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Tissue-specific DNA-PK-dependent H2AX phosphorylation and γ-H2AX elimination after X-irradiation *in vivo*

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ABSTRACT

Histone H2AX rapidly undergoes phosphorylation at Ser139 (γ-H2AX) in response to DNA double-strand breaks. Although ATM kinase and DNA-PK phosphorylate Ser139 of H2AX in culture cells, the regulatory mechanism of γ-H2AX level remains unclear *in vivo*. Here, we detected the phosphorylation of H2AX and the elimination of γ-H2AX in the mouse skin after X-irradiation. Furthermore, following X-irradiation, the level of γ-H2AX also increased in mice lacking either ATM or DNA-PK. Although the elimination after X-irradiation was detected in the skin of these mutant mice, the elimination in DNA-PK-deficient mice was slower than that in C3H and ATM knockout mice, suggesting that a fraction of γ-H2AX in the skin is eliminated in a DNA-PK-dependent manner. Although the DNA-PK-dependent elimination of γ-H2AX was also detected in the liver, kidney, and spleen, the DNA-PK-dependent phosphorylation of H2AX was detected in the spleen only. These results suggest that the regulatory mechanism of γ-H2AX level is tissue-specific.

DNA double-strand break (DSB) is the most dangerous type of DNA damage [1]. In mammalian cells, two major pathways exist for the repair of DSB: nonhomologous DNA-end-joining (NHEJ) repair and homologous recombination (HR) [1,2]. H2AX is phosphorylated at Ser139 (γ-H2AX) at sites of DSB, where it is considered to restructure chromatin and assist in the recruitment of DNA repair and signaling factors [3]. Many components of the DNA damage response including BRCA1, the MRN complex, 53BP1, MDC1, and Rad51, which are involved in DSB repair by HR form foci that colocalize with γ-H2AX foci [3]. DNA-damage-induced phosphorylation of H2AX at Ser139 is mediated by the phosphoinositide 3-kinase-like kinases ataxia telangiectasia mutated (ATM) and DNA-PK, which are activated by DSB, in culture cells [3]. In addition, mice lacking H2AX show radiation sensitivity [4]. Therefore, it is considered that H2AX phosphorylation and γ-H2AX elimination contribute to DSB repair, although the mechanisms involved are not fully clear.

It has been debated as to whether Ser139 of H2AX is phosphorylated by ATM alone or both ATM and DNA-PK. Burma et al. reported that H2AX phosphorylation at Ser139 after X-irradiation is ATM-dependent in mouse embryonic fibroblasts [5]. On the other hand, Stiff et al. showed that ATM or DNA-PK can promote IR-induced H2AX phosphorylation redundantly in human and rodent culture cells [6]. There have been only few reports on the phosphorylation of H2AX or the elimination of γ-H2AX in mammalian tissues *in vivo* [7–11], because nearly all the studies on γ-H2AX were carried out using culture cells. Gavrilov et al. reported that there are significant variations between nonproliferating mammalian tissues, *i.e.*, heart and kidney, in the initial H2AX phosphorylation rate as well as in the rate of γ-H2AX elimination after X-irradiation [9]. However, the pattern of γ-H2AX elimination and the mechanism underlying this elimination *in vivo* are not understood presently.

Here, we examined the phosphorylation of H2AX and elimination of γ-H2AX in four tissues, namely, skin, liver, kidney, and spleen, after X-irradiation.

Materials and methods

Mice. Five-week-old SCID mice, ATM knockout mice with the C3H background, and C3H mice were purchased from a colony at the animal production facility of the National Institute of Radiological Sciences in Chiba. C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All mice were reared and handled in accordance with the guidelines on the care and use of laboratory animals of the National Institute of Radiological Sciences [10,11].

X-irradiation. The mice were exposed to X-rays at 5 Gy at a dose rate of 0.63 Gy/min or 20 Gy at a dose rate of 1.90–2.04 Gy/min [8,10–12]. X-rays were generated at 200 kVp/20 mA and filtered through 0.5 mm Cu and Al filters using Pantak HF320S or HF320 (Shimadzu, Kyoto, Japan).

Abbreviations: DSB, DNA double-strand break; ECL, enhanced chemiluminescence; γ-H2AX, phosphorylated H2AX.

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Western blot analysis. Total lysates from the mouse skin, liver, spleen, or kidney were extracted using MM-300 (QIAGEN Inc., Chatworth, CA) in accordance with the manufacturer's protocol and cleared by centrifugation, and the supernatants containing 20 μg of proteins were electrophoresed on 5–20% SDS–polyacrylamide gels [11,13]. Western blot analysis was performed as previously described with slight modification [11,13]. In brief, fractionated products were electroblotted onto Hybond-P membranes (GE Healthcare Bio-Sci. Corp.). After blocking nonspecific binding sites with an enhanced chemiluminescence (ECL)-blocking reagent (GE Healthcare Bio-Sci. Corp.), the membranes were incubated with a rabbit anti-H2AX polyclonal antibody (Bethyl Lab. Inc.), a mouse anti-γ-H2AX monoclonal antibody (Upstate Biotechnology Inc.), or a mouse anti-PCNA monoclonal antibody (Santa Cruz Biotechnology, Inc). The blots corresponding to proteins were visualized using an enhanced ECL Western blotting detection system (GE Healthcare Bio-Sci. Corp.), in accordance with the manufacturer's instructions [11,13].

Immunohistochemical analysis. For immunohistochemical staining, freshly excised samples from mice were frozen in the O.C.T. compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan) in liquid nitrogen and sectioned with a cryostat microtome [10–12]. Immunostaining was performed using a rabbit polyclonal anti-Ki67 antibody at 1:2000 dilution (NovoCastra) and the DAKO Envision Plus HRP system (DAKO). PFA (4%)-fixed sections of mouse skin, liver or spleen samples were antigen-retrieved with or without heating using a microwave oven for 8 min in citrate buffer (pH 6.5), prior to peroxidase blocking (0.03%, v/v hydrogen peroxide). The primary antibody and horseradish peroxide-labeled polymer were used in accordance with the instructions included in the DAKO Envision kit used, and 3,3-diaminobenzidine treatment and counterstaining with hematoxylin before mounting were then carried out.

Results and discussion

The post-translational modification of H2AX, *e.g.*, phosphorylation, plays a central role in responses to radiation, including the repair of DSB [3]. However, the phosphorylation of H2AX and γ-H2AX elimination pattern *in vivo* is not understood. To investigate the phosphorylation of H2AX at Ser139 and its elimination after X-irradiation *in vivo*, we first examined the X-ray-induced H2AX phosphorylation and γ-H2AX elimination in the C57BL/6 mouse skin by Western blot analysis using the anti-γ-H2AX antibody. We detected γ-H2AX and its elimination in the mouse skin after X-irradiation. As shown in Fig. 1, extracts from the skin at 1 h postirradiation exhibited a strong induction of γ-H2AX compared with those from the unirradiated skin. Afterwards, γ-H2AX level decreased in a time-dependent manner. The disappearance of γ-H2AX indicates that DSB was repaired by a DSB repair process in culture cells [3]. Thus, these data suggest that a fraction of X-ray-induced DSB was repaired by a DSB repair process(s) in the mouse skin *in vivo*.

The mechanism of γ-H2AX elimination is not clear *in vivo*, although the disappearance of γ-H2AX shows that DSB was repaired by a DSB repair process. It is suggested that ATM promotes the HR repair of DSB [14]. It is also suggested that DNA-PKcs is coregulated HR in cooperation with ATM [14]. In fact, HR is repressed when DNA-PKcs is chemically inhibited or expressed as a kinase-inactive splice variant [14]. To examine the roles of ATM and DNA-PK in γ-H2AX elimination after X-irradiation *in vivo*, we examined X-ray-induced H2AX phosphorylation and γ-H2AX elimination in extracts from the skin of DNA-PK-deficient SCID mice and ATM knockout mice by Western blot analysis using the anti-γ-H2AX antibody. Following X-irradiation, the high level of γ-H2AX was detected in extracts at 2 h postirradiation from the mouse skin lacking either ATM or DNA-PK as well as in extracts from the C3H mouse skin (Fig. 2). Although the elimination during from 2 to 5 h after X-irradiation was detected in the skin of these mutant mice, the elimination in DNA-PK-deficient SCID mice was slower than that in ATM knockout mice and C3H mice (Fig. 2). These findings suggest that the elimination of γ-H2AX in the skin observed in this study, at least in part, proceeded in a DNA-PK-dependent manner, although the elimination proceeded in both DNA-PK-dependent and -independent manner.

The elimination of γ-H2AX at DSB can occur either by dephosphorylation by protein phosphatase or by histone exchange in culture cells [9]. However, the elimination of γ-H2AX *in vivo* has not been clarified, because there is only one report about the mechanism of γ-H2AX elimination in tissues *in vivo*. Gavrilov et al. reported that the elimination in the heart is much slower than that in the kidney from mice after whole-body irradiation [9]. They suggested that the low rate of γ-H2AX elimination in the heart results from the expression of the histone acetyltransferase TIP60 gene, which contributes to the elimination of γ-H2AX in culture cells; the rate in a normal heart is significantly lower than that in a normal kidney [9]. Recently, we have reported that the level of γ-H2AX in extracts 2 h after X-irradiation differs between the kidney or liver and the ear [11]. To examine whether there is tissue specificity in the elimination of γ-H2AX, we examined

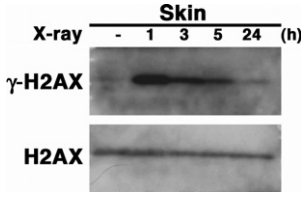

Fig. 1. Detection of γ-H2AX and γ-H2AX elimination in skin of X-irradiated mice. Each of the total skin protein samples was prepared from X-irradiated C57BL/6 mice (5 Gy) at indicated times after the start of irradiation. γ-H2AX protein level was determined by Western blot analysis using specific antibodies against γ-H2AX (top) and H2AX (bottom). H2AX was used a loading control.

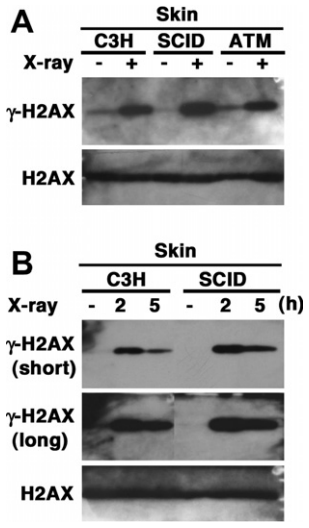

Fig. 2. Western blot analysis of γ-H2AX and γ-H2AX elimination in skin from C3H mice, DNA-PK-deficient SCID mice (SCID), and ATM knockout mice (ATM). The mice on day 35 were either nonirradiated or irradiated with 20 Gy X-rays. Each of the total protein samples from the skin was prepared 2 or 5 h after treatment. γ-H2AX protein level was determined by Western blot analysis using specific antibodies against γ-H2AX (top, short exposure; middle, long exposure) and H2AX (bottom). H2AX was used a loading control.

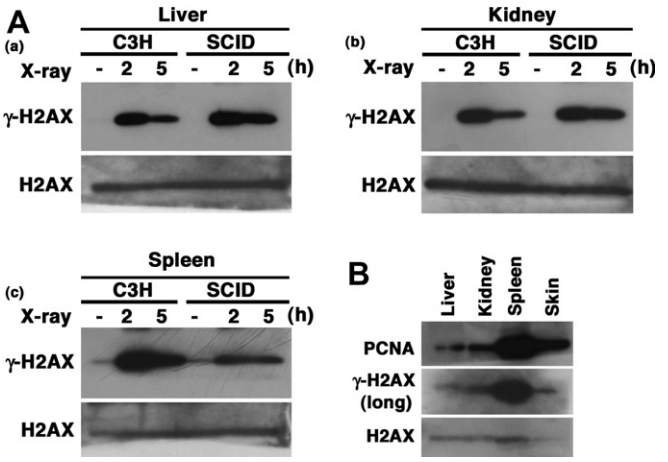

Fig. 3. Western blot analysis of γ-H2AX elimination in tissues from C3H mice and DNA-PK-deficient SCID mice (SCID). (A) Both mice on day 35 were either nonirradiated or irradiated with 20 Gy X-rays. Each of the total protein samples from the liver (a), kidney (b), and spleen (c) was prepared 2 or 5 h after treatment. γ-H2AX protein level was determined by Western blot analysis using specific antibodies against γ-H2AX (top) and H2AX (bottom). (B) Western blot analysis of cell proliferation activity in tissues. Cell proliferation activity in tissues prepared from unirradiated mice was determined by Western blot analysis using specific antibodies against PCNA (top), γ-H2AX (middle, long exposure), and H2AX (bottom). H2AX was used a loading control.

X-ray-induced H2AX phosphorylation and γ-H2AX elimination in extracts from the liver, kidney, or spleen of SCID and C3H mice by Western blot analysis using the anti-γ-H2AX antibody. As shown in Fig. 3A, the DNA-PK-dependent elimination was detected in the liver, kidney, and spleen as well as in the skin, suggesting that the molecular mechanism of underlying γ-H2AX elimination is, at least in part, dependent on a DNA-PK-dependent repair process in all tissues examined. We also found that there are significant differences between the spleen and the other tissues, *i.e.*, the liver, the kidney, and the skin, in the initial γ-H2AX level (Fig. 3B). Next, we examined the induction of γ-H2AX in the spleen of C3H and DNA-PK-deficient SCID mice after X-irradiation. As shown in Fig. 3A, extracts from the spleen of C3H mice at 2 h postirradiation exhibited a strong induction of γ-H2AX compared with those from the unirradiated spleen. Although the phosphorylation level of H2AX after X-irradiation also increased in the spleen of the DNA-PK-deficient SCID mice, the level of γ-H2AX in DNA-PK-deficient SCID mice was very low than that in C3H mice (Fig. 3A-c), suggesting that the phosphorylation of H2AX at Ser139 after X-irradiation in the spleen is mainly mediated by the DNA-PK. Altogether, the DNA-PK-dependent phosphorylation of H2AX was detected in the spleen only, although the DNA-PK-dependent elimination of γ-H2AX was detected in the liver, kidney, skin, and spleen. These findings suggest that the phosphorylation mechanism of H2AX is tissue-specific.

We also found that the phosphorylation level of H2AX after X-irradiation in the spleen of the C3H mice was very high than that in the other tissues (Fig. 3A). These results suggest that the regulatory mechanism of γ-H2AX level is tissue-specific.

It is considered that NHEJ, which is a DNA-PK-dependent repair process, can repair X-ray-induced DSB throughout the cell cycle [3,14]. On the other hand, DNA-PK activity, which is essential for the NHEJ, is dependent of cell proliferation activity. Therefore, it is a possibility that cell proliferation activity is an important factor for selecting either the DNA-PK or other kinase in H2AX-phosphorylation process *in vivo*. To compare cell proliferation activity between the spleen and other tissues, we carried out Western blot analysis using an antibody against a marker of proliferation, the anti-PCNA antibody, and immunohistochemistry using an antibody against a marker of proliferation, Ki67. As shown in Fig. 4, in all tissues examined, Ki67-positive cells were detected by immunohistochemistry. Interestingly, spleen cells were highly proliferative in both the red pulp and the white pulp. On the other hand, the cells of other tissues, *i.e.*, kidney, liver, and skin, were slow-growing, in accordance with our previous report [11] (Fig. 4) (data not shown). By Western blot analysis, all tissues examined expressed PCNA, and the level of PCNA was significantly higher in extracts from spleen than in those from other tissues (Fig. 3B). These results suggest that the difference in the γ-H2AX level between the spleen and other tissues might correlate with cell proliferation activity. Therefore, we speculate that the regulatory mechanism of γ-H2AX *in vivo* is, at least in part, influenced by cell proliferation activity, although further studies are necessary to confirm this.

In conclusion, our findings demonstrate that the phosphorylation of H2AX and the elimination of γ-H2AX at X-ray-induced DSB proceeds in both DNA-PK-dependent and -independent manner *in vivo*. The phosphorylation of H2AX at Ser139 after X-irradiation in the spleen is mainly mediated by the DNA-PK. In addition, the regulatory mechanism of γ-H2AX level might be tissue-specific. To date, γ-H2AX is widely used as a sensitive indicator of DNA damage, especially DSB. The H2AX phosphorylation induced by DSB is induced not only by ionizing radiation, but also by chemotherapeutics including etoposide and breomycin in human and rodent cells [15] (data not shown). Further studies to elucidate the molecular mechanism underlying of H2AX phosphorylation and H2AX elimination *in vivo* will lead to a better understanding of not only the physiological functions and regulatory mechanisms of this protein but also the development of new tissue-specific radiotherapy protocol and chemotherapeutics.

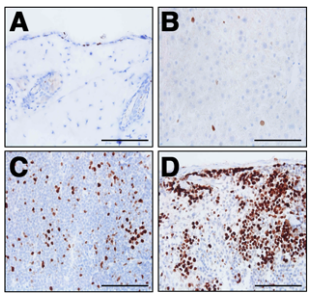

Fig. 4. Immunohistochemical analysis of cell proliferation activity in tissues. An image of cryostat sections labeled for Ki67 (A,B,C,D) is shown. Each skin (A), liver (B), or spleen (C, red pulp; D, white pulp) sample was prepared from unirradiated mice. Nuclei were visualized by counterstaining with hematoxylin before mounting. Scale bars: 100 μm.

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