p53 Phosphorylation in Mouse Skin and In vitro Human Skin Model by High-dose-radiation Exposure

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ATM/DNA-PKcs/p53/Skin/X-ray.

The skin is an external organ that is most frequently exposed to radiation. High-dose radiation initiates and promotes acute radiation injury. Thus, it is important to investigate the influence of high-dose radiation exposure on the skin at the molecular level. The post-translational modification of p53 plays a central role in radiation responses, including apoptosis and cell growth arrest. Although it is well known that ataxia telangiectasia mutated (ATM) kinase and DNA-dependent protein kinase (DNA-PK) can phosphorylate Ser15/Ser18 of p53 in vitro, the post-translational modification pattern and the modifier of p53 in the skin after exposure to high-dose X-rays are not yet well understood. Here we show that the phosphorylation of p53 on Ser15/Ser18, as well as the phosphorylation of histone H2AX on Ser139, was detected in the keratinocytes of the mouse skin and human skin models after high-dose X-ray irradiation. Following high-dose X-ray irradiation, both proteins were also phosphorylated in the skin keratinocytes of both ATM gene knockout mice and DNA-PK-deficient SCID mice.

INTRODUCTION

Ionizing radiation initiates and promotes carcinogenesis, apoptosis, aging, and immune suppression. The skin is an external organ that is most frequently exposed to radiation. DNA damage activates a variety of cellular cascades leading to cell cycle arrest, DNA repair, or apoptosis. Depending on the nature of DNA damage, defined nuclear pathways transduce signals towards a specific response. p53 has key roles in the cellular response to DNA-damage-inducing agents such as ionizing radiation.1–4) The phosphorylation of human p53 at serine 15 and murine p53 at the homologous serine 18 residue (designated below as Ser15/Ser18) promotes both the accumulation and functional activation of p53 in response to DNA damage by ionizing radiation. Ionizing radiation induces DNA-double strand breaks in cells, and then the stabilization and accumulation of the p53 protein by phosphorylation of Ser15/Ser18. In response to DNA damage, activated p53 binds to specific DNA sequences and acts as a transcription factor whose target genes, e.g., p21 and GADD45, are mainly involved in cell cycle arrest and apoptosis. On the other hand, p53 pathways in the human skin after exposure to high-dose X-ray radiation are still poorly understood, but appear to be activated by high-energy radiation and play important roles in radiation injuries.

Ataxia telangiectasia mutated (ATM) kinase, ataxia telangiectasia-Rad3-related protein (ATR) kinase, and DNA-dependent protein kinase (DNA-PK), which are members of the phosphatidylinositol 3 (PI3) kinase family that can phosphorylate Ser15/Ser18 of p53 in vitro, are candidate upstream activators or regulators of p53.5,6) The p53 phosphorylation mechanism by irradiation in vivo is still controversial. Many groups have reported that ATM and not DNA-PK functions as the major activator of p53 in response to DNA damage in vivo.6,7–12) On the other hand, Wang et al. (2000) have proposed that DNA-PK and ATM are similar in their selective activation of p53, but are dissimilar in that DNA-PK induces apoptosis but not cell cycle arrest, whereas ATM induces cell cycle arrest but not apoptosis.13) It was reported that ATM is rapidly activated by ionizing radiation and phosphorylates Ser15/Ser18 of p53, whereas ATR is slowly activated and is involved in maintaining p53 phosphorylation.10–14)

The clarification of molecular signal transduction mechanisms in keratinocytes following high-dose irradiation is necessary for understanding the mechanism of acute radiation skin injury. An increased p53 protein level was observed in the mouse skin after exposure of mice to 5 Gy of ionizing radiation, suggesting that p53 in skin cells is stabilized by irradiation.15) Recently, we have reported that p53 accumulates within the nuclei of keratinocytes and fibroblasts in the...
skin of the irradiated (high-dose X-rays (40 Gy)) mice. Therefore, we hypothesized that in skin cells, the post-translational modification of p53 plays a central role in radiation responses, including apoptosis and cell growth arrest. However, the stabilization mechanism and the post-translational modification pattern of p53 in the skin after exposure to high-dose X-rays are not yet well understood. In this study, we performed a histological study of the phosphorylation of Ser15/Ser18 of p53 after X-irradiation of the mouse skin and human skin model.

**MATERIALS AND METHODS**

**Mice**

Seven to nine-week-old female C57BL mice, CB.17 mice, SCID mice with the CB.17 background and ATM knockout mice with the C3H background were purchased from a colony at the animal production facility of the National Institute of Radiological Sciences in Chiba. All mice were reared and handled in accordance with the guidelines governing the care and use of laboratory animals of the National Institute of Radiological Sciences.

**X-irradiation**

Keratinocytes in the human skin model (Toyobo Co., Ltd., Osaka, Japan) and the mice were exposed to various doses (10, 20, 40 Gy) of X-rays at dose rates of 0.9 Gy/min at room temperature. X-rays were generated at 200 kVp/20 mA and filtered through 0.5 mm each of Cu and Al using Pantak HF320S (Shimadzu, Kyoto, Japan).

**Western blot analysis**

Total lysates from the mouse skin were extracted by MM-300 (QIAGEN Inc, Chatworth, CA) according to the manufacturer’s protocol and cleared by centrifugation, and the supernatants were electrophoresed on 4–20% SDS-polyacrylamide gels. Western blot analysis was performed as previously described. In brief, the fractionated products were electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking nonspecific binding sites with 1% bovine serum albumin, the membranes were incubated with a rabbit anti-phospho-p53(Ser15/Ser18) antibody (Cell Signaling Technology Inc., Beverly, MA) or a mouse anti-p53 antibody (Novocasta Lab. Ltd., Newcastle, UK) at 1:1500 dilution was used as primary antibody. The specimen was fixed in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. Three µm-thick sections were deparaffinized in xylene and rehydrated through graded alcohol solutions. Endogenous peroxidase activities were blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were blocked with normal goat serum at 1:10 dilution for 15 min to block nonspecific protein binding, followed by incubation with the primary antibody overnight at 4°C. After treatment with a secondary biotinylated antibody (Nichirei Bioscience Inc., Tokyo, Japan) for 30 min, the sections were incubated with streptavidin-biotin-peroxidase complex for 20 min at room temperature. After the incubation time of each step, the sections were washed in PBS three times for 3 min each. Color reaction was performed with 3,3’-diaminobenzidine as chromogen, then the sections were counterstained with hematoxylin. A negative control reaction with no primary antibody was performed alongside the reaction-containing sample.

**RESULTS**

It is well known that the post-translational modification of p53 plays a key role in radiation responses and the phosphorylation of p53 on Ser15/Ser18 is important for p53 stabilization on the basis of many in vitro experiments using cultured cells. We and others showed that p53 accumulates within the nuclei of cells in the mouse skin exposed to ionizing radiation, i.e., γ- and X-rays. These studies suggest that p53 in skin cells is also phosphorylated and stabilized by irradiation in vivo. To confirm this possibility, we first investigated the phosphorylation and stabilization of p53 in the irradiated C57BL mouse skin by Western blot analysis using anti-phospho-p53(Ser15/Ser18) antibody. As shown in Fig. 1A, the levels of p53 phosphorylated on Ser15/Ser18 increased in a dose dependent manner when the protein was extracted 5 hr after X-irradiation. In addition, the levels of p53 phosphorylated on Ser15/Ser18 increased with time after high-dose X-irradiation (40 Gy) (Fig. 1B), suggesting that the modification caused by high-dose X-irradiation is not transient but lasts at least 8 hr after irradiation. These results indicate that p53 is modified and stabilized by high-dose X-irradiation in normal mouse skin cells. Although ATM kinase and DNA-PK can phosphorylate Ser15/Ser18 of p53 in vitro, the post-translational modification pattern and the modifier of p53 in the skin after exposure to high-dose X-rays are not yet well understood. We next investigated whether ATM kinase or DNA-PK is essential for the phosphorylation of p53 on Ser15/Ser18. To analyze this, we used DNA-PK-deficient SCID mice with the CB.17 background and ATM knockout mice with the C3H background. Western blot analysis using the anti-phospho-p53(Ser15/Ser18) antibody demonstrated that in response to high-dose X-irradiation, p53 was accumulated and phosphorylated on Ser15/Ser18 in extracts from the skin of three mouse strains:
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C57BL, CB.17, and C3H (Fig. 1C). Furthermore, in response to high-dose X-irradiation, p53 was accumulated and phosphorylated on Ser15/Ser18 in both DNA-PK-deficient SCID mice and ATM knockout mice (Fig. 1C). These results indicate that in response to high-dose X-ray radiation, both ATM kinase and DNA-PK are not essential for the phosphorylation of p53 on Ser15/Ser18 in mouse skin cells.

In addition to p53, ATM kinase and DNA-PK phosphorylate other proteins important for radiosensitivity. ATM kinase and DNA-PK can phosphorylate serine 139 in a variant form of the histone H2A designated as H2AX.\(^{21}\) \(\gamma\)H2AX forms foci at DNA double-strand breaks (DSBs) induced by ionizing radiation.\(^{22}\) \(\gamma\)H2AX plays a critical role in the retention of repair factors at sites of DSBs.\(^{23, 24}\) On the basis of the results of experiments using ATM knockout cell lines or A-T lymphoblastoid cell lines, ionizing radiation-induced-H2AX foci formation was shown to be ATM-dependent.\(^{25, 26}\) On the other hand, another study reported a defective H2AX phosphorylation in the DNA-PK-defective tumor line M059J.\(^{27}\) To investigate whether DNA-PK or ATM kinase is indispensable for the phosphorylation of H2AX on Ser139 in irradiated skin cells, we used DNA-PK-deficient SCID mice and ATM knockout mice with the C3H background. As shown in Fig. 1A, the levels of H2AX phosphorylated on Ser139 increased in a dose dependent manner when the protein was extracted 5 hr after X-irradiation in C57BL. The levels of \(\gamma\)H2AX reached the maximum 2 h after high-dose X-irradiation (40 Gy) (Fig. 1B), suggesting that the modification by a high-dose X-ray is at least in part transient. These results indicate that H2AX is modified by high-dose X-irradiation in normal mouse skin cells. Western blot analysis using the anti-\(\gamma\)-H2AX antibody demonstrated that in response to high-dose X-irradiation, H2AX was phosphorylated on Ser139 in extracts from the skin of the three mouse strains, C57BL, CB.17, and C3H (Fig. 1C). Furthermore, in response to high-dose X-irradiation, H2AX was phosphorylated on Ser139 in both DNA-PK-deficient SCID mice and ATM knockout mice (Fig. 1C). These results indicate that in response to high-dose X-irradiation, both ATM kinase and DNA-PK are not essential for the phosphorylation of H2AX on Ser139 in mouse skin cells.

As described above, the levels of p53 phosphorylated on Ser15/Ser18 and H2AX phosphorylated on Ser139 increased in a dose dependent manner in C57BL in response to X-irradiation (Fig. 1A). We also found that after 40 Gy the skin shows no visible signs of radiation damage at 7 h and 8 h (Fig. 2A and data not shown). Therefore, we used 40 Gy of X-rays in subsequent experiments to show the effect of X-irradiation on phospho-p53 expression unambiguously. To examine the effect of high-dose radiation on p53 phosphorylation in individual cells of the skin, we stained skin sections immunohistochemically using the anti-phospho-p53(Ser15/Ser18) antibody. First, 40 Gy of X-irradiation was delivered to C57BL mice. There are two types of epithelial cell in the skin, i.e., epidermal and hair follicular cells. As shown in Fig. 2A, exposure to ionizing radiation induced the phosphorylation of p53 on Ser15/Ser18 in the nucleus of both epidermal and hair follicular cells. Three hours and seven hours after the X-irradiation, we detected the phosphory-
lation of p53 on Ser15/Ser18 in about 45% and 55%, respectively, of the cells in the skin epidermal cells (Fig. 3A). Without the X-irradiation, we hardly detected the phosphorylation of p53 on Ser15/Ser18 in the epidermal cells.

As described above, the results of Western blot analysis using SCID mice and ATM knockout mice suggest the possibility that ATM kinase and DNA-PK are not absolutely required for the phosphorylation of p53 on Ser15/Ser18 in mouse skin keratinocytes after high-dose X-irradiation. To confirm and extend this, we next carried out an immuno-

Fig. 2. Immunohistochemical analysis of phosphorylated-p53 level in mouse skin and human skin model. (A, B, C) p53 is phosphorylated by X-irradiation and is accumulated within the nuclei of keratinocytes in the skin of irradiated normal mice (C57BL (A), C3H (B), CB.17 (C)), irradiated ATM knockout mice (B), and irradiated DNA-PK-deficient SCID mice (C). The sections of X-irradiated (40 Gy) mouse skin (3 h (A), 5 h (B, C), or 7 h (A)) and control mouse skin (0 h) were immunostained with antibodies to phospho-p53(Ser15/Ser18) antibody. Nuclei were visualized by counterstaining with haematoxylin before mounting. (D) p53 is phosphorylated by X-irradiation and is accumulated within the nuclei of keratinocytes in the irradiated human skin model. The sections of X-irradiated (40 Gy) human skin model (3 h) and unirradiated model (0 h) were immunostained with antibodies to the phospho-p53(Ser15/Ser18) antibody. Nuclei were visualized by counterstaining with haematoxylin before mounting. Serial sections were stained with hematoxylin and eosin to analyze their histopathological characteristics (HE staining and data not shown).
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histochemical analysis using DNA-PK-deficient SCID mice with the CB.17 background and ATM knockout mice with the C3H background. Immunohistochemical analysis using the anti-phospho-p53(Ser15/Ser18) antibody demonstrated that in response to high-dose X-irradiation, p53 was accumulated and phosphorylated on Ser15/Ser18 in the nuclei of both epidermal and hair follicular cells in the skin of both DNA-PK-deficient SCID mice and ATM knockout mice, as well as in that of the CB.17 and C3H mice, respectively (Fig. 2 and Fig. 3). Without X-irradiation, we hardly detected the phosphorylation of p53 on Ser15/Ser18 in the epidermal cells of all the mouse strains examined (Fig. 2 and Fig. 3). Taken together, these results support the idea that in response to high-dose X-irradiation, both ATM kinase and DNA-PK are not essential for the phosphorylation of p53 on Ser15/Ser18 in two types of skin epithelial cell. Recently, we showed that following irradiation, p53 protein levels were increased in a time dependent manner in epidermis of the C57BL.16 Next, we examined the level of p53 in irradiated mouse skin by immunohistochemical analysis using the anti-p53 antibody. As shown in Table 1, in response to high-dose X-irradiation, p53 was accumulated in the epidermis in the skin of both DNA-PK-deficient SCID mice and ATM knockout mice, as well as in that of the CB.17 and C3H mice, respectively, in similar to the data by using the anti-phospho-p53(Ser15/Ser18) antibody.

The effects of high-dose radiation exposure on the mechanisms underlying the intracellular and intercellular transmissions of signals in keratinocytes need to be clarified for the medical treatment of acute radiation injury. However, the effect of high-dose X- or gamma-ray radiation exposure of the human skin at the molecular level has been hardly analyzed due to the limitations in the use of human skin materials. To examine the effect of the high-dose radiation on p53 phosphorylation in individual cells of a human skin model, i.e., three-dimensionally cultured human skin, we stained the human skin model sections immunohistochemically using the anti-phospho-p53(Ser15/Ser18) antibody. The skin model was an organotypic cocultured skin composed of human

Table 1. Induction of p53 in the epidermis after X-irradiation

| Mouse strain | Irradiation* | % p53-positive cells** |
|--------------|--------------|------------------------|
| CB.17        | –            | 1.33±1.15              |
| CB.17        | –            | 0                      |
| CB.17        | +            | 29.33±6.66             |
| CB.17        | +            | 35.00±3.61             |
| SCID         | –            | 1.33±0.58              |
| SCID         | –            | 1.67±1.53              |
| SCID         | +            | 24.67±8.62             |
| SCID         | +            | 24.00±6.08             |
| C3H          | –            | 1.33±0.58              |
| C3H          | –            | 3.67±2.08              |
| C3H          | +            | 28.67±3.79             |
| C3H          | +            | 41.33±4.04             |
| ATM–/–       | –            | 1.00±1.00              |
| ATM–/–       | –            | 2.33±1.53              |
| ATM–/–       | +            | 38.00±1.00             |
| ATM–/–       | +            | 27.67±7.23             |

**Average numbers ± SE from skin sections.
*5 hours after irradiation (40 Gy).
The epidermis is a physiological barrier that protects the body against pathogens and chemical or physical damage. High-dose radiation causes acute radiation injury to the skin. It is important to investigate the influence of high-dose radiation exposure on the skin at the molecular level. In this study, we demonstrated that the phosphorylation of p53 on Ser15/Ser18, as well as the phosphorylation of histone H2AX on Ser139, was detected in the keratinocytes of the mouse skin and human skin models after high-dose X-irradiation. These results suggest that the post-translational modification of p53 and H2AX, which play a central role in radiation responses, is important in the understanding of the influence of high-dose-radiation exposure on the human skin.

In this study, we demonstrated that H2AX phosphorylation on Ser139 after exposure to high-dose X-rays occurs in the keratinocytes of the mouse skin lacking either DNA-PK or ATM. Since Ser139 lies within the consensus phosphorylation sequence of the PI-3 family of kinases, ATM, DNA-PK, and ATR, which are kinases activated by DNA DSBs, have been considered as prime candidates for this phosphorylation.27–30 Burma et al. (2001) in their study using fibroblasts from knockout mice with defects in DNA-PK or ATM, showed that ATM is the major H2AX kinase responsible for about 95% of H2AX phosphorylation.36 On the other hand, Stiff et al. (2004) have recently shown the redundant operation of DNA-PK and ATM in H2AX phosphorylation in different biological systems using in vitro-cultured cells.37 In addition, they clearly demonstrated that possibly an additional kinase, ATR kinase, does not contribute to ionizing radiation-induced H2AX phosphorylation in nonreplicating primary fibroblasts. Altogether, although we need further studies to confirm this, we hypothesize that high-dose-X-ray-induced phosphorylation of histone H2AX on Ser139 can be carried out by DNA-PK or ATM redundant operation in the skin in vivo, because almost all keratinocytes in vivo are nonreplicating.

ATM phosphorylates and activates key proteins, e.g., p53 in radiation response signaling pathway. Many studies indicated that the ATM and the not DNA-PK functions as the major activator of p53 in response to DNA damage using cultured cells.7–12 In agreement with these reports, we found in this study that the phosphorylation of p53 after exposure to high-dose X-rays occurred in the keratinocytes of the mouse skin lacking DNA-PK, supporting the hypothesis that ATM is major kinase for the phosphorylation of Ser15/Ser18 on p53 in response to DNA damage in the skin in vivo. On the other hand, following high-dose X-ray irradiation, p53 was phosphorylated in the skin keratinocytes of ATM gene knockout mice, demonstrating clearly that protein kinases other than ATM can also carry out the phosphorylation of...
p53 in skin cells in vivo. Thus, we conclude that another kinase in addition to ATM kinase plays a key role in activating p53 in response to high-dose radiation in vivo. It appears that the responses to ionizing radiation in human skin cells are dependent on cell type or the type of derived tissues, although the molecular mechanisms for these responses are still not elucidated. For example, in the p53-dependent response of the skin, two epithelial cell types respond to radiation by different pathways that are governed in part by the differential p53- and p21-dependent responses of these cells; the high level induction of p53 in the absence of p21 induction leads to apoptosis of hair follicle cells, whereas the intermediate-level induction of both p53 and p21 leads to growth arrest of epidermal cells. On the other hand, Yan et al. (2005) reported that activating transcription factor 3 (ATF3) activates p53 by a novel post-translational mechanism, i.e., blockade of its ubiquitination. ATF3 might play a role in influencing the apoptosis pathway in radiation injuries caused by high-dose radiation exposure of human skin cells. In addition, the mechanism of ATF3 induction by X-irradiation might differ between epidermal keratinocytes and dermal fibroblasts. Therefore, we speculate that the activation mechanism of p53 differs depending on the cell type of the skin, and that ATF3-dependent activation mechanisms besides phosphorylation also occur in skin cells.

p53 pathways in the human skin after exposure to high-dose X-rays are poorly understood, but appear to be activated by high-energy radiation and play important roles in radiation injuries. One reason is that the influence of high-dose X- or gamma-ray radiation exposure on human skin at the molecular level has been hardly analyzed due to the limited use of human skin materials. It was suggested that threedimensionally cultured human skin model is unique in that the regulatory mechanisms of growth and differentiation of keratinocytes can be investigated under conditions mimicking those in vivo. In this study, using a similar model, we detected the phosphorylation of p53 on Ser15/Ser18 in the keratinocytes of the human skin model, as well as in the mouse skin, after high-dose X-irradiation. In addition, normal keratinocytes formed a three-dimensional structure of the epithelium that closely resembled the epidermis in vivo. Therefore, we hypothesize that the skin model is effective for experimentally analyzing and understanding the molecular mechanism of the influence of high-dose radiation exposure on the human skin.

Comparative high single doses (10, 20, 40 Gy) were applied to mouse skin to show the effect of X-irradiation on phospho-p53 and H2AX expression unambiguously. Similar and further high-doses have been utilized to demonstrate the effects of ionizing radiation on the mouse skin. Thirty Gy caused less damage to the skin resulting in only acanthosis of the epidermis at 6 weeks with none of the ulceration that had been observed at the 50 Gy dose. In this study, we observed that after 40 Gy the mouse skin shows no visible signs of radiation damage at 7 h and 8 h.

In conclusion, p53 and H2AX are modified and accumulated with high-dose X-irradiation in the nuclei of keratinocytes of the skin in vivo. Thus, p53 and H2AX might play an important role in the phosphorylation pathway in radiation injury in the human skin caused by the high-dose radiation exposure. ATM is essential for mediating checkpoint control in cells exposed to ionizing radiation. On the other hand, DNA-PK, which is made from Ku70, Ku80 and DNA-PKcs, is essential for the DNA repair system and nonhomologous end joining of radiation-induced DNA double strand breaks. Further studies to elucidate the molecular mechanism underlying the modification of these proteins in keratinocytes will lead to a better understanding of not only the physiological functions of these proteins but also the intracellular signal transmission among cells.

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