases function primarily as regulators of cell fate and differentiation. Interestingly, we found a decrease in the expression of DNA methyltransferases involved in *de novo* methylation, DNMT3a and 3b, in both irradiated and bystander tissues. It is well established that clinical exposure to radiation therapy can induce cutaneous injury, which involves complex physiological changes. Thus, one possibility is that the suppressed levels of DNMT3a and 3b reflect an early injury response. Interestingly, previous studies show a correlation between DNA hypomethylation and suppression of DNMT3a and 3b in mouse liver, spleen and thymus following whole-body exposure to X-rays (Raiche *et al.*, 2004; Pogribny *et al.*, 2005).

In contrast to the suppression of *de novo* methyltransferase levels, we observed an increase in the levels of the methyltransferase responsible for maintaining DNA methylation patterns, DNMT1, only in bystander tissues. It is interesting that global methylation was suppressed in directly irradiated tissue, but not in bystander. One possibility is that increased levels of DNMT1 may offset the radiosuppression of DNA methylation that was observed in directly irradiated tissue. Along with the increased levels of DNMT1 in bystander tissue, we also found that the levels of MeCP2 and MBD2, proteins involved in transcriptional silencing, were increased in bystander but not irradiated skin. Although additional studies are necessary to delineate the potential biological significance and persistence of these shifts in protein levels, these results demonstrate that there are significant physiological changes in distant unexposed tissue of animals exposed to X-irradiation.

Materials and methods

Model and irradiation of animals

In this study, we examined genetic and epigenetic alterations in mouse skin following *in vivo* radiation exposure. Mice were randomly assigned to different treatment groups. Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use (1993). The procedures have been approved by the University of Lethbridge Animal Welfare Committee. Animals were housed in a virus-free facility and given food and water *ad libitum*.

The exposed cohort (25 animals) received 1 Gy (2cGy/s) of X-rays (90 kV, 5 mA). In the bystander group (25 animals) each animal was exposed to 1 Gy (2cGy/s) of X-rays (90 kV, 5 mA) having half of its body protected by an ~2.5 mm thick lead shield, the same type as used for the human body protection in diagnostic radiology (Figure 1) The protection of

shielded 'bystander' tissue was complete, as verified by careful dosimetry using RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen Inc., FL, USA). Control mice were sham treated. All animals were humanely killed 6 or 4 days (precisely 96 h) upon completion of the treatment protocol. On the basis of previous research, these time points are sufficient to see the induction and persistence, if any, of the radiation-induced epigenetic changes (Kovalchuk *et al.*, 2004; Koturbash *et al.*, 2005). Cutaneous tissue was sampled upon killed and processed for further molecular studies.

A cohort of five animals was exposed to 1 Gy of X-rays while their bodies were completely shielded with a ~2.5 mm lead shield. Protection by the lead shielding was complete as verified by RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen Inc., FL, USA).

Another cohort of five animals was exposed to an approximate scatter dose of ~1.3 mGy. To determine the bystander ventral/thigh skin dose resulting from photon scatter within the mouse itself, a Monte Carlo simulation was performed (X-5 Monte Carlo Team, 2003). The skin was assumed to be 0.1 cm thick and a 2 mm thick lead shield covered one-half of the mouse. Absorbed dose was tallied within the skin region in several locations in both the unshielded dorsal regions and the shielded ventral/thigh regions. Absorbed dose to the ventral skin from a 90 kVp X-ray spectrum was determined to be approximately 0.014–0.017 Gy for a 1 Gy dose delivered to the dorsal skin.

Cytosine extension assay to detect sequence-specific changes in DNA methylation

Total DNA was prepared from thymus tissues using Trizol™ Reagent (Amersham, Baie d'Urfé, Québec, Canada) according to the manufacturer's protocol.

DNA (0.5 µg) was digested overnight with a 10-fold excess of *HpaII* endonuclease according to the manufacturer's protocol (New England Biolabs, Beverly, MA, USA). A second DNA aliquot (0.5 µg) was incubated without restriction enzyme addition and served as a background control. The single nucleotide extension reaction was performed in a 25 µg of DNA, 1 × PCR bufferII, 1.0 mM MgCl₂, 0.25 units of Taq DNA polymerase (Fisher Scientific, Ottawa, ON, USA), [³H]dCTP (57.4 Ci/mmol) (Perkin-Elmer, Boston, MA, USA) and incubated at 55°C for 1 h, then immediately placed on ice. Duplicate aliquots (25 µl) from each reaction were placed on Whatman DE-81 ion-exchange filters and washed three times 10 min with gentle agitation with sodium phosphate buffer (0.5 M, pH 7.0) at room temperature. The filters were dried and processed by scintillation counting (Beckman Coulter). Background label incorporation was subtracted from enzyme-digested samples and results were expressed as relative [³H]dCTP incorporation/1 µg of DNA or as percent change from control (Pogribny *et al.*, 1999, 2004, 2005; Raiche *et al.*, 2004).

DNA strand break measurement

A modification of the ROPS assay was used to detect the presence of DNA strand-breaks in high molecular weight DNA (Basnakian and James, 1996; Pogribny *et al.*, 2004). The assay is based on the ability of Klenow fragment polymerase to initiate ROPS from the reannealed 3'-OH ends of single-stranded DNA. Briefly, 3'-OH DNA fragments present in the high molecular weight DNA are separated into single-strand fragments by heat denaturation and subsequently reassociated by cooling. The resulting random reassociation of DNA strands consists primarily of single-stranded DNA fragments primed by their own tails or by other DNA fragments. These

fragments serve as random primers and the excess of DNA serves as template for Klenow fragment polymerase. DNA was denatured by exposure at 100°C for 5 min, and then immediately cooled on ice. The mixture contained 0.25 µg heat-denatured DNA, 0.1 µl [³H]dCTP (57.4 Ci/mmol) (Perkin-Elmer, Boston, MA), 0.05 mM concentrations of each dGTP, dATP, TTP, 0.6 µM dCTP, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 7.5 mM DTT, and 0.5 U Klenow polymerase (New England Biolabs, Beverly, MA, USA) in a total volume of 25 µl. After incubation for 30 min at 16°C, the reaction was stopped by the addition of an equal volume of 12.5 mM EDTA. The samples were subsequently applied on Whatman DE-81 ion-exchange filters and washed three times with sodium phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed by scintillation counting. The results were expressed as the percent difference in [³H]dCTP incorporation relative to control values.

Western immunoblotting

Western immunoblotting for RAD51, γH2AX, DNMT1, DNMT3a, DNMT3b, MeCP2 and MBD2 was conducted using cutaneous tissue. Tissue samples were sonicated in 0.4–0.8 ml of ice-chilled 1% sodium dodecyl sulphate (SDS) and boiled for 10 min. Small aliquots (10 µl) of homogenate were reserved for protein determination using protein assay reagents from BioRad (Hercules, CA, USA). Equal amounts of proteins (20 µg) were separated by SDS–polyacrylamide electrophoresis (PAGE) in slab gels of 8 or 12% polyacrylamide, made in duplicates, and transferred to PVDF membranes (Amersham, Baie d’Urfé, Québec, Canada). Membranes were incubated with antibodies against RAD 51 (1:1000, BD Biosciences, Mountain View, CA, USA), γH2AX (1:1000, Cell Signaling, Danvers, MA, USA), DNMT1 (1:1000, Abcam, Cambridge, MA, USA), DNMT3a, DNMT3b (1:500, Abgent, San Diego, CA, USA), MeCP2 (1:1000, Abcam, Cambridge, MA, USA) and MBD2 (1:500,

Abgent, San Diego, CA, USA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham, Baie d’Urfé, Québec, Canada) and the ECL Plus immunoblotting detection system (Amersham, Baie d’Urfé, Québec, Canada). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT, USA). Unaltered PVDF membranes were stained with Coomassie Blue (BioRad, Hercules, CA, USA) and the intensity of the Mr 50 000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software and normalized to both GAPDH and the Mr 50 000 protein, which gave consistent results (values relative to Mr 50 000 are plotted).

Statistical analysis

Statistical analysis was performed using the MS Excel 2000, Sigma Plot and JMP5 software packages.

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