p53-dependent delayed effects of radiation vary according to time of irradiation of p53+/- mice

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(Received 28 March 2013; revised 10 May 2013; accepted 16 May 2013)

We previously reported that in p53+/- mice that had been given a whole-body dose of 3 Gy at 8 weeks of age, p53-dependent delayed effects of radiation, as manifested in T-cell receptor (TCR) variant fractions (VF) instability in mouse splenocytes, were biphasic, namely, induction of TCR-VF mutation reappeared at 44 weeks. The manifestation of the delayed effects and the measures of biological markers varied according to the timing of irradiation. We also reported that the decrease in function of the p53 gene was related to the effects of a delayed mutation. In the present study, we investigated the functions and mutations of the p53 gene in old age for p53+/- mice following irradiation at various ages. p53+/- mice were given a whole-body dose of 3 Gy at 8, 28 or 40 weeks of age. There were significant differences for all variables tested at 8 weeks of age. This was similarly the case for mice irradiated at 28 weeks of age, in which there were also significant differences in TCR VF and the percentage of apoptosis. In mice irradiated at 40 weeks of age, there were significant differences for all considered variables except for the p53 allele. We demonstrated that the different patterns of delayed mutation of the p53 gene at 56 weeks of age depended on the age at which mice had undergone 3-Gy whole-body irradiation. Our conclusions are limited to variation in p53-dependent delayed effects according to the time of irradiation.

Keywords: p53; irradiation; age; delayed effects

INTRODUCTION

Many papers have reported the induction of delayed effects of ionizing radiation [1–3]. Moreover, ionizing radiation at a young age induces delayed mutation [4, 5]. The effects vary depending on age at the time of exposure. The life-shortening effect of radiation decreased with increase in age of exposure to radiation [6]. The risk of cataract was higher among individuals who were younger at the time of exposure in rats [7, 8] and in the Chernobyl clean-up workers [9]. In a study of atomic bomb survivors, the number of both solid cancers and leukemias increased with decrease in age of irradiation [1, 10–12]. Furthermore, the excess absolute risk (EAR)/Gy for all solid cancers has been found to be higher with exposure to radiation at a younger age [12, 13].

In a previous in vivo study, we reported that X-irradiation at a young age caused a delayed re-increase in the fraction of T-cell receptor (TCR) variant fractions (VF) [14]. Moreover, we showed that the expression of p53 increased spontaneously with age in non-irradiated mice and that the function of p53 was lost in older mice that were exposed to whole-body 3 Gy irradiation at 8 weeks of age [15]. These data suggest that p53 is related to cellular senescence [16], genomic instability [17] and carcinogenesis [17, 18]. Therefore, in atomic bomb survivors, the greater EAR observed after radiation exposure at a younger age may be related to p53 function.

In this study, p53+/- mice were exposed to 3 Gy of whole-body irradiation at a range of ages. The delayed re-increase in TCR VF was detected in p53+/- mice at an earlier age than in wild-type p53 mice [14]. In this manner, p53+/- mice (as...
opposed to p53-deficient mice) are very useful for investigating aging events. We subsequently evaluated the TCR VF, the expression of p53 protein and the p53 instabilities of these mice after they had reached old age. We demonstrated that the different patterns of delayed mutation of the p53 gene observed in old age depended on the age at which they had received whole-body 3-Gy irradiation.

MATERIALS AND METHODS

Experimental animals

Mice carrying a disrupted, non-functional p53 gene (p53<sup>−/−</sup>) were obtained from the transgenic facility at the Center for Laboratory Animal Care at the University of Occupational and Environmental Health, Japan, as previously described [19]. Wild-type mice of the parental wild-type inbred strain were used as controls for the p53<sup>+/−</sup> mice. The p53<sup>−/−</sup> mice were obtained by crossing male p53<sup>−/−</sup> mice with female p53<sup>+/+</sup> mice. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the University of Occupational and Environmental Health, Japan, Kitakayushu, Japan.

In a previous study [14], after p53<sup>+/−</sup> mice received a whole-body dose of 3-Gy X-rays at 8 weeks of age, the TCR VF increased to the maximum level at nine days, decreased to background levels at 20 weeks of age and increased again at 44 weeks of age. In p53<sup>+/−</sup> mice, the TCR VF second increase occurred at 64 weeks of age. The dose of p53 in p53<sup>+/−</sup> mice was almost half that of p53<sup>+/+</sup> mice. We used p53<sup>+/−</sup> mice to obtain data earlier than the data obtained for p53<sup>+/+</sup> mice in this study.

Radiation treatments

In the present study, p53<sup>+/−</sup> mice were randomly allocated to either the ‘irradiated’ group or the ‘non-irradiated’ group. The irradiated group was given a whole-body dose of 3-Gy X-rays at 8, 24 or 40 weeks of age. In p53<sup>+/−</sup> mice, the TCR VF second increase occurred at 64 weeks of age. The dose of p53 in p53<sup>+/−</sup> mice was almost half that of p53<sup>+/+</sup> mice. We used p53<sup>+/−</sup> mice to obtain data earlier than the data obtained for p53<sup>+/+</sup> mice in this study.

TCR VF assay

TCR VF assays have been described previously [14]. The spleens of the mice were immediately resected. The spleen cells were gently dissociated and were subsequently filtered through a stainless steel mesh. After hemolysis using modified Gey’s solution, which contained NaCl instead of NH₄Cl, the T-cells were concentrated using nylon wool columns, and 500 000 were kept on ice for 30 min. Subsequently, T-cells were stained with PE-anti CD4 and FITC-anti CD3 antibodies (Pharmingen, San Diego, CA, USA) and were analyzed with an EPICS-XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). We counted 50 000 events for each measurement. Lymphocytes were examined by arranging bit map gates on a plot of forward and 90° light scatter, and by arranging a window for CD3<sup>−</sup>CD4<sup>+</sup> mutant T-cells in the region where the surface CD3 level was less than one-twenty-fifth that of normal CD4<sup>+</sup> cells [14]. The TCR VF was calculated as the number of events in the CD3<sup>−</sup>CD4<sup>+</sup> T-cells window divided by the total number of events in the CD3<sup>+</sup>CD4<sup>+</sup> T-cells.

Apoptosis assay

To clarify the relationship between TCR VF and p53-dependent apoptosis, the frequencies of radiation-induced apoptosis were analyzed. The mice were exposed to 3 Gy again at each age point to evaluate the radiation-induced apoptosis activity.

The percentage of apoptotic cells in a spleen was evaluated with a modified TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method. The spleen was removed 4 h after the final irradiation. Specimens were fixed in 10% formalin for 48 h, dehydrated, and embedded in paraffin. After deparaffinization and rehydration, serial 4-μm sections were incubated with proteinase K (20 mg/ml) at room temperature for 15 min. Following washing in distilled water four times for 2 min each, tissue sections were exposed to 2% H₂O₂ in PBS for 5 min at room temperature and then rinsed with PBS. The sections were stained with an ApopTag peroxidase in situ apoptosis detection kit (Chemicon, Inc., Temecula, CA, USA), according to the manufacturer’s protocol. Briefly, terminal deoxynucleotidyl transferase labeling reaction mix was added inside a humidity chamber at 37°C for 1 h. Subsequently, the tissues were exposed to a stop solution at room temperature for 10 min, washed three times in PBS for 1 min per wash, and exposed to anti-digoxigenin peroxidase at room temperature for 30 min. The sections were subsequently exposed to 0.02% H₂O₂ and dianaminobenzidine tetrachloride (1 mg/ml) in PBS for 1 min using a DAB substrate kit (Vector Laboratories, Inc. Burlington, CA) and were then counterstained with hematoxylin. TUNEL-positive cells were assessed and determined to amount to approximately 3000 cells in three different areas.

Western blot analysis

Spleen samples were then electrophoresed on acrylamide gels (NuPage 10% Bis-Tris gel, Invitrogen, Carlsbad, CA, USA) with MES running buffer (Invitrogen) using an XCell surelock electrophoresis cell tank (Invitrogen). After electrophoresis, proteins were transferred to 0.45-μm nitrocellulose membranes (Invitrogen). The SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA) was used according to the manufacturer’s instructions. Briefly, membranes were placed in the blot holder and were blocked for 20 sec with 0.5% skimmed milk in tris-buffered saline/tween 0.1% (TBS-T) before they were incubated with the primary antibody for 10 min. The primary antibodies utilized in this study included anti-p53 antibody (BD biosciences, San Jose, CA), anti-p21 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-MDM2 (Santa Cruz Biotechnology, Inc.) and anti-MDM2 (Santa Cruz Biotechnology, Inc.)
anti-actin (Santa Cruz Biotechnology) (1:500 dilution in 0.1% TBS-T). The membranes were washed three times in 0.1% TBS-T for 20 sec per wash and treated with a horseradish peroxidase-conjugated secondary antibody (1:5000, Amersham Bioscience, Piscataway, NJ) for 10 min. After the washes, proteins were detected by the enhanced chemiluminescence system (ECL advance, Amersham Biosciences).

**Determination of p53 allele loss and wild-type p53/p53-pseudogene ratio**

For the determination of the loss of p53 alleles, we used the wild-type p53/p53-pseudogene ratio as per the method of Hulla et al [20]. The primers were designed for exon 5 (5'-TGC CCT GTG CAG TTG TGG GTC A -3') and exon 6 (5'-ATT TCC TTT CAC CCG GAT AAG ATG -3'). The thermal cycling conditions were as follows: one cycle at 95°C for 1 min; thirty cycles at 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 3 min. We observed a 258-bp amplicon from the wild-type p53 gene and a 179-bp amplicon from the p53-pseudogene. After the wild-type p53 gene and p53-pseudogene were amplified, the amount of fluorescence in the agarose gels was measured with Image J software (NIH), and the wild-type p53/p53-pseudogene ratio was calculated as wild-type p53/(wild-type p53 + p53-pseudogene).

**Bisulfite conversion and methylation-specific PCR (MSP)**

Approximately 1 µg of DNA from spleen cells was treated with sodium bisulfite using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Briefly, DNA, bisulfite mix and DNA protect buffer were mixed together. The bisulfite conversion thermal cycling conditions were at 99°C for 5 min, 60°C for 25 min, 99°C for 5 min, 60°C for 85 min, 99°C for 5 min and 60°C for 175 min. Finally, bisulfite-converted DNA was cleaned up with a spin column and eluted with 20 µl of EB buffer.

We previously reported on the establishment of MSP for mouse p53 [21]. CpG islands in the p53 gene were searched and PCR primer sets were designed by Methyl Primer Express Software v1.0 (Applied Biosystems Inc.). PCR primer sets for p53-UnMethylation were sense 5'-ATC GTT ATTd CGG TTC TTT GTC -3' and antisense 5'-CGA ACA CGA CTC CCA ACT AA, whereas those for p53-Methylation were sense 5'-ATC GTT ATT CGG TTC TTT GTC -3' and antisense 5'-CGA ACA CGA CTC CCA GCT AA-3'. PCR amplification was performed using the EpiTect MSP kit (Qiagen). The thermal cycling conditions were as follows: one cycle at 95°C for 10 min; thirty cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min.

**Statistical analysis**

Data were expressed as mean ± SD and evaluated by analysis of variance. For the cell number assay, the cell cycle assay and the cell proliferation assay, a Student’s t-test was utilized to determine the differences between irradiated mice and non-irradiated mice at each time point. A P-value < 0.05 was considered statistically significant.

**RESULTS**

**Time-course of TCR mutation frequencies in p53 +/- mice after 3-Gy whole-body irradiation at 8, 28 or 40 weeks of age**

Previously, we have reported that in p53 +/- mice irradiated at 8 weeks of age, the TCR VF increased rapidly and reached a maximum level 9 days after irradiation; after that, the TCR VF gradually decreased to background levels at 20 weeks of age [14]. In this study, the TCR VF in p53 +/- mice irradiated at 28 weeks of age reached a maximum level 1–3 weeks after irradiation before decreasing gradually, although these levels did not decrease to background levels (Fig. 1). In mice irradiated at 40 weeks of age the TCR VF displayed maximum levels until 10 weeks after irradiation and then decreased until 20 weeks after irradiation. The TCR VF of these mice also failed to decrease to background levels. The periods of decreasing TCR VF post-irradiation were longer in older mice.

**Frequencies of apoptotic cells in the spleens of p53 +/- mice at 56 weeks of age after 3-Gy whole-body irradiation at 8, 28 or 40 weeks of age**

In p53 +/- mice that underwent 3-Gy irradiation at 8 weeks of age, at 56 weeks of age the percentage frequencies of apoptotic cells in the irradiated mice were approximately half...
those of the non-irradiated mice [14]. In this study, we obtained similar data for mice given 3 Gy irradiation at 8 weeks of age and non-irradiated mice at 56 weeks of age ($P < 0.001$, Fig. 2). Moreover, the percentage frequencies of apoptotic cells in other mice irradiated at 28 or 40 weeks of age were significantly decreased compared with those in non-irradiated mice. The percentage frequencies of apoptotic cells in mice irradiated at 28 weeks of age were significantly higher than those of the mice irradiated at 8 or 40 weeks of age ($P < 0.05$).

**Protein expression of p53, phospho-p53 and p21 in the spleens of p53 $^{+/−}$ mice at 56 weeks of age after 3-Gy whole-body irradiation at 8, 28 or 40 weeks of age**

The expression levels of p53, phospho-p53 and p21 proteins in mice exposed to 3 Gy at 28 or 40 weeks of age were significantly lower than those in non-irradiated mice, except for the protein expression levels of phospho-p53 in mice exposed to 3 Gy at 28 weeks of age (Fig. 3A). Additionally, the protein expression levels of p53, phospho-p53 and p21 in mice that underwent 3 Gy at 40 weeks of age were significantly lower than those in mice exposed to 3 Gy at 28 weeks of age ($P < 0.05$, Fig. 3A).

We previously showed that the expression of phospho-p53 at 56 weeks of age in mice previously given 3-Gy irradiation at 8 weeks of age was dramatically suppressed compared with that
in non-irradiated mice [15]. In the present study, the expression of phospho-p53 at 56 weeks of age in mice given 3 Gy at 28 weeks of age showed a tendency to decrease compared with the expression in non-irradiated mice. Figure 3B was made from a combination of the previous data and the present data. The expression of phospho-p53 at 56 weeks of age in mice given 3 Gy at 28 weeks of age was significantly higher than that in mice given 3 Gy at 8 or 40 weeks of age (Fig. 3B).

p53 allele loss in p53 
+−/− mice at 56 weeks of age after 3-Gy whole-body irradiation at 8, 28 or 40 weeks of age

The loss of the p53 allele in mice exposed to 3 Gy at 8 weeks of age was significantly lower compared with the loss of this allele in mice given 3-Gy irradiation at 28 or 40 weeks of age and that in non-irradiated mice (P < 0.05, Fig. 4). There were no significant differences between non-irradiated mice, mice given 3 Gy at 28 weeks and mice given 3 Gy at 40 weeks.

p53 methylation frequency in p53 
+−/− mice at 56 weeks of age after 3-Gy whole-body irradiation at 8, 28 or 40 weeks of age

Among the non-irradiated mice at 56 weeks of age, 30% displayed p53 methylation (Fig. 5). After 3-Gy whole-body irradiation at 8 or 40 weeks of age, 100% of the p53 
+−/− mice displayed p53 methylation at 56 weeks of age. After 3-Gy whole-body irradiation at 28 weeks of age, 60% of the p53 
+−/− mice displayed p53 methylation at 56 weeks of age.

DISCUSSION

In the present study, we demonstrated that the age at which mice underwent 3-Gy whole-body irradiation leads to differences in TCR VF, percentage of apoptosis, expression of phospho-p53, ratio of the p53 allele and p53 methylation levels at 56 weeks of age (Table 1). For all variables, there were significant differences at 56 weeks of age between the p53 
+−/− mice after irradiation at 8 weeks of age and the non-irradiated mice. Regarding TCR VF and the percentage of apoptosis in p53 
+−/− mice at 56 weeks of age there were significant differences when comparing non-irradiated mice with mice that had undergone irradiation at 28 weeks of age. For all considered variables, except the p53 allele, at 56 weeks of age there were significant differences in p53 
+−/− mice after irradiation at 40 weeks of age when compared with non-irradiated mice. Taken together, the delayed effects of irradiation in old age might be more severe at a younger age, especially if mice are exposed to 3-Gy whole-body irradiation at a younger age.

At 56 weeks, the TCR VF after irradiation at 40 weeks of age was higher than that in all other irradiated groups. Previously, we showed that TCR VF rapidly increased to a maximum level 7–9 days after irradiation. The time needed to decrease to spontaneous levels was greater in p53 
+−/− mice than in p53 
+−/+ mice, while the TCR VF after irradiation was also greater in p53 
−−/− mice [14, 22]. The reason for the higher values of TCR VF in p53-deficient mice that received 3-Gy whole-body irradiation could be that p53 function may be gradually lost with age. Declining p53 responses to stresses have been observed in cultured splenocytes obtained from aging mice [23]. We suggest that effectiveness of the repair system of p53 might decrease with age, thereby leading to decreased elimination of injured cells via p53-dependent apoptosis. As such, TCR VF was higher in mice that received irradiation at 40 weeks of age.

We showed, at 56 weeks of age, that the frequencies of the apoptotic cells and the protein expressions of p53 and phospho-p53 in mice after irradiation at any age were lower than the values in non-irradiated mice. Different frequencies...
of apoptotic cells were observed, depending on the age at which radiation was administered. One of the major functions of p53 is to induce apoptosis in response to stress. We suggest that declining p53 function at old age may be influenced by the age at the time of irradiation. In particular, significantly fewer apoptotic cells were observed in the spleens of older mice compared with younger mice [23]. In this study, the frequencies of apoptotic cells were higher in mice that received radiation at 28 weeks of age compared with those that received radiation at 40 weeks of age. We suggest that the effects of irradiation on p53 might be more severe when mice are exposed to radiation at an older age because p53 function declines with age.

We demonstrated the different patterns of p53 allele loss and p53 methylation after irradiation at different ages. Previously, we showed several types of p53 mutations (e.g. allele loss, methylation and sequence transition) after irradiation at 8 weeks of age [15]. In the present study, we obtained consistent data regarding p53 allele loss and 100% p53 methylation after irradiation at 8 weeks of age. These data seemed to suggest that irradiation at 28 weeks of age had a decreased effect on p53 disorder relative to the irradiated group. A likely explanation is that proteins other than p53 also influence mutation and carcinogenesis. Even in the same carcinomas, p53 mutations may show different patterns, as in the case with malignant salivary gland neoplasms [24] and in head and neck cancers [25]. A previous study of atomic bomb survivors showed that ERR/Gy was 30% higher in patients who were exposed to radiation when 10 years younger [13]. In summary, ERR/Gy may be dependent on the age at the time of irradiation. We suggest that p53 function may decline more in samples receiving radiation at 40 weeks of age than those that received radiation at 28 weeks of age; hence, p53 mutations were more severe in mice that received radiation at 40 weeks of age compared with those that received radiation at 28 weeks of age.

It is well known that p53 relates to cellular senescence [16], and p53 itself decreases in function with aging [21, 23]. In the present study, we showed that the differences in expression of phospho-p53, the ratio of the p53 allele and p53 methylation levels at 56 weeks of age after 3 Gy whole-body irradiation at the different ages

Table 1. A summary of differences for the values of TCR VF, percentage of apoptosis, expression of phospho-p53, ratio of p53 allele and p53 methylation levels at 56 weeks of age after 3 Gy whole-body irradiation at the different ages

| Age at irradiated (weeks) | control | 8     | 28    | 40     |
|---------------------------|---------|-------|-------|--------|
| TCR VF ($\times 10^{-4}$) | 6.9 ± 2.7 | 13.2 ± 3.2* | 22.5 ± 2.3* | 38.8 ± 12.4* |
| apoptosis                 | 12.8 ± 1.6% | 5.6 ± 1.6%*** | 10.7 ± 1.1%* | 8.1 ± 0.6%*** |
| phospho-p53 (relative amount) | 1 ± 0.42 | 0.09 ± 0.04* | 0.74 ± 0.21 | 0.26 ± 0.09*** |
| p53 allele (ratio)        | 0.29 ± 0.053 | 0.20 ± 0.02* | 0.25 ± 0.05 | 0.3 ± 0.03 |
| p53 methylation           | 35% 100% | 60% 100% |

*P < 0.05 vs control, **P < 0.01, ***P < 0.001 vs control.
ACKNOWLEDGEMENTS

The authors thank the following UOEH medical students for technical assistance: Mr Yuya Saito, Mr Kuniki Moriwaki, Mr Yuki Maruno and Mr Masaya Yasutake.

FUNDING

This work was supported in part by a UOEH Research Grant for Advanced Research from the University of Occupational and Environmental Health, Japan (H23-1#1301), and by Grants-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (#23510070).

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