
Fractionated Low-Dose Radiation Exposure Leads to Accumulation of DNA Damage and Profound Alterations in DNA and Histone Methylation in the Murine Thymus

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Abstract

Thymus, an important component of hematopoietic tissue, is a well-documented “target” of radiation carcinogenesis. Both acute and fractionated irradiation result in a high risk of leukemia and thymic lymphoma. However, the exact mechanisms underlying radiation-induced predisposition to leukemia and lymphoma are still unknown, and the contributions of genetic and epigenetic mechanisms in particular have yet to be defined. Global DNA hypomethylation is a well-known characteristic of cancer cells. Recent studies have also shown that tumor cells undergo prominent changes in histone methylation, particularly a substantial loss of trimethylation of histone H4-Lys²⁰ and demethylation of genomic DNA. These losses are considered a universal marker of malignant transformation. In the present study, we investigated the effect of low-dose radiation exposure on the accumulation of DNA lesions and alterations of DNA methylation and histone H4-Lys²⁰ trimethylation in the thymus tissue using an *in vivo* murine model. For the first time, we show that fractionated whole-body application of 0.5 Gy X-ray leads to decrease in histone H4-Lys²⁰ trimethylation in the thymus. The loss of histone H4-Lys²⁰ trimethylation was accompanied by a significant decrease in global DNA methylation as well as the accumulation of DNA damage as monitored by persistence of histone γ H2AX foci in the thymus tissue of mice exposed to fractionated irradiation. Altered DNA

methylation was associated with reduced expression of maintenance (DNMT1) and, to a lesser extent, *de novo* DNA methyltransferase DNMT3a in exposed animals. Expression of another *de novo* DNA methyltransferase DNMT3b was decreased only in males. Irradiation also resulted in ~20% reduction in the levels of methyl-binding proteins MeCP2 and MBD2. Our results show the involvement of epigenetic alterations in radiation-induced responses *in vivo*. These changes may play a role in genome destabilization that ultimately leads to cancer. (Mol Cancer Res 2005;3(10):553–61)

Introduction

Ionizing radiation is a well-known genotoxic agent and a human carcinogen, and hematopoietic tissue is one of the main “targets” of radiation carcinogenesis. Radiation-induced leukemia in humans was first reported early in the 20th century (1-4). Since then, numerous studies of atomic bomb survivors, people working or living near nuclear power plants, radiologists, and radiotherapy patients have shown that certain doses of both acute and fractionated radiation exposure result in a high risk of leukemia and lymphoma (1-7). Furthermore, the risk of secondary radiation treatment-related lymphoma and leukemia is a clinical problem (6). The exact mechanisms of radiation-induced leukemia and lymphoma, particularly the contributions of genetic and epigenetic mechanisms, remain unknown. In recent years, the role of epigenetics in the etiology of disease, including cancer, has been increasingly recognized (8, 9). Epigenetic changes are meiotically heritable and mitotically stable alterations in gene expression that include DNA methylation, RNA-associated silencing, and histone modification. Cytosine DNA methylation was the first identified epigenetic alteration and is the best-studied epigenetic mechanism (8-10). Cytosine DNA methylation is crucially important for the normal development, cell proliferation, and proper maintenance of genome stability of a given organism (9-12). Aberrant DNA methylation is well documented in cancer development and is linked to the phenomenon of genomic instability and increased mutation rates (11-17).

A variety of DNA-damaging agents, including ionizing radiation, are known to affect genome stability by altering

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Note: I. Pogribny and I. Koturbash contributed equally to this work.

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DNA methylation patterns (18, 19). Radiation-induced global genome DNA hypomethylation is linked to genome instability in the target tissue (20-22). There is a tight link between DNA methylation and histone modifications. Because of that, changes in DNA methylation in exposed cells are not isolated events and occur in a context of global chromatin deregulation (23, 24). Although attention has been given to radiation-induced changes in DNA methylation, histones, also key players, have been largely overlooked. Among histone modifications, phosphorylation of histone H2AX, a variant histone H2A, on radiation exposure is the most widely studied. Histone H2AX is rapidly phosphorylated at Ser¹³⁹ on induction of DNA strand breaks by irradiation and can be effectively visualized within repair foci using phosphospecific antibodies (25-27). Analysis of histone H2AX phosphorylation is widely used to assess the extent of radiation damage to cellular DNA (27-29).

Studies of histone modifications on genotoxic stress exposure and the involvement of histone methylation in the neoplastic cell transformation and progression of cancer just started to emerge (24). Fraga et al. (24) have shown recently that tumors undergo a massive loss of trimethylation at histone H4-Lys²⁰. This loss, along with DNA hypomethylation, is considered by many to be a universal marker for malignant transformation (24). Furthermore, histone H4-Lys²⁰ trimethylation is important for coordinating the DNA damage checkpoint in yeast (30). The role of histone modifications in radiation-induced transformation *in vivo* are still to be defined and the interrelationship between radiation-induced changes in DNA methylation and histone modifications are still to be elucidated.

Due to the paucity of data in this area, in the present study, we investigated the effect of low-dose radiation exposure on accumulation of DNA lesions and alterations of DNA and histone methylation in thymus tissue using an *in vivo* murine model (31-33). Radiation exposure induces leukemia and lymphoma in rodents; therefore, the rodent model has become a valuable system for delineating molecular mechanisms of radiation-induced leukemia and lymphoma predisposition (31-33).

For the first time, we show that fractionated whole-body low-dose radiation leads to more prominent decrease of trimethylation of histone H4-Lys²⁰ in thymus compared with acute low-dose radiation exposure. The loss of histone

H4-Lys²⁰ methylation was accompanied by a significant decrease in global DNA methylation, reduced levels of DNMT1, DNMT3a, DNMT3b, and methyl-binding proteins MeCP2 and MBD2, and accumulation of DNA damage monitored by persistence of histone γ H2AX foci in the thymus tissue of mice subjected to fractionated exposure.

Results

Radiation-Induced Decrease in Global DNA Methylation in the Murine Thymus

In this study, we used an *in vivo* murine model to characterize and compare the epigenetic changes in thymus on acute and fractionated radiation exposure in males and females. During the study, experimental animals (female and male C57/BL6 mice of comparable weight) were divided into three groups: control group, acutely exposed group, and group subjected to fractionated exposure for 10 days. Animals from the fractionated group were exposed to whole-body irradiation of 0.05 Gy X-ray daily for 10 consecutive days and received a cumulative dose of 0.5 Gy. The "acute" group of mice was exposed to 0.5 Gy X-ray only once, coinciding with day 10 of treatment in the "fractionated" group. Control animals were sham treated. To determine the status of DNA methylation in the thymus of female and male mice after acute and fractionated whole-body low-dose radiation exposure, we used the sensitive *HpaII*-based cytosine extension assay that measures the proportion of unmethylated CCGG sites. *HpaII* is a methylation-sensitive restriction enzyme that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands and leaves a 5'-guanine overhang after DNA cleavage that can be used for subsequent single nucleotide extension with labeled [³H]dCTP. The extent of [³H]dCTP incorporation opposite the exposed guanine is directly proportional to the number of cleaved and thus unmethylated CpG sites and inversely proportional to the levels of methylation (i.e., the higher the methylation, the less [³H]dCTP is incorporated; refs. 12, 20, 21, 34). We found that acute application of 0.5 Gy X-ray led to a 1.8- and 2.3-fold ($P < 0.05$) increase in the [³H]dCTP incorporation in female and male thymus, respectively, indicative of hypomethylation of DNA (Fig. 1). Notably, fractionated low-dose radiation exposure resulted in even more

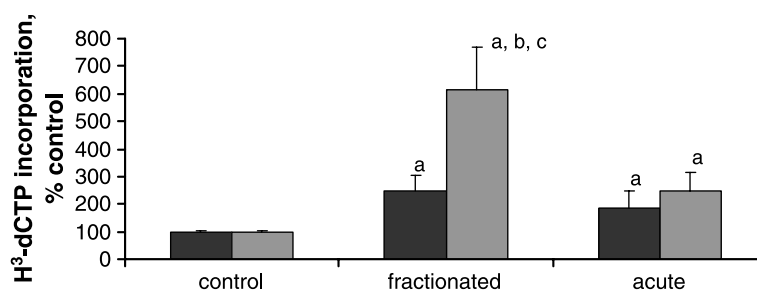


FIGURE 1. Low-dose radiation exposure induces significant loss of DNA methylation in the murine thymus. Levels of global genome DNA methylation in the thymus tissue of irradiated mice were measured by the cytosine extension assay on treatment of DNA with a methylation-sensitive restriction enzyme *HpaII* that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. The enzyme leaves a 5'-guanine overhang after DNA cleavage that is used for subsequent single nucleotide extension with labeled [³H]dCTP. The extent of [³H]dCTP incorporation opposite the exposed guanine is directly proportional to the number of cleaved and thus unmethylated CpG sites and inversely proportional to the levels of methylation (i.e., the higher the methylation, the lower is the incorporation of [³H]dCTP). Columns, mean levels of [³H]dCTP ($n = 6$); bars, SE. a, $P < 0.05$, significantly different from control; b, $P < 0.05$, significantly different from female mice; c, $P < 0.05$, significantly different from male mice exposed to acute low-dose radiation, ANOVA. Black columns, females; gray columns, males.

substantial decrease in global DNA methylation than acute irradiation, causing 2.5- and 6.1-fold ($P < 0.05$) reduction in global DNA methylation in females and males, respectively (Fig. 1).

The observed decrease in global DNA methylation on low-dose radiation exposure agrees with results obtained previously by our laboratory that showed a loss of DNA methylation in spleen tissue (20, 21) and supports the hypothesis that low-dose radiation exposure leads to DNA hypomethylation in radiation target organs. In the current study, an even more considerable loss of methylation was observed in the thymus tissue. The spleen and thymus are important parts of hematopoietic and lymphatic tissue and are profoundly affected in lymphomas and leukemias (31-33).

Mechanisms of Radiation-Induced DNA Hypomethylation—Role of Radiation-Induced DNA Damage

The mechanisms underlying the observed decrease in global DNA methylation after radiation exposure remain unclear. Based on the results of our previous study (20), we hypothesized that one of the possible mechanisms that lead to the observed decrease in global DNA methylation after radiation exposure might be related to radiation-induced DNA damage. Radiation is a potent damaging agent that induces double-strand breaks and other lesions in DNA, activating a battery of DNA repair mechanisms. Because DNA lesions can interfere with activity of DNA methyltransferases (35, 36), and during repair DNA synthesis polymerases incorporate cytidine but not methyl cytidine, the presence of DNA lesions and activation of DNA repair mechanisms may result in DNA hypomethylation. In light of these considerations, we measured the levels of DNA damage (radiation-induced strand breaks) in the thymus tissue of animals subjected to acute and fractionated low-dose radiation exposure. It has been shown previously that the initial response to formation of DNA breaks, especially double-strand breaks, is a rapid phosphorylation of Ser¹³⁹ of histone H2AX in the chromatin microenvironment surrounding a DNA double-strand break (25, 27, 37). Indeed, within minutes after exposure to radiation, the phosphorylated H2AX can be visualized within repair foci using phosphospecific antibodies (25, 26). Quantification of phosphorylated histone H2AX foci (γ H2AX foci) constitutes a sensitive and reliable measure of radiation-induced DNA damage (28, 29, 38). We applied this method to estimate the levels of DNA damage in control and exposed murine thymus tissues. Acute exposure of mice to 0.5 Gy X-ray resulted in a significant ($P < 0.05$) 3.3-fold elevation of γ H2AX foci per cell in both sexes (Fig. 2E-G). Quite striking, a considerable 2.9- and 3.1-fold increase of the number of γ H2AX foci per cell was observed on fractionated irradiation in female and male thymocytes, respectively (Fig. 2C, D, and G).

In present study, both acute and, most importantly, fractionated low-dose radiation exposure led to accumulation of γ H2AX foci in thymus cells. It should be pointed out that we cannot exclude the possibility that the persistence of γ H2AX foci of the fractionated exposure group may suggest that they represent DNA lesions with unreparable double-strand breaks (28). Persistence of γ H2AX foci may also be due to clustering of DNA damage during fractionated irradiation (39). The observed persistence of γ H2AX supports our previous findings that

fractionated radiation exposure profoundly affects the balance of DNA repair systems in cells (22), possibly leading to damage accumulation/clustering. Accumulation of γ H2AX foci in this study was not linked to changes in cellular proliferation, as fractionated exposure did not lead to any changes in proliferating cell nuclear antigen expression (data not shown). A single

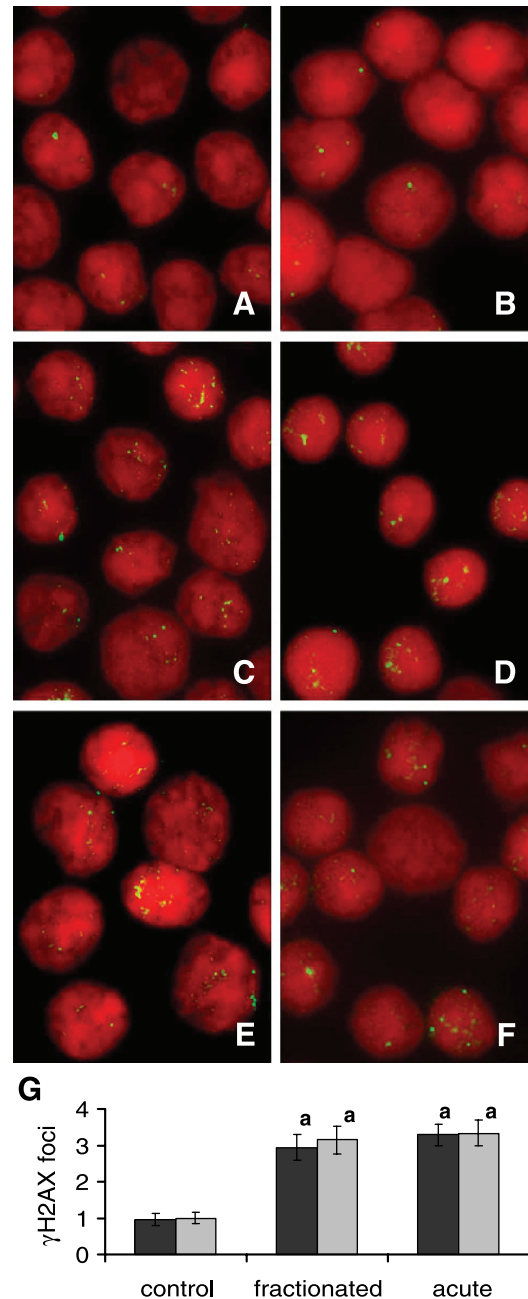


FIGURE 2. Radiation-induced accumulation of γ H2AX foci in murine thymus. Histone H2AX is phosphorylated at Ser¹³⁹ immediately on induction of DNA damage, forming γ H2AX foci, which revealed DNA double-strand breaks. **A.** Control female. **B.** Control male. **C.** Female, fractionated exposure. **D.** Male, fractionated exposure. **E.** Female, acute exposure. **F.** Male, acute exposure. **G.** Levels of γ H2AX foci per cell. Columns, mean ($n = 240$); bars, SE. a, $P < 0.05$, significantly different from control. Black columns, females; gray columns, males.

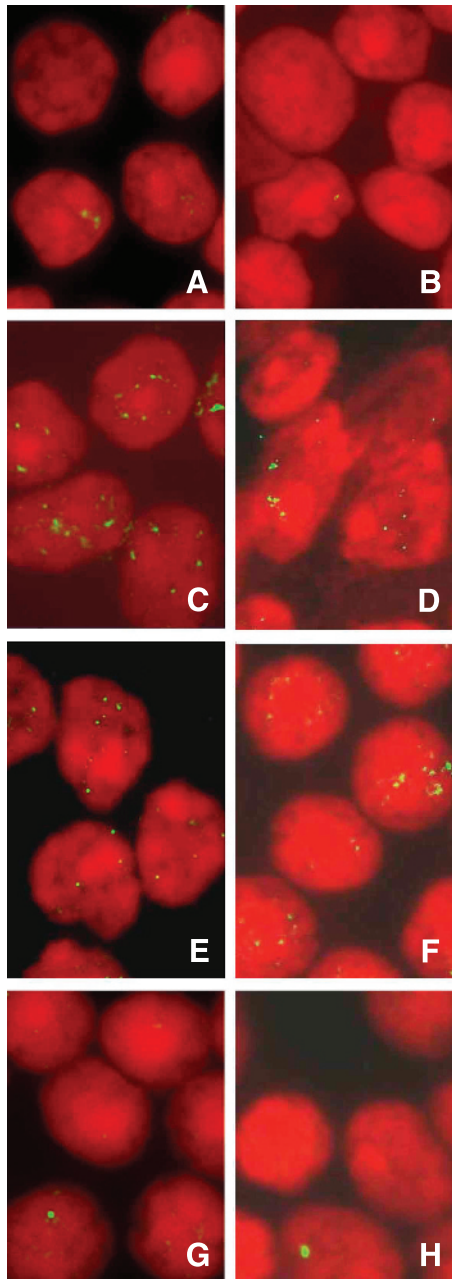


FIGURE 3. Induction and persistence of fractionated radiation-induced γ H2AX foci in murine thymus. **A.** Control female. **B.** Control male. **C.** Female, fractionated exposure, 0.5 Gy total dose, 4 hours after the last fraction. **D.** Male, fractionated exposure, 0.5 Gy total dose, 4 hours after the last fraction. **E.** Female, fractionated exposure, 0.5 Gy total dose, 24 hours after the last fraction. **F.** Male, fractionated exposure, 0.5 Gy total dose, 24 hours after the last fraction; **G.** Female exposed to a single fraction of 0.05 Gy, 4 hours after exposure. **H.** Male exposed to a single fraction of 0.05 Gy, 4 hours after exposure.

fraction of 0.05 Gy delivered alone did not lead to significant elevation of γ H2AX foci 4 hours after exposure. On the other hand, we noted that γ H2AX foci induced by fractionated exposure persisted 24 hours on the completion of fractionated irradiation protocol (Fig. 3). Interestingly, recent studies also showed that prolonged accumulation of phosphorylated γ H2AX

is often found in MCT-1 and E7 oncoprotein-transformed cells (27) and may be an early sign of predisposition to carcinogenesis (40). In the light of these findings, the molecular mechanisms and biological significance of γ H2AX foci persistence on fractionated irradiation deserve special attention in the future.

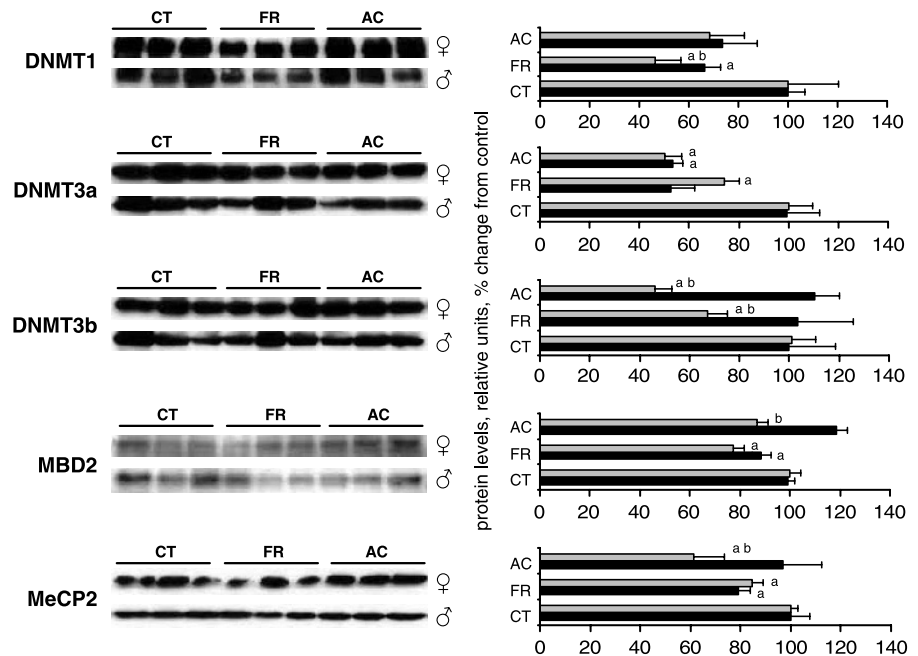
Mechanisms of Radiation-Induced DNA Hypomethylation—Roles of DNA Methyltransferases and Methyl-Binding Proteins

DNA methyltransferases DNMT1, DNMT3a, and DNMT3b are the three main functional enzymes that are responsible for setting and maintaining DNA methylation patterns in mammalian cells. These enzymes have different abilities to catalyze maintenance and *de novo* methylation. Deregulation of any or all of their activities may result in perturbations of DNA methylation (41). Therefore, we evaluated the effects of acute and fractionated low-dose radiation exposure on the expression of DNMT1, DNMT3a, and DNMT3b in thymus of female and male mice as another possible contributing mechanism of radiation-induced hypomethylation of DNA.

Expression of DNMT1 was not affected by acute low-dose radiation. In contrast, fractionated irradiation led to a significant $\sim 34\%$ and $\sim 54\%$ decrease ($P < 0.05$) in expression of DNMT1 in female and male thymus, respectively (Fig. 4). The expression of DNMT1 in the thymus of male mice exposed to fractionated low-dose radiation was significantly lower compared with female mice ($P < 0.05$) and slightly yet statistically insignificantly lower compared with male mice exposed to acute low-dose radiation. Acute radiation exposure led to a modest reduction of DNMT3a levels in both sexes and a reduction of DNMT3b protein levels only in males (Fig. 4). Expression of DNMT3a was decreased in females and males that were exposed in a fractionated fashion. Levels of DNMT3b were also decreased, on fractionated exposure, but only in male thymus (Fig. 4).

Notably, fractionated low-dose radiation exposure not only down-regulated the expression of both maintenance and *de novo* DNA methyltransferases but also led to slightly yet statistically significant decrease ($P < 0.05$) in the expression of methyl-binding proteins MeCP2 and MBD2 in thymus of exposed females and males compared with respective controls. Radiation-induced loss of MeCP2 and MBD2 expression is an important finding of this study. MeCP2 is a transcriptional repressor that selectively recognizes methylated DNA. The methyl-binding domain of MeCP2 recognizes symmetrically methylated CpG dinucleotides through contact with the major groove of the double helix in the nucleosome and, under certain circumstances, can even displace histone H1 from chromatin (14, 42). Its transcription repression domain interacts with corepressor complexes containing histone deacetylases (43, 44). The properties of MeCP2 are central to understanding the mechanisms of methylation-mediated gene silencing and chromatin remodeling. The other recently identified MeCP2 homologue, methyl-binding protein MBD2, also constitutes important component of chromatin remodeling machinery and selectively interact with methylated DNA (14). The methyl-binding proteins do not have prescribed locus specificity and participate in chromatin remodeling (14). A decrease in their expression may lead to alterations in the global genome expression patterns leading to genome destabilization (10, 14).

FIGURE 4. Radiation exposure alters the expression of DNA (cytosine-5) methyltransferases and methyl-binding proteins in thymus of female and male mice. Lysates from thymus tissue were subjected to immunoblotting using antibodies against DNMT1, DNMT3a, DNMT3b, MBD2, and MeCP2. Columns, mean protein levels relative to those of control animals; bars, SE. a, $P < 0.05$, significantly different from control; b, $P < 0.05$, significantly different from female mice, Student's *t* test. Black columns, females; gray columns, males. CT, control animals; FR, animals subjected to fractionated exposure; AC, acutely exposed animals. All sample loading was normalized to protein content. Representative Western blots from three independent experiments are shown; each lane represents a protein extract of a thymus of one animal.



Interestingly, fractionated low-dose radiation-induced reduction in DNMT1, DNMT3b, and MBD2 protein levels was much more pronounced in males than in females as was the fractionated low-dose radiation-induced DNA methylation loss (Figs. 1 and 4). This link still has to be further elucidated to uncover the roles of the aforementioned proteins in DNA methylation dynamics on irradiation.

Overall, fractionated low-dose radiation exposure led to a significant accumulation of DNA damage and a significant loss of DNA methylation paralleled by decreases in the expression of DNA methyltransferases and methyl-binding proteins, all of which are important modulators of chromatin dynamics (10, 14). The latter changes were particularly interesting as methyl-binding proteins serve as “linkers” conveying messages from methylated DNA to histones (10).

Radiation-Induced Changes in Histone H4-Lys²⁰ Trimethylation

The interplay between radiation-induced DNA methylation changes and histone modifications is still underinvestigated (24, 45). Recent studies indicate that global loss of DNA methylation may be paralleled by changes in histone methylation (23, 24).

To determine whether the radiation-induced DNA hypomethylation is accompanied by alterations in histone methylation, we analyzed the changes in histone H4-Lys²⁰ trimethylation. The level of histone H4-Lys²⁰ trimethylation in thymus tissue of female mice exposed to acute and fractionated low-dose radiation exposure was significantly lower than in control group (Fig. 5). Remarkably, radiation exposure of male mice led to more prominent changes of histone H4-Lys²⁰ methylation resulting in reduction of methylation by 50%.

This radiation-induced loss of trimethylation at histone H4-Lys²⁰ is another outcome of this study. Recent studies have

shown that loss of histone H4-Lys²⁰ trimethylation could result in a more “relaxed” heterochromatin organization, which markedly impairs genome stability and viability of the cells (46, 47) and may lead to the malignant transformation (24).

In addition, in yeast, histone H4-Lys²⁰ trimethylation was shown to play an important role in DNA damage response and the loss of histone H4-Lys²⁰ trimethylation compromised the cell's ability to maintain the cell cycle checkpoint response to DNA damage (30). These studies provide an important link between histone H4-Lys²⁰ trimethylation and genome stability (30, 42, 43).

Discussion

In the present study, we have shown that fractionated whole-body low-dose radiation exposure resulted in epigenetic alterations in the murine thymus. The main findings of the present study are the following: (a) fractionated/repetitive low-dose radiation exposure of C57/BL6 mice led to more prominent genetic and epigenetic changes in the thymus than acute exposure, especially in male mice; (b) fractionated genotoxic stress exposure resulted in a significant decrease in global DNA methylation, which was accompanied by decreased levels of DNMT1, DNMT3a, and DNMT3b and reduction in the levels of methyl-binding proteins MeCP2 and MBD2; (c) fractionated radiation exposure led to the accumulation and/or persistence of DNA damage as monitored by γ H2AX foci accumulation; and (d) fractionated radiation exposure altered histone H4-Lys²⁰ trimethylation levels. For the first time, we show that genotoxic stress exposure led to a loss of trimethylation of histone H4-Lys²⁰. These epigenetic changes may therefore constitute one of the initial steps in radiation-induced tumorigenic transformation.

An interesting and important outcome of this study is the observed interrelationship between accumulation of γ H2AX foci (histone modification in response to DNA damage) and

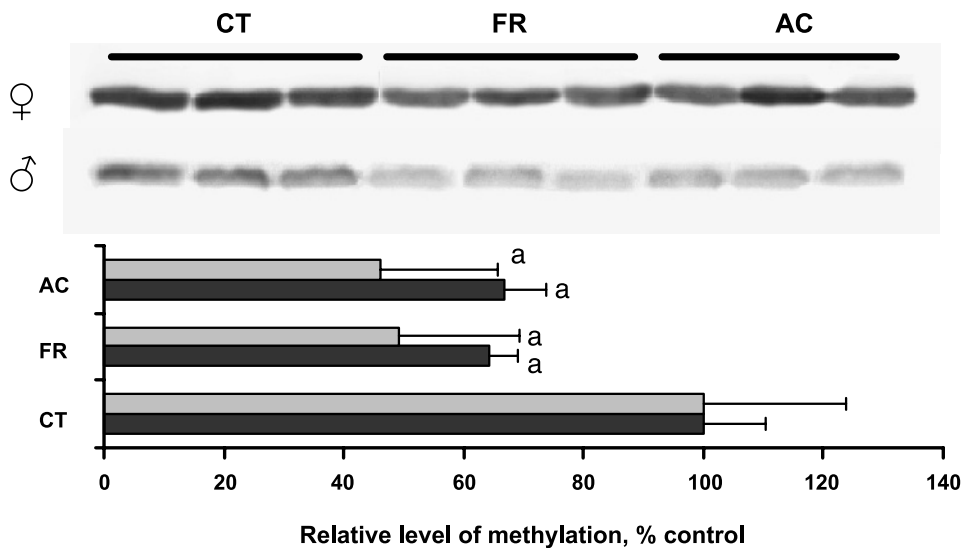


FIGURE 5. Radiation-induced loss of histone H4-Lys²⁰ trimethylation in thymus tissue. Acidic thymus tissue lysates enriched in histones were subjected to immunoblotting using anti-trimethyl histone H4-Lys²⁰ antibodies. Columns, mean levels relative to those of control animals; bars, SD. a, $P < 0.05$, significantly different from control, Student's t test. Black columns, females; gray columns, males. All sample loading was normalized for protein content. Representative blots from two independent experiments are shown.

other epigenetic (DNA methylation) changes. Presence of DNA lesions has been reported to affect the methylation ability of DNA methyltransferases (35, 36). Furthermore, during repair, DNA synthesis polymerases incorporate cytidine but not methyl cytidine; thus, the elevated levels of DNA lesions seen in this study and activation of DNA repair may be one of the factors contributing to decrease in DNA methylation.

Changes in DNA methylation were accompanied by the decreased expression of DNA methyltransferases DNMT1, DNMT3a, and DNMT3b, especially in male mice. In the present study, fractionated low-dose radiation exposure slightly but statistically significantly affected expression of the maintenance DNA methyltransferase DNMT1 and *de novo* methyltransferases DNMT3a and DNMT3b. This finding supports the results of our previous study on the role of *de novo* methyltransferases in methylation patterning in radiation-exposed tissues (21). Recent results showed that DNMT3a is distributed in the nucleoplasm and is not associated with nuclear DNA replication sites during S phase (48). This enzyme, together with DNMT3b, likely contributes to DNA repair synthesis-related DNA methylation patterning as reported previously by our group (21). Recent data suggest that the inactivation of *de novo* methyltransferases leads to hypomethylation of DNA and chromosome instability (49). The exact relationship between radiation-induced repair DNA synthesis and the action of methyltransferases has yet to be established.

Our study also showed a radiation-induced decrease in the levels of important methyl-binding proteins MeCP2 and MBD2. These proteins selectively interact with methylated DNA and play pivotal roles in methylation-mediated chromatin remodeling and gene silencing (14). The contribution of MeCP2 and MBD2 as well as the other methyl-binding proteins to low-dose radiation-induced genome instability has yet to be elucidated.

Another important outcome of the current study is the loss of histone H4-Lys²⁰ trimethylation in the exposed thymus. Emerging evidence provided links between histone H4-Lys²⁰ trimethylation and oncotransformation (24, 30, 46, 47). Interestingly, Fraga et al. (24) have observed a decrease in trimethylated histone H4-Lys²⁰ in leukemia cell lines when

compared with normal lymphocytes. They also noted that four human primary lymphomas exhibited a significant decrease in histone H4-Lys²⁰ methylation when compared with normal tissues. In light of these findings, our observation of the fractionated low-dose radiation exposure induced histone H4-Lys²⁰ trimethylation loss may be viewed as one of the important steps that could lead to radiation-induced malignant transformation (24).

Our data revealed that fractionated low-dose radiation-induced decrease in DNA methylation and loss histone H4-Lys²⁰ trimethylation were more pronounced in male than in female thymus. It has been reported previously that the incidence of radiation-induced thymic lymphoma is much higher in male mice (50). Furthermore, radiation-induced leukemia and lymphoma in humans is also more frequent in males (51, 52). Further studies are clearly needed to analyze the mechanisms and biological significance of these sex differences.

Our data support the notion that there is an interrelation between histone modifications and DNA methylation. Changes in histone modifications may affect DNA methylation, but DNA methylation changes may reciprocally influence the histone modifications (53). The exact role of alterations in histone H4-Lys²⁰ trimethylation and DNA methylation in radiation carcinogenesis, particularly radiation-induced lymphomagenesis and leukemogenesis, requires further study, as it potentially serves as an early marker of radiation-induced secondary malignization.

Conclusions and Outlook

This is the first study showing genetic and, most importantly, epigenetic alterations on fractionated low-dose radiation exposure in thymus tissue. Future studies are clearly needed to address the effects of the other doses and different exposure regimens on the status of DNA and histone methylation. The persistence of the radiation-induced epigenetic changes in thymus also remains to be uncovered. Mechanisms (direct and indirect) and significance of the striking sex differences in the radiation-induced epigenetic changes in mice have to be further substantiated. The exact role of global DNA and histone hypomethylation in radiation carcinogenesis, particularly in

radiation-induced lymphomagenesis and leukemogenesis, must be further elucidated as these changes might serve as early markers of radiation-induced malignization. Moreover, DNA and histone methylation changes induced by radiation exposure may hold the keys to understanding radiation carcinogenesis, which, although widely studied, remains enigmatic.

Materials and Methods

Model

In this study, we used an *in vivo* murine model to study genetic and epigenetic alterations in thymus following radiation exposure. The murine model is widely used, well characterized, and generally accepted for studies of radiation-induced changes in hematopoietic and lymphatic tissue (31-33). Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures have been approved by the University of Lethbridge Animal Welfare Committee.

Effects of Acute and Fractionated Low-Dose Exposure

Eighteen test animals, C57/BL6 mice (9 males and 9 females, Charles River, Toronto, Ontario, Canada), were divided into three groups: control, acutely exposed, and fractionated exposure for 10 days. All animals were 45 days old at day 1 of the experiment, had comparable body weights, were kept in a virus-free facility, and given food and water *ad libitum*. Animals were monitored daily by a trained technician. No signs of pain or distress were observed during the experiment. The fractionated groups received a repetitive fractionated whole-body irradiation of 0.5 Gy applied as 5 cGy X-ray daily for 10 days. The acute mouse group received 0.5 Gy X-ray in a single dose on the 10th day of treatment for the fractionated group. Control mice were sham treated. All animals were sacrificed 4 hours after the last treatment on day 10. Thymus tissue was sampled immediately following sacrifice, divided into two equal parts, frozen using liquid nitrogen, and stored at -80°C until processed for DNA and protein analysis. This experiment was independently replicated twice according to the same experimental scheme using a cohort of 18 C57/BL6 mice each time (3 animals per group). All three experiments yielded congruent data.

DNA Extraction

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

Cytosine Extension Assay to Detect Sequence-Specific Changes in DNA Methylation

DNA (0.5 μg) was digested overnight with a 10-fold excess of *Hpa*II endonuclease according to manufacturers protocol (New England Biolabs, Beverly, MA). 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 done in 25 μg DNA, $1\times$ PCR buffer II, 1.0 mmol/L MgCl_2 , 0.25 units Taq DNA polymerase (Fisher Scientific, Ottawa, Ontario, Canada), [^3H]dCTP (57.4 Ci/mmol; Perkin-Elmer, Boston, MA), incubated at 55°C for 1 hour, and immediately placed on ice. Duplicate aliquots (25 μL) from each reaction were placed on Whatman DE-81 ion-exchange filters

and washed thrice for 10 minutes with gentle agitation with sodium phosphate buffer (0.5 mol/L, pH 7.0) at room temperature. The filters were dried and processed by scintillation counting (Beckman Coulter, Fullerton, CA). Background label incorporation was subtracted from enzyme-digested samples and results were expressed as relative [^3H]dCTP incorporation per 1 μg DNA or as percent change from control (12, 20, 21, 34).

DNA Damage Analysis—Accumulation of γH2AX Foci

The thymus tissue was touch printed onto positively charged slides (VWR, Mississauga, Ontario, Canada), air-dried, and fixed in 2% paraformaldehyde in PBS. On fixation, slides were immunostained using antibodies against phosphorylated histone H2AX as described (28).

Western Immunoblotting

Western immunoblotting for DNMT1, DNMT3a, DNMT3b, MBD2, and MeCP2 was conducted using the thymus tissue. Tissue samples were sonicated in 0.4 to 0.8 mL ice-chilled 1% SDS and boiled for 10 minutes. Small aliquots (10 μL) of homogenate were reserved for protein determination using protein assay reagents from Bio-Rad (Hercules, CA). Equal amounts of proteins (10 μg) were separated by SDS-PAGE in slab gels of 8% or 12% polyacrylamide, made in duplicates, and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). Membranes were incubated with primary antibodies against DNMT1 (1:1,000, Abcam, Cambridge, MA), DNMT3a and DNMT3b (1:500, Abgent, San Diego, CA), MBD2 (1:500, Abcam), and MeCP2 (1:1,000, Abcam). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies and the Enhanced Chemiluminescence Plus immunoblotting detection system (Amersham Biosciences). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Unaltered polyvinylidene difluoride membranes were stained with Coomassie blue (Bio-Rad) to confirm equal protein loading on unaltered membrane. Signals were quantified using NIH Image J 1.63 software and normalized relative to glyceraldehyde-3-phosphate dehydrogenase or the M_r 50,000 protein band, which gave internally consistent results (values relative to M_r 50,000 are plotted).

Analysis of Histone H4-Lys²⁰ Trimethylation

Histone H4-Lys²⁰ trimethylation status was analyzed using the total histone extracts of thymus tissue. The acid extracts were prepared from frozen thymus tissues using lysis buffer containing 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl_2 , 10 mmol/L KCl, 0.5 mmol/L DTT, and 1.5 mmol/L phenylmethylsulfonyl fluoride followed by addition of HCl to a final concentration of 200 mmol/L according to manufacturer's protocol (Upstate, Charlottesville, VA). Cell lysates were centrifuged at $14,000\times g$ for 10 minutes at 4°C , and the acid-insoluble pellets were discarded. The supernatant fractions, which contain the acid-soluble proteins, were purified by sequential dialysis against 100 mmol/L acetic acid and H_2O . Protein concentrations were determined by Bradford assay (Pierce, Rockford, IL). Equal amounts of total histones (40 μg) were mixed with two volumes of gel loading buffer [250 mmol/L Tris-HCl (pH 8.0), 20% β -mercaptoethanol,

40% glycerol, 8% SDS, 1.2 mg/mL bromophenol blue], heated for 5 minutes at 95°C, and resolved on 15% polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 4 hours in TBS buffer containing 5% nonfat dry milk. Anti-trimethyl histone H4-Lys²⁰ primary antibodies were diluted 1:2,000 according to manufacturer's recommendations (Upstate). Primary antibody binding was done at 4°C overnight with constant shaking. Secondary antibodies labeled with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA) were applied at 1:5,000 dilutions, and binding was carried out at room temperature for 1.5 hours. Chemifluorescence detection was done with the ECF substrate for Western blotting (Amersham Biosciences) and detected directly by Storm imaging system (Molecular Dynamics, Sunnyvale, CA). Images are representative of three independent immunoblots and were analyzed by ImageQuant software. All membranes were stained with Coomassie blue to confirm equal loading.

Statistical Analysis

The statistical procedures are described by Sokal and Rohlf (54) Statistical treatment and plotting of the results were done using the Sigma Plot and Excel for Windows XP software. The results are presented as mean ± SE.

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