

Histone H2AX Phosphorylation Independent of ATM after X-irradiation in Mouse Liver and Kidney *in situ*

Manabu KOIKE*[†], Minako MASHINO[†], Jun SUGASAWA and Aki KOIKE

ATM/Histone H2AX phosphorylation/Ionizing radiation/Liver/Kidney.

Histone H2AX undergoes phosphorylation at Ser-139 (γ -H2AX) rapidly in response to DNA double-strand breaks (DSBs) induced by ionizing radiation. The post-translational modification of H2AX plays a central role in responses to radiation, including the repair of DSBs. Although ataxia telangiectasia mutated (ATM) kinase phosphorylates Ser-139 of H2AX *in vitro*, the post-translational modification pattern and the modifier of H2AX in organs *in vivo* are not yet well understood. In this study, we detected phosphorylation of H2AX at Ser-139 in cells of the mouse ear, liver, and kidney after X-irradiation. Moreover, the phosphorylation of H2AX was regulated depending on not only the cell type, but also the organ type and the localization of a cell type in an organ. Following X-irradiation, H2AX was phosphorylated in the liver and kidney of ATM gene knockout mice, suggesting that ATM kinase is not essential for phosphorylation of H2AX in these organs after X-irradiation *in vivo*.

INTRODUCTION

Higher-order chromatin structure presents a barrier to the recognition and repair of DNA damage. It is considered on the basis of the data obtained from cell cultured systems that DNA double-strand breaks (DSBs) induce histone H2AX phosphorylation by a phosphoinositide 3-kinase-like kinase, ATM kinase.¹⁾ Phosphorylated H2AX (γ -H2AX) accumulates rapidly at sites of DNA DSBs and H2AX phosphorylation contributes to the restructuring of chromatin and assists in the recruitment of DNA repair and signaling factors.¹⁾ Therefore, it is considered that the post-translational modification of H2AX plays a central role in responses to DNA damage including the repair and/or the signaling of DSBs. On the other hand, the mechanisms involved are not yet fully understood. In addition, the phosphorylation pattern and modifier of H2AX *in vivo* remain unclear.

p53 plays a key role in cellular responses to genotoxic stress by inducing cell-cycle arrest, DNA repair, senescence, or apoptosis. It is well known that ATM kinase phosphorylates Ser-139 of H2AX as well as Ser-15/18 of p53 in various types of cultured mammalian cell exposed to geno-

toxic insults such as ionizing radiation (IR).²⁾ The phosphorylation of H2AX as well as p53 in response to genotoxic stress *in vitro* is now well established in cell culture systems. Previously, we detected γ -H2AX in the extracts of the back skin of X-irradiated mice by western blot analysis.³⁾ Furthermore, γ -H2AX was detected in the extracts of the back skin of X-irradiated mice lacking ATM as well as in those of irradiated wild-type mice.³⁾ These observations suggest that this kinase is not essential for phosphorylation in the skin of irradiated mice. On the other hand, previous *in vivo* studies of p53 expression after X- or γ -irradiation have shown profound induction and phosphorylation of p53 at Ser-18 in the mouse back skin but not in the liver,^{2,3)} indicating that the phosphorylation of p53 by ATM kinase and stabilization of p53 after irradiation are regulated in an organ-specific manner. Therefore, it is important to clarify whether or not ATM kinase is essential for the phosphorylation of H2AX in organs other than the back skin of irradiated mice. However, the phosphorylation pattern and modifier of H2AX remain unclear in almost all organs, such as the liver and kidneys of irradiated mice *in situ*.

Here, by immunohistochemistry and Western blot analysis, we examined the expression of γ -H2AX and localization of γ -H2AX-positive cells in three organs, namely, ear, liver, and kidney, after X-irradiation.

MATERIALS AND METHODS

Mice

Five-week-old female ATM knockout mice with the C3H

*Corresponding author: Phone: +81-43-206-3139,

Fax: +81-43-206-3139,

E-mail: m_koike@nirs.go.jp

DNA Repair Gene Research, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan.

[†] These authors contributed equally to this work.

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background and C3H mice were purchased from a colony at the animal production facility of the National Institute of Radiological Sciences in Chiba. Five-week-old female C57BL mice and pregnant C57BL 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.

X-irradiation

The mice were exposed to 20 Gy of X-rays at a dose rate of 1.90–2.04 Gy/min at room temperature.^{3,4)} X-rays were generated at 200 kVp/20 mA and filtered through 0.5 mm Cu and Al filters using Pantak HF320S (Shimadzu, Kyoto, Japan).

Western blot analysis

Total lysates from the mouse ear, liver, or kidney were extracted using MM-300 (QIAGEN Inc., Chatworth, CA) according to the manufacturer's protocol and cleared by centrifugation, and the supernatants containing 20 µg were electrophoresed on 5–20% SDS-polyacrylamide gels.³⁾ Western blot analysis was performed as previously described with slight modification.^{3,5)} In brief, the 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 mouse anti-γ-H2AX monoclonal antibody (Upstate Biotechnology Inc.) or a mouse anti-β-actin monoclonal antibody (Sigma).³⁾ 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.

Immunohistochemistry

For immunohistochemical staining, freshly excised samples from C57BL/6J 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.⁴⁾ Immunostaining was performed using a rabbit polyclonal anti-γ-H2AX antibody (Cell Signaling Technology Inc.) at 1:200 dilution or 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 samples were antigen-retrieved with or without heating using a microwave oven for 7 min in citrate buffer (pH 6.5), prior to peroxidase blocking (0.03%, v/v hydrogen peroxide). The primary antibody and horseradish peroxide-labelled polymer were used in accordance with the instructions included in the DAKO Envision kit, and 3,3-diaminobenzidine treatment and counterstaining with haematoxylin before mounting were then carried out.

RESULTS AND DISCUSSION

It is well known that some organs including the liver and brain are much more resistant to the effects of irradiation. Although a dramatic accumulation of p53, which is mediated by the phosphorylation of Ser-15/18, is apparent in cells of radiosensitive organs, such as skin cells, splenocytes, and thymocytes of irradiated mice, no p53 accumulation is detected in hepatocytes,^{2,3)} suggesting that the phosphorylation and accumulation of p53 are regulated in an organ-specific manner. To date, there have been only few reports on the phosphorylation of H2AX in mammalian organs *in vivo*;^{3,6–8)} almost all of the studies on γ-H2AX were carried out using cultured cells. Previously, we detected γ-H2AX in the extracts of the back skin of X-irradiated mice.³⁾ To determine whether H2AX undergoes phosphorylation at Ser-139 in some organs other than the back skin of X-irradiated mice, we examined the phosphorylation of H2AX in three organs, namely, the ear, liver, and kidney of the X-irradiated C57BL mice by Western blot analysis using the anti-γ-H2AX antibody. In our previous study, we detected using western blot analysis and immunohistochemistry the phosphorylation of H2AX that is induced by X-irradiation (20 Gy) in mouse skin *in vivo*. We also found that at 5 h after X-irradiation γ-H2AX increased in a dose dependent manner (10, 20, 40 Gy) by Western blot analysis.³⁾ Therefore, in the present study, 20 Gy of X-ray was applied to mouse to show the effect of X-irradiation on phospho-H2AX expression unambiguously. As shown in Fig. 1, we found that X-irradiation induced a strong phosphorylation of H2AX at Ser-139 in extracts from all the three organs examined. We also found that the level of H2AX phosphorylated at Ser-139 of the extracts after X-irradiation differed between the kidney or liver and the ear (Fig. 1). Recently, it

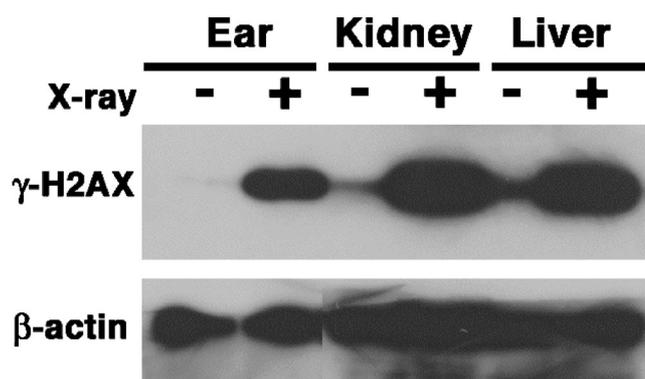


Fig. 1. Detection of γ-H2AX in organs of mouse after X-irradiation. C57BL mice were either nonirradiated or irradiated with 20 Gy X-rays. Total proteins from the ear, liver, or kidney were prepared 2 h after treatment. Phosphorylated H2AX (γ-H2AX) protein level was determined by Western blot analysis using specific antibodies against γ-H2AX (top) and β-actin (bottom).

has been reported that there is a significant difference in the induction of γ -H2AX between the heart and kidney of X-irradiated mice.⁷⁾ Taken together, these data suggest that the kinase for the phosphorylation of H2AX is controlled in an organ-specific manner. We also detected γ -H2AX in extracts of all organs from non-X-irradiated mice, although its level was low (Fig. 1). Recently, Gavrinov *et al.* (2006) have reported that the unirradiated kidney and heart have 3–5% γ -H2AX-positive cells.⁷⁾ Therefore, we consider that the endogenous γ -H2AX we detected is a common physical aspect of normal metabolism.

We next determined whether ATM kinase is essential for the phosphorylation of H2AX at Ser-139 in mouse liver and kidney extracts on day 35 using ATM knockout mice with the C3H background. Western blot analysis using the anti- γ -H2AX antibody demonstrated that after X-irradiation, γ -H2AX was easily detected in extracts from the liver and kidney of ATM knockout mice and C3H mice (Fig. 2). Although it is well known that ATM kinase phosphorylates Ser-139 of H2AX in cultured cells in response to X-irradiation,^{9–12)} ATM kinase is not essential for the phosphorylation of H2AX at Ser-139 in the back skin extracts from X-irradiated mice.³⁾ Taken together, our present and previous studies³⁾ suggest that ATM kinase is not essential for the phosphorylation of H2AX at Ser-139 in various mouse organs with purposefully introduced DSBs *in vivo*. On the other hand, we easily detected γ -H2AX in extracts from the liver and kidney of X-irradiated SCID mice (DNA-PK-deficient mutant) by Western blot analysis using the anti- γ -H2AX antibody (data not shown).

Cell populations in organs are heterogeneous. To identify the type of γ -H2AX-positive cell in the ear, liver, and kidney with purposefully introduced DNA DSBs, we next carried out an immunohistochemical analysis using X-irradiated and non-X-irradiated C57BL mice. Immunohistochemical

analysis using the anti- γ -H2AX antibody demonstrated that in response to X-irradiation, γ -H2AX accumulated in the nuclei of various types of cell, such as hepatocytes, endothelial cells around the central vein, and cells in interlobular connective tissues of the liver from X-irradiated mice. In addition, γ -H2AX was detected in the nuclei of various types of cell, such as the simple cuboidal epithelium in renal tubules and collecting ducts, and capsule cells of the kidney of X-irradiated mice. Furthermore, it was detected in the nuclei of various types of cell, such as keratinocytes in the epidermis, fibroblasts in the dermis, dermal papilla cells, and myocytes of the ear of X-irradiated mice (Fig. 3). Interestingly, we observed that γ -H2AX was detected easily in the nuclei of cells (*e.g.*, fibroblasts and condrocytes) of connective tissues in the ear of irradiated mice, but not in the liver and kidney. These results suggest that the phosphorylation of H2AX after X-irradiation is regulated depending on the organ type, although further studies in detail need to understand this difference quantitatively in detail. Previously, Yoshida *et al.* (2003) reported that H2AX in epithelial cells in the small intestine is not highly phosphorylated even after X-irradiation.⁶⁾ On the other hand, our data showed that H2AX in epithelial cells in the kidney, liver, and ear is highly phosphorylated after X-irradiation. Although the reason for the discrepancy between our observation of epithelial cells and that of Yoshida *et al.* (2003) remains unclear, this may be due to the differences in the organ studied.⁶⁾

We examined the type of γ -H2AX-positive cell in the ear, liver, and kidney without the purposeful introduction of DNA DSBs using non-X-irradiated C57BL mice on day 21. In hepatocytes of the liver and in the epithelium of the cortex and medulla of the kidney of non-X-irradiated mice, a population of γ -H2AX-positive cells was observed (Fig. 3). These results support the results of Western blot analysis (Fig. 1). As shown in Fig. 3, in the kidney, the ratio of γ -H2AX-positive cells was obviously different between the epithelium of the cortex and that of the medulla, suggesting that the phosphorylation of H2AX is regulated depending on the localization of a cell type in an organ. On the other hand, in the ear of the non-X-irradiated mice, γ -H2AX-positive cells were hardly detected in hair follicles and the epidermis, in addition to the dermis and cells around the germ cell cap (Fig. 3). Most recently, we have detected γ -H2AX-positive cells around the germ cell cap in the back skin of the non-X-irradiated mice on day 21 *in vivo*.⁸⁾ These data suggest that the phosphorylation of H2AX is regulated depending on the organ type. Altogether, the phosphorylation of H2AX might be regulated strictly depending on not only the cell type, but also the organ type and the localization of a cell type in an organ, regardless of the presence or absence of DNA damage responses. On the other hand, further studies in detail need to understand the effects of difference of absorbed dose of each organ on phosphorylation of H2AX in order to elucidate the pattern of H2AX phosphorylation. In addition, further

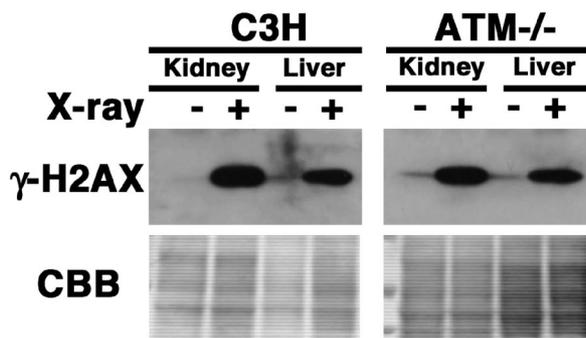


Fig. 2. Western blot analysis of liver or kidney of ATM knockout mice (ATM^{-/-}) and C3H mice. Both mice on day 35 were either nonirradiated or irradiated with 20 Gy X-rays. Total proteins from the liver or kidney were prepared 5 h after treatment. Phosphorylated H2AX (γ -H2AX) protein level was determined by Western blot analysis using specific antibodies against γ -H2AX (top). The Coomassie brilliant blue (CBB)-stained band is a loading reference (bottom).

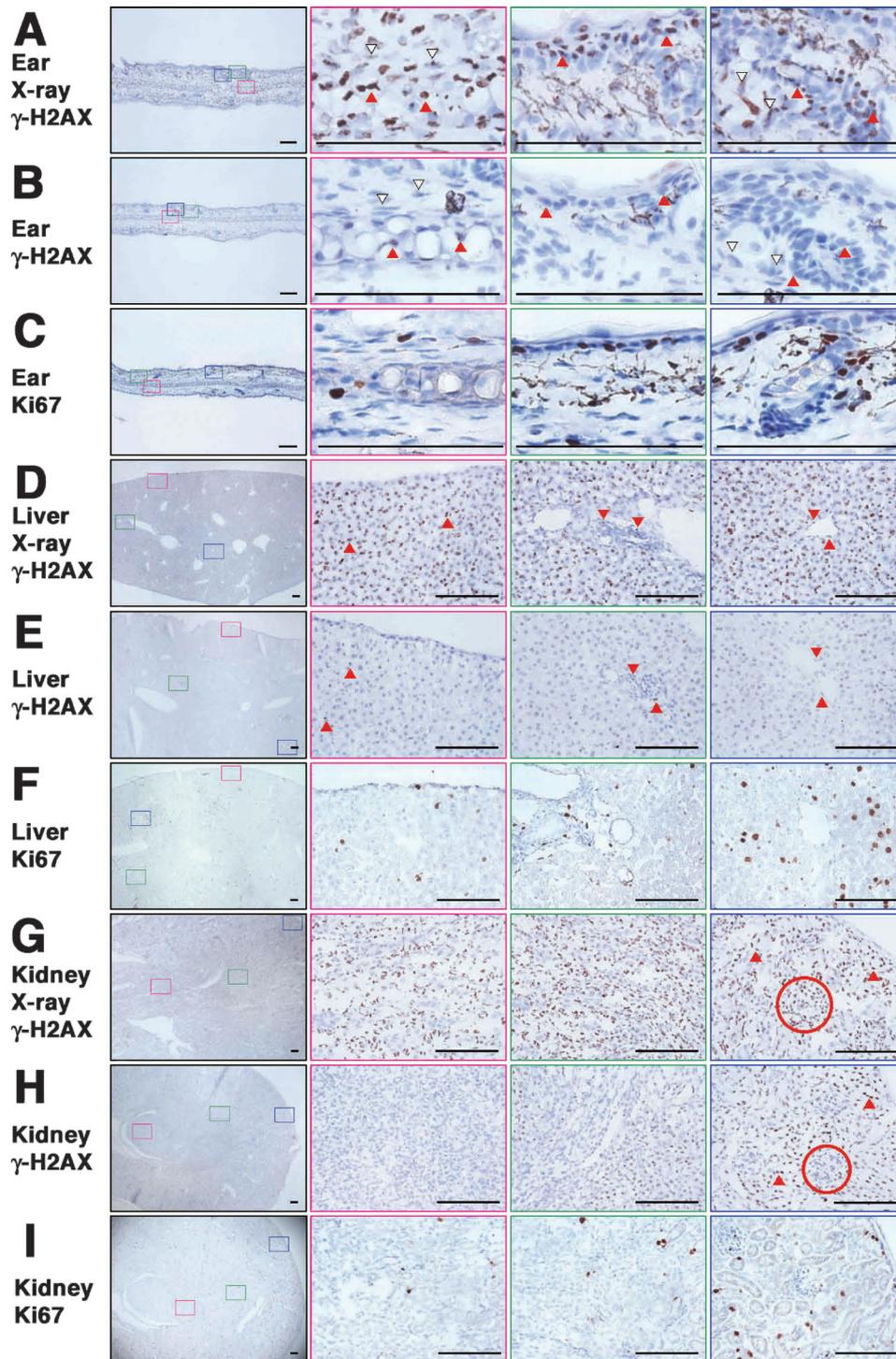


Fig. 3. Immunohistochemical analysis of γ -H2AX in tissues from X-irradiated mice. An image of cryostat sections labeled for γ -H2AX (A, B, D, E, G, H) or Ki67 (C, F, I) is shown. Each ear (B, C), liver (E, F), or kidney (H, I) section was prepared from non-X-irradiated mice on day 21. Each ear (A), liver (D), or kidney (G) section was prepared at 2 h after treatment from X-irradiated mice (20 Gy) on day 21. Nuclei were visualized by counterstaining with haematoxylin before mounting. Red, green and blue panels are high magnifications of corresponding images (left, black panels). (A, B) Arrowheads indicate condrocytes (red panel, red arrowhead), myocytes (red panel, white arrowhead), keratinocytes (green panel, red arrowhead), hair follicles (blue panel, red arrowhead), or fibroblasts (blue panel, white arrowhead). (D, E) Arrowheads indicate a connective tissue (green panel), endothelial cells (blue panel), or γ -H2AX-positive hepatocytes in X-irradiated mice (D) (red panel) and non-X-irradiated mice (E) (red panel). (G, H, I) an inner site of medulla (red panel); an outer site of medulla (green panel); cortex (blue panel). A red circle indicates a renal corpuscle. Arrowheads indicate urinary tubules. Scale bars: 100 μ m.

studies using various doses and time points in detail contribute to elucidate the pattern of H2AX phosphorylation.

Burma *et al.* (2001) showed in their study using a cell culture system with fibroblasts from knockout mice with defects in DNA-PK or ATM that ATM kinase is the major kinase responsible for about 95% of H2AX phosphorylation.¹⁰⁾ As described above, in this and previous studies, we demonstrated that H2AX phosphorylation at Ser-139 occurs in cells of the mouse back skin, liver, and kidney lacking either ATM or DNA-PK with purposefully introduced DNA DSBs *in vivo* (Fig. 2) (data not shown).^{3,8)} On the other hand, it was reported that H2AX phosphorylation in cultured cells subjected to replication stress is mediated by ATR kinase.¹³⁾ Therefore, we hypothesized that some of the γ -H2AX-positive cells are replicating in non-X-irradiated mouse organs examined. To test this hypothesis, we carried out an immunohistochemical analysis using an antibody against a marker of proliferation, Ki67. In all organs examined, Ki67-positive cells were detected (Fig. 3). Therefore, we consider that the phosphorylation of H2AX at Ser-139 is, at least in part, catalyzed by ATR kinase in replicating cells in non-X-irradiated mouse organs examined, although further studies are necessary to confirm this. To date, no published data have shown that ATM kinase, as well as ATR kinase or DNA-PK, phosphorylates H2AX directly *in vivo*. Recently, Lu *et al.* (2006) have reported that UVA irradiation strongly induces H2AX phosphorylation mediated by c-Jun N-terminal kinase (JNK) in culture cells.¹⁴⁾ Therefore, we speculate that there might still be unknown kinases that phosphorylate H2AX *in vivo*. Alternatively, we cannot exclude completely the possibility that the phosphorylation catalyzed by DNA-PK or ATM kinase is a redundant operation in the liver and kidney *in vivo*.

In conclusion, our data demonstrates that H2AX is phosphorylated in cells in the X-irradiated mouse liver and kidney independent of ATM kinase physiologically. Furthermore, our results in this study together with our earlier observations in hair follicles⁸⁾ demonstrate that the phosphorylation of H2AX *in vivo* might be regulated strictly depending on not only the cell type, but also the organ type and tissue type, and the localization of each cell, regardless of the presence of purposefully introduced DNA DSBs. Further studies to elucidate the molecular mechanism underlying the phosphorylation of H2AX in various cell types and organs *in vivo* will lead to a better understanding of not only the physiological functions of this protein but also the modifier of H2AX and the radio- or chemo sensitivities of each cell type and tissue type.

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