Influences of p53 deficiency on the apoptotic response, DNA damage removal and mutagenesis in UVB-exposed mouse skin

Hironobu Ikehata*, Ryuhei Okuyama1,4, Eisaku Ogawa1, Shingo Nakamura5, Atsuko Usami, Toshiro Mori2, Kiyoji Tanaka1, Setsuya Aiba1 and Tetsuya Ono

Department of Cell Biology, Tohoku University Graduate School of Medicine, Seiryo-machi 1-1, Aoba-ku, Sendai 980-8574, Japan. 1Department of Dermatology, Tohoku University Graduate School of Medicine, Seiryo-machi 1-1, Aoba-ku, Sendai 980-8574, Japan. 2Radioisotope Research Center, Nara Medical University, Shijo-machi 840, Kashihara, Nara 634-8521, Japan and 3Laboratories of Organismal Biosystems, Graduate School of Frontier Biosciences, Osaka University, Yamadaoka 1-3, Suita, Osaka 565-0871, Japan

*Present address: Department of Dermatology, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

†Present address: Department of Radiobiology, Institute for Environmental Sciences, Rokkasho, Aomori 039-3212, Japan

*To whom correspondence should be addressed. Tel: +81 22 717 8134; Fax: +81 22 717 8136; Email: ikehata@mail.tains.tohoku.ac.jp

Received on February 24, 2010; revised on March 25, 2010; accepted on March 30, 2010

p53 suppresses the genomic instability provoked by genotoxic agents. Ultraviolet (UV) B induces skin cancers by producing DNA damage and mutations in the skin genome, whereas the skin tissue responds to the UVB insult with cell cycle arrest and apoptosis as well as damage exclusion by DNA repair. To address the p53 contribution to these skin responses in vivo, we analyzed the time course of DNA damage removal, apoptosis induction and hyperplasia in the skin after UVB irradiation in p53-knockout mice. We also examined UVB-induced mutations in the skin. We found that p53 deficiency does not abolish the UVB-induced apoptotic response in the epidermis but delays the process and the following hyperplasia 12–24 h. Regardless of the p53 genotype, 1 kJ/m² UVB induced a total replacement of the epidermal layer by destroying the damaged epidermis by apoptosis and rebuilding a new one through hyperplasia. We failed to detect a clear defect in removal of UVB-induced DNA photolesions from the genome of the p53-deficient skin except for a delay in the epidermis, which seemed to result from the delay in the apoptotic response. However, we found that p53 deficiency enhanced UVB-induced mutagenesis. Furthermore, in a genetic study using Xpa-knockout mice, we showed that the enhanced mutagenic response depends on the activity of nucleotide excision repair (NER), which was also supported by the mutation spectrum observed in the UVB-exposed p53-knockout mice. These results indicate that p53 protects the skin genome from the UVB genotoxicity by facilitating NER, whereas its contribution to the UVB-induced apoptosis is limited.

Introduction

The gene of the tumour suppressor p53 is known to mutate frequently in various types of human tumours (1), including skin cancers (2), which are among the most frequent types of human cancers (3), suggesting an important role of this protein in skin carcinogenesis (4). The p53 mutations found in human non-melanoma skin cancers, which usually occur in sun-light-exposed areas, show ultraviolet (UV) light-specific patterns: C → T base substitution at dipyrimidine sites and CC → TT tandem substitutions, both of which are also called UV signature mutations, indicating that solar UV genotoxicity is the main cause of human skin carcinogenesis (2). The UV genotoxicity results from the photochemical formation of DNA damage such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (64PPs), which can cause mutations in the process of DNA replication (5–7). These photolesions can be removed from the damaged genome by a cellular mechanism of DNA repair, especially by a pathway called nucleotide excision repair (NER) in mammalian cells (8). The p53 protein is known to contribute to one of the NER subpathways, global genome repair (GGR) (9–11), by inducing the expression of DDB2 and XPC genes in human cells, both of which code GGR factors (12,13). Another role is also suggested for p53 as a chromatin accessibility factor (14,15), which would also facilitate GGR.

p53 is also known to be involved in cell cycle arrest and apoptosis after genotoxic insults (16). The cell cycle arrest could allow damaged cells time enough to repair DNA, and the apoptotic response would selectively eliminate heavily damaged cells from the insulted population, thus repressing genotoxic effects. UV induces both cell cycle arrest and apoptosis in mammalian cells and skin tissues (17–19), though the relevance of p53 to these UV-induced responses has not been conclusively demonstrated. UV retards cell cycle progression at the S phase by producing DNA photolesions that block DNA replication (17), whereas p53 mediates an earlier arrest at the G1 phase of the cell cycle by inducing the expression of a cyclin-dependent kinase inhibitor p21 (20). Deficiency in p53 was found to rather prolong the UV-induced S phase block (21). Some studies supported a contribution of p53 to the promotion of UV-induced apoptosis (22), though several studies have claimed that UV-induced apoptosis is not necessarily mediated by p53 (23–27).

It is certain that p53 protects the skin from UV genotoxicity as evidenced by the p53-dependent suppression of UVB light-induced skin cancer (28). To address how p53 contributes to the suppression of UV genotoxicity in vivo, we studied the effects of a deficiency of p53 in UVB-exposed mouse skin, using p53-knockout (p53−/−) mice, by analyzing the time course of DNA damage retention, apoptosis induction and hyperplasia in the skin after UVB irradiation. We also compared the frequencies and molecular changes of mutations induced by UVB in the skin epidermis among different p53 genotypes.
Materials and methods

Mice

All experimental procedures, including the animal husbandry, were conducted according to the Guidelines for Animal Welfare and Experimentation at Tohoku University. A transgenic mouse strain harbouring λ-phage-based lacZ mutational reporter genes (29) was crossed with p53-knockout mice homozygous for a Trp53g129aa allele (20) (http://www.cdb.riken.jp/arg/mutant%20mice%20list.html, Acc. No. CD80001K). The resulting F1 mice were interbred and the genotypes of their offspring were determined using tail DNA samples. Genotyping for the Trp53 allele was performed by polymerase chain reaction (94°C for 4 min; followed by 40 cycles of 94°C for 30 sec, 52°C for 1 min and 72°C for 1 min; and a final incubation of 72°C for 4 min) with a primer set described before (31). Transmission of the lacZ transgene was confirmed as described (32). lacZ-transgenic Xpa−/−p53+/+ and Xpa−/−p53−/− mice were produced by interbreeding the F1 mice derived from crossing of the transgenic p53−/− mice constructed as above and the transgenic Xpa-knockout (Xpa−/−) mice described before (32).

Irradiation and skin DNA preparation

UVB irradiation was performed as described (33). Briefly, the depilated dorsal skin of 8- to 12-week-old mice was irradiated under anesthesia to UVB from a broadband fluorescent FL20.S.E lamps (peak emission 313 nm; Toshiba, Tokyo, Japan). UVB dosimetry was performed with a UVS radiometer equipped with a UVX-31 sensor (UVP, San Gabriel, CA, USA). The preparation of the epidermal and dermal genomic DNA of the irradiated skin was described previously (32).

Histological analysis of irradiated skin

Fourteen each of male wild-type and p53−/− mice were exposed to 1.5 kJ/m2 of UVB, and two of each genotype were sacrificed at 0, 6, 12, 24, 36, 48 and 72 h after the irradiation, and the exposed skin areas were excised, fixed in 10% phosphate-buffered formalin, dehydrated and embedded in paraffin wax. The skin specimens were sectioned at a 4-μm thickness, placed on glass slides, deparaffinized, rehydrated and stained for morphological analysis with hematoxylin and eosin (HE) or for immunohistochemical apoptotic cell detection using diaminobenzidene using an antibody for cleaved caspase-3 (#9661, Cell Signaling Technology, Inc. Danvers, MA, USA), which is an activated form of this protein. The immunohistochemically stained samples were counterstained with hematoxylin. The numbers of total and apoptotic cells in the epidermis were scored by scanning the whole epidermal region in a microscopic field of the activated caspase-3-stained samples with 400× magnification. Total cell numbers were scored by counting hematoxylin-stained nuclei. Activated caspase-3-positive cells were scored as apoptotic. For the total cell number scoring, at least 20 fields were examined for each section. For the apoptotic cell scoring, each section was scanned for every field from end to end. In the scoring, cells in the epidermis pre-existing at the time of irradiation were discriminated from the cells in the epidermal layer newly appearing by post-irradiation hyperplasia, based on the morphological distinction between the two epidermal layers.

DNA damage assay

Twelve each of the wild-type and the p53−/− mice were exposed to 1.5 kJ/m2 of UVB, and two of each genotype were sacrificed 0, 6, 12, 24, 36, 48 and 72 h later. The epidermal and dermal genomic DNA was isolated separately from the exposed skin area and assayed for the quantification of CPD and 64PP with an enzyme-linked immunosorbent assay (ELISA) as described (32) using monoclonal antibodies specific to each photolesion, TDM-2 and 64M-2, respectively.

Mutation analysis

Mutation analysis with lacZ-transgenic mice was described previously (32). Four weeks after irradiation, in which mutations were assumed to be fully induced (33), mice were sacrificed and the exposed skin areas were excised. The epidermal and dermal genomic DNA isolated separately from the skin sections were used for the detection of lacZ mutants, the evaluation of mutant frequencies (MFs), and the analysis of DNA sequence changes by mutation.

Statistical analyses

Differences in the dose-dependent induction kinetics of MFs were evaluated by two-way or one-way analysis of variance (ANOVA) and a post hoc Tukey-Kramer’s test. UVB dose and genotype were set as the independent variables and the MF as the dependent variable. Differences in the mutation spectrum were estimated with Student’s t-test, Fisher’s exact probability test and Adams-Skopek test (34).

Results

Induction kinetics of apoptosis and hyperplasia in the skin epidermis after UVB irradiation

Mice with the p53+/+ and p53−/− genotypes were depilated at their back and exposed to 1 kJ/m2 of UVB. The irradiated dorsal skin tissues were excised in the post-irradiation time course (0, 6, 12, 24, 36, 48 and 72 h) and examined histologically for hyperplasia with HE staining and for apoptosis with immunohistochemical staining of activated caspase-3 (Figure 1). Apoptotic and total cell numbers in the epidermis in each microscopic field were scored at each time point and their time-dependent changes are shown in Figure 2. In the scoring, cells in the epidermal layer newly appearing by post-irradiation hyperplasia were separately counted. In the epidermis of wild-type mice, activated caspase-3-positive apoptotic cells started to appear at 6 h after irradiation, then increased in number, reaching the maximum at 36 h, and decreased thereafter, disappearing by 72 h post-irradiation (Figure 2A). In the dermis, the time-dependent increase in apoptosis was not evident although a small number of apoptotic cells appeared sparsely after irradiation (data not shown). At 48 h after UVB exposure, hyperplasia of the epidermis occurred obviously at the basal cell layer along with hyperkeratosis in the upper layer and continued till 72 h post-irradiation (Figure 1). Apoptotic cells were found to clearly localize in the upper epidermal layers with hyperkeratosis above the newly appearing hyperplastic epidermal layers, in which few apoptotic cells were observed (Figure 1, activated caspase-3, p53+/+, UV 48 h). Accordingly, total cell numbers in the pre-existing epidermis began to decrease from 36 h post-irradiation, the apoptotic cells appeared maximally, and at the same time, cells appearing in the newly grown hyperplastic lower epidermis started to increase in number (Figure 2A, upper). This observation revealed a UVB-triggered renewal of the total epidermal layer: the pre-existing epidermal keratinocytes died mostly by apoptosis, transited to the upper layers differentiating into cornified cells, and were replaced by newly appearing keratinocytes proliferated by hyperplasia. The total cell death in the pre-existing epidermal layer during the 36–72 h post-irradiation time was supported by the remarkably higher ratio of apoptosis during this duration (Figure 2B).

In the epidermis of p53−/− mice, apoptotic cells started to appear at 6–12 h after irradiation, with a slight delay compared to the wild type. The number of the apoptotic cells increased thereafter, peaked at 48 h as high as the wild-type maximal level but 12 h later, and then decreased to nearly the background level (Figure 2A, lower). The UVB-triggered renewal of the total epidermal layer, which was evident in the wild-type skin, was still observed in the irradiated skin of p53−/− mice, though the response was delayed 12–24 h compared to the wild-type mice (Figure 2A and B). The localization of apoptotic cells in the upper hyperkeratotic epidermal layer was observed again in the p53−/− epidermis along with little apoptosis in the newly grown hyperplastic lower layer (Figure 1, activated caspase-3, p53−/−, UV 72 h). This result clearly showed that the p53 defect does not abolish the apoptotic or hyperplastic response to UVB of the epidermis; the responses were only delayed 12–24 h but still fully occurred.

DNA damage removal kinetics in the UVB-exposed skin

Wild-type and p53−/− mice depilated on the back were exposed to 1.5 kJ/m2 of UVB, and the exposed skin areas were excised immediately (time 0) or 6–72 h later and separated into...
the epidermis and dermis, from each of which the genomic DNA was prepared. Amounts of the remaining UV photoproducts, CPD and 64PP, in a unit amount of the DNA were estimated by ELISA, and shown as a ratio to that of the initially produced photolesions (time 0) in Figure 3. In both the epidermis and dermis of p53+/+ mice, the amounts of CPD and 64PP, respectively, decreased to less than half the initial amounts by 48 and 12 h after irradiation. The initial rates of decrease of both lesions were faster in the dermis than in the epidermis (see Figure 3, 24 h), although the removal of CPD at

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>p53+/+ (HE)</th>
<th>p53+/+ (Activated caspase-3)</th>
<th>p53−/− (HE)</th>
<th>p53−/− (Activated caspase-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV 0h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV 6h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV 12h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV 24h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV 36h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV 48h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV 72h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Histological time-course analysis of the induction of apoptosis and hyperplasia in the UVB-exposed mouse skin with different p53 genotypes. Histological images of the epidermis and associated dermis from p53-proficient (p53+/+) and -deficient (p53−/−) mice unirradiated (no UV) and irradiated with 1 kJ/m² UVB (UV) are shown. The irradiated skin tissues were sampled from mice at the post-irradiation time points shown on the left (0–72 h). The images of the tissues stained with HE for the detection of hyperplasia were taken at 200-fold magnification (HE, the left part), and the images immunohistochemically stained for the detection of apoptosis at 400-fold magnification (activated caspase-3, the right part). Arrows show some of the apoptotic cells. Vertical bars with arrowheads on both ends indicate epidermal thickening by hyperplasia. Scale bars show 50-μm length.
later time points was incomplete in the dermis compared to the epidermis (see Figure 3, 72 h). In the \( p53^{+/+} \) mice, the kinetics of damage removal in the dermis were quite similar to those in the \( p53^{-/-} \) mice for both CPD and 64PP (Figure 3, lower), whereas, in the epidermis, a delay was evident for both lesions for the first 48 h after irradiation although the remaining amount of each lesion finally reached at 72 h the same decreased level as in the wild-type mice (Figure 3, upper). Regardless of the genotype, much faster removal was detected for 64PP than for CPD in either tissue, consistent with the fact that CPD is a poor substrate for GGR compared to 64PP in murine cells (35).

The observed reduction in the amount of photolesions in the skin tissues should occur when the lesions in DNA are excluded by repair, diluted by cell proliferation, or decomposed through apoptosis or differentiation into cornified cells. Because massive death and the corresponding proliferation of cells started at 36 h after irradiation in the \( p53^{+/+} \) epidermis (Figure 2A, upper), a large portion of the lesion decrease in the same epidermis, which was evident later than 24 h post-irradiation (Figure 3, upper, closed circles), is attributable to the apoptotic and hyperplastic response. In the \( p53^{-/-} \) epidermis, a 24-h delay was observed for CPD removal compared to the wild type (Figure 3, upper). This delay is easily explainable by the delay of the apoptotic and hyperplastic response found in \( p53^{-/-} \) mice in the histological analysis (Figure 2A) because the time courses of delay were well consistent between the damage-removal and apoptotic kinetics. The delay in the apoptotic response also accounts, at least partially, for the delay in the removal of 64PP observed in the \( p53^{-/-} \) epidermis, although some contribution of NER deficiencies in the \( p53^{-/-} \) mice could not be ruled out as another possible mechanism for the delay in lesion removal. However, the latter possibility seems unlikely because, in the dermis, few differences in the lesion removal were detected between the two genotypes (Figure 3, lower), though the kinetics of apoptotic and cell proliferating responses there are unknown. In addition, the difference in the kinetics of damage removal between the epidermis and dermis observed in Figure 3 might suggest some
Influences of p53 deficiency on UVB-exposed mouse skin

Mutation induction in UVB-exposed skin

lacZ-transgenic mice homozygous and heterozygous for the p53 null mutation were exposed to UVB (at doses of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 1.5 kJ/m² for the p53−/− mice, and 0.3, 0.5, 1.0 and 1.5 kJ/m² for the heterozygote p53+/−) or mock exposed (0 kJ/m²) and mutation induction in the epidermis and dermis was evaluated 4 weeks later using the lacZ transgene as a mutational reporter. The observed frequencies of lacZ animals. Error bars indicate standard deviations.

Each data point represents the mean of the MF values derived from at least four epidermis (circles) and dermis (triangles) is shown for the Xpa−/− transgene mutants in the epidermis (upper) and dermis (lower) is shown for the p53 genotypes (Figure 4A, upper), in the same manner as already reported for the wild-type mouse epidermis (33). For the p53−/− mice, mutations were induced by UVB at frequencies significantly higher in both the epidermis and dermis than for the wild-type and p53+/− mice (33). For the p53−/− mice, mutations were induced by UVB at frequencies significantly higher in both the epidermis and dermis than for the wild-type and p53+/− mice (33). For the p53−/− mice, mutations were induced by UVB at frequencies significantly higher in both the epidermis and dermis than for the wild-type and p53+/− mice (33).

Dose-dependent linear increases in MF were observed in the dermis at the entire dose range examined for all the p53 genotypes (Figure 4A, lower), and in the epidermis at doses up to 0.4 kJ/m² for p53−/− and up to 0.5 kJ/m² for the wild-type and heterozygote (Figure 4A, upper). The slopes were ~4-fold steeper in the epidermis than in the dermis irrespective of the genotype (Figure 4A). However, in the epidermis, the MF increase was suppressed at higher doses and switched to another linear slope with a much smaller increment regardless of the genotype (Figure 4A, upper), in the same manner as already reported for the wild-type mouse epidermis (33). For the p53−/− mice, mutations were induced by UVB at frequencies significantly higher in both the epidermis and dermis than for the wild-type and p53+/− mice (P < 0.0001, two-way ANOVA for the epidermis and one-way ANOVA for the dermis), whereas the mutation induction was not significantly different between the wild type and heterozygote.

Independent comparisons of the initial slope and the second slope of the mutation induction curves observed in the epidermis (Figure 4A, upper) also showed significant differences for the p53−/− mice compared with the p53+/+ and p53+/− mice (P < 0.0001, one-way ANOVA for the initial slope and two-way ANOVA for the second slope).

Induced and background mutation spectra in the p53−/− epidermis

In total, 48 background and 100 induced mutants of the lacZ transgene were isolated from the epidermis of p53−/− mice eight unexposed and four exposed to 0.5 kJ/m² of UVB, respectively. The entire coding region of the lacZ gene of these mutants was sequenced, and mutations were detected for all the mutants (see supplementary Tables S1 and S2, available at Mutagenesis Online). For the background mutants, we excluded redundant mutants with the same mutations as those detected in the same animal and scored 45 independent mutants, thus avoiding the possibility of repetitive counting of propagated mutants of the same origin pre-existing in the unexposed epidermis. The obtained mutation spectra for the background and the UVB-exposed epidermis are summarized in Table I and compared with those for the wild-type epidermis, which we reported before (36). For the p53−/− epidermis, we found that all of the induced mutants had base substitutions, which included eight mutants with a tandem or alternate base substitution and four multiple mutants with two or three separate base substitutions (Table I and supplementary Table S1—available at Mutagenesis Online) and that 87% (n = 39) of the background mutants had a single base substitution (Table I). The other background mutants were three frameshift mutants with a single base deletion, one deletion mutant, one complex mutant, which is a frameshift

differences between epidermal keratinocytes and dermal fibroblasts in the responses to UVB genotoxicity such as DNA repair, cell proliferation control and apoptosis.

Fig. 4. Dose-dependent response of mutation induction in the mouse skin exposed to UVB. (A) UVB-dose dependent increase in the frequency of lacZ transgene mutants in the epidermis (upper) and dermis (lower) is shown for p53−/− (closed circles), p53+/− (open triangles) and p53−/− mice (open circles). (B) UVB-dose dependent increase in the lacZ mutant frequency in the epidermis (circles) and dermis (triangles) is shown for the Xpa−/− mice with different p53 genotypes (p53+/+, closed symbols; p53−/−, open symbols). Each data point represents the mean of the MF values derived from at least four animals. Error bars indicate standard deviations.
associated with a base change and one multiple mutant with a base substitution and a deletion (Table I and supplementary Table S2—available at Mutagenesis Online). The mutation spectra shown in Table I are significantly different between the induced and background mutations in the p53+/−/− epidermis (P < 0.01, Adams–Skopek test), but not between the wild-type and p53+/−/− epidermis either for the induced or for the background mutation.

The base substitutions detected in the 100 induced mutants in the p53+/−/− epidermis were 96 single base substitutions, 8 of which were derived from the 4 multiple base substitution mutants, and 9 tandem or alternate base substitutions, 1 of which was from 1 of the multiple mutants (Table I and supplementary Table S1 — available at Mutagenesis Online).

### Table I. Mutation spectra in mouse skin epidermis of p53+/+ and p53+/−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Background (Number of mutants (%))</th>
<th>UVB (Number of mutants (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mutants (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base substitution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>41 (95)</td>
<td>39 (87)</td>
</tr>
<tr>
<td>Tandem/alternate</td>
<td>1 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Multiple</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Frameshift</td>
<td>1 (2)</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Deletion</td>
<td>0 (0)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Duplication</td>
<td>1 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Complex</td>
<td>0 (0)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Multiple</td>
<td>0 (0)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>45</td>
</tr>
</tbody>
</table>

Data from Ikehata et al. (36).

Alternate substitutions are double base substitutions whose mutations are separated by one unchanged base.

Mutants with two or more base substitutions separated by more than one nucleotide from each other.

Mutants with base changes associated with a frameshift.

Mutants with two or more different-type mutations at separate sites.

### Table II. Base substitutions in mouse skin epidermis of p53+/+ and p53+/−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Background (Number of mutants (%))</th>
<th>UVB (Number of mutants (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C → T (CpG)</td>
<td>24 (57)</td>
<td>63</td>
</tr>
<tr>
<td>C → T (non-CpG)</td>
<td>2 (5)</td>
<td>50</td>
</tr>
<tr>
<td>T → C</td>
<td>6 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C → G</td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td>C → A</td>
<td>5 (12)</td>
<td>80</td>
</tr>
<tr>
<td>T → G</td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td>T → A</td>
<td>2 (5)</td>
<td>100</td>
</tr>
<tr>
<td>Tandem/alternate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC → TT</td>
<td>2 (3)</td>
<td>100</td>
</tr>
<tr>
<td>Other two-base tandem</td>
<td>1 (2)</td>
<td>100</td>
</tr>
<tr>
<td>Three-base</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>40</td>
</tr>
</tbody>
</table>

Data from Ikehata et al. (36).

Percentage of total mutations occurring at dipyrimidine sites.

Alternate substitutions are double base substitutions that are separated by one unchanged nucleotide.

For one of the two mutations, one of the affected dinucleotides did not reside in a dipyrimidine site.

For two of the three mutations, one of the affected triplet nucleotides did not reside in a dipyrimidine site.
known to facilitate NER (9,10), we evaluated the relevance of NER to this pathway by examining the effect of NER deficiency on the enhancement of UVB-induced mutation by p53 deficiency. In the genetic background with the homozygous Xpa null allele (Xpa/−/−), in which NER is completely deficient, p53+/+/ and p53−/− mice were exposed to 10, 25, 50, 100 and 200 J/m² of UVB and analysed for the MFs of the lacZ transgene in the dorsal skin epidermis and dermis 4 weeks later, along with unexposed control mice (0 J/m²) (Figure 4B). The background MFs were not significantly different between the Xpa/−/−p53+/+ and Xpa/−/−p53−/− mice for either the epidermis or dermis. With increases in the UVB dose, MFs increased roughly linearly in the dermis but to a lesser degree than in the epidermis, in which MFs increased drastically up to 50 J/m² but were thereafter suppressed completely to a constant unchanging level. These dose-dependent mutation induction kinetics were observed both for the Xpa−/−p53+/+ and for the Xpa−/−p53−/− mice and were quite similar between the two genotypes. No significant differences were detected between them in either the epidermis or dermis (two-way ANOVA). That there was no enhancement of the UVB-induced mutagenesis by p53 deficiency in the Xpa−/− genetic background indicates that p53 suppresses the UVB genotoxicity by facilitating the NER pathway.

Discussion

p53-dependent suppression of UV mutagenesis in skin

The present in vivo study directly showed that p53 suppresses UVB-induced mutation in the mouse skin, which is consistent with the well-known role of this protein as a tumour suppressor (15) and its relevance to the prevention of solar UV-induced skin cancers (2,28). The p53-dependent suppression of UV mutagenesis was also reported previously in vitro using cultured mouse cells (40,41). As a possible mechanism for the p53-dependent mutation suppression in UV-exposed skin, apoptosis, cell cycle arrest, DNA repair and combinations thereof could have been suggested because p53 is known to be involved in these biological responses (9,16). However, in the present study, we observed a full manifestation of the apoptotic response in the UVB-damaged epidermis even for the p53-deficient mice, though the response was delayed 12–24 h compared to the wild type. The following reciprocal hyperplasia completed the epidermal renewal by 72 h after irradiation regardless of the p53 genotype (Figures 1 and 2). Seventy-two hours would seem swift enough to eliminate the damaged cells that could otherwise acquire mutations in the following proliferating steps because full expression of UVB-induced mutations in mouse skin requires at least 3–7 days after irradiation (33). Moreover, since the epidermis pre-existing at the time of irradiation should be shed off the skin by the massive apoptotic cell death and differentiation into cornified layers, no mutations could have been recovered there. The mutations recovered in the present study should have originated from the epidermis newly grown by the post-irradiation hyperplasia because apoptosis rarely occurred there (Figure 2) and because the fixation of mutation occurs after progression through the S phase of the cell cycle following UV irradiation (5,6). Therefore, p53 should exert its ability of suppressing UV-induced mutation in the newly propagated epidermis.

Significance of the p53-dependent apoptosis and cell cycle control against UV genotoxicity in skin

Although the p53 dependence of the UV-induced apoptotic response in the mouse skin was reported previously (22), apoptosis was scored only at 24 h after UVB irradiation, when such a difference was evident even in our study, although it resulted from the delay of the apoptotic response in the p53+/− skin (see Figures 1 and 2). Another study also supported a role of p53 in the UVB-induced apoptosis in skin, although the kinetics of apoptosis induction shown there seemed to be much faster than ours irrespective of the p53 genotype (42). Nevertheless, both studies have suggested that the role of p53 is not essential but stimulatory for the UVB-induced apoptosis in the epidermis as they showed that the apoptotic defect by p53 deficiency can be recovered by repressing the expression of E2F1, which is an activator of the apoptosis inhibitor BCL-2 and is inactivated by p53 (43), or can be counteracted by topical application of caffeine (42). Accordingly, a stimulatory effect of p53 on expediting the apoptosis progression in the UVB-exposed epidermis was clearly observed in the present study (Figure 2). On the other hand, an inverse activity of suppressing UV-induced apoptosis was reported for p53 in normal human fibroblasts and keratinocytes (11,23,25). In addition, the UV-induced apoptosis observed in human cells deficient in transcription-coupled nucleotide excision repair (TCR), which are known to be extremely sensitive to UV in terms of the apoptotic response, has been proved to be p53 independent (24,26). These studies could only suggest, at best, a regulatory but not an indispensable role for p53 in the UV-induced apoptosis.

p53 is known to mediate cell cycle arrest at the G1 phase after genotoxic insults, thereby allowing time to repair damaged DNA (16,20). Consistent with this, no sign of cell proliferation was evident in the epidermis until 36 h at the earliest after UVB irradiation in the present study (Figures 1 and 2). However, the appearance of hyperplasia was rather delayed in the p53−/− epidermis (Figures 1 and 2), suggesting a stimulatory role in cell proliferation for p53. This contradiction would weaken the grounds for suggesting a contribution of the p53-dependent G1 arrest to the p53-dependent suppression of UVB-induced mutation observed in the present study. UV is known to induce replicational block, thereby arresting cells at the S phase (18). The present study suggests that p53 would facilitate the recovery of cell proliferation from the S arrest, which has already been mentioned before in another study (21).

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>Position</th>
<th>Triplet sequence change</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>gtGGCat</td>
<td>637-9</td>
<td>GCC – TCT</td>
<td>Ia</td>
</tr>
<tr>
<td>gtCTGa</td>
<td>1294-5</td>
<td>TCT – TTA</td>
<td>Va</td>
</tr>
<tr>
<td>GAACGC</td>
<td>1332-3</td>
<td>GTT – AAT</td>
<td>IV</td>
</tr>
<tr>
<td>GCCGCCG</td>
<td>1610-2</td>
<td>GCC – CTC</td>
<td>Ia</td>
</tr>
<tr>
<td>cTGGAAAG</td>
<td>2447-9</td>
<td>TCC – CCT</td>
<td>Ia</td>
</tr>
</tbody>
</table>

*aWild-type sequence of the coding strand (5′ – 3′) is shown. Bases affected by mutation are underlined, and affected codons are capitalized.
*bNumbering of the positions starts at the first nucleotide of the start codon in the lacZ gene.
*cSequences of the strand containing the affected dipyrimidines are shown. In wild-type triplet sequences, dipyrimidines are italicized. In the mutant sequences, bases changed by mutations are underlined.
*dDefined as in Ikeda et al. (37).

Table III. Triplet mutations detected in the UVB-exposed skin epidermis of p53−/− mice

Influences of p53 deficiency on UVB-exposed mouse skin

**Discussion**

**p53-dependent suppression of UV mutagenesis in skin**

The present in vivo study directly showed that p53 suppresses UVB-induced mutation in the mouse skin, which is consistent with the well-known role of this protein as a tumour suppressor (15) and its relevance to the prevention of solar UV-induced skin cancers (2,28). The p53-dependent suppression of UV mutagenesis was also reported previously in vitro using cultured mouse cells (40,41). As a possible mechanism for the p53-dependent mutation suppression in UV-exposed skin, apoptosis, cell cycle arrest, DNA repair and combinations thereof could have been suggested because p53 is known to be involved in these biological responses (9,16). However, in the present study, we observed a full manifestation of the apoptotic response in the UVB-damaged epidermis even for the p53-deficient mice, though the response was delayed 12–24 h compared to the wild type. The following reciprocal hyperplasia completed the epidermal renewal by 72 h after irradiation regardless of the p53 genotype (Figures 1 and 2). Seventy-two hours would seem swift enough to eliminate the damaged cells that could otherwise acquire mutations in the following proliferating steps because full expression of UVB-induced mutations in mouse skin requires at least 3–7 days after irradiation (33). Moreover, since the epidermis pre-existing at the time of irradiation should be shed off the skin by the massive apoptotic cell death and differentiation into cornified layers, no mutations could have been recovered there. The mutations recovered in the present study should have originated from the epidermis newly grown by the post-irradiation hyperplasia because apoptosis rarely occurred there (Figure 2) and because the fixation of mutation occurs after progression through the S phase of the cell cycle following UV irradiation (5,6). Therefore, p53 should exert its ability of suppressing UV-induced mutation in the newly propagated epidermis.

**Significance of the p53-dependent apoptosis and cell cycle control against UV genotoxicity in skin**

Although the p53 dependence of the UV-induced apoptotic response in the mouse skin was reported previously (22), apoptosis was scored only at 24 h after UVB irradiation, when such a difference was evident even in our study, although it resulted from the delay of the apoptotic response in the p53+/− skin (see Figures 1 and 2). Another study also supported a role of p53 in the UVB-induced apoptosis in skin, although the kinetics of apoptosis induction shown there seemed to be much faster than ours irrespective of the p53 genotype (42). Nevertheless, both studies have suggested that the role of p53 is not essential but stimulatory for the UVB-induced apoptosis in the epidermis as they showed that the apoptotic defect by p53 deficiency can be recovered by repressing the expression of E2F1, which is an activator of the apoptosis inhibitor BCL-2 and is inactivated by p53 (43), or can be counteracted by topical application of caffeine (42). Accordingly, a stimulatory effect of p53 on expediting the apoptosis progression in the UVB-exposed epidermis was clearly observed in the present study (Figure 2). On the other hand, an inverse activity of suppressing UV-induced apoptosis was reported for p53 in normal human fibroblasts and keratinocytes (11,23,25). In addition, the UV-induced apoptosis observed in human cells deficient in transcription-coupled nucleotide excision repair (TCR), which are known to be extremely sensitive to UV in terms of the apoptotic response, has been proved to be p53 independent (24,26). These studies could only suggest, at best, a regulatory but not an indispensable role for p53 in the UV-induced apoptosis.

p53 is known to mediate cell cycle arrest at the G1 phase after genotoxic insults, thereby allowing time to repair damaged DNA (16,20). Consistent with this, no sign of cell proliferation was evident in the epidermis until 36 h at the earliest after UVB irradiation in the present study (Figures 1 and 2). However, the appearance of hyperplasia was rather delayed in the p53−/− epidermis (Figures 1 and 2), suggesting a stimulatory role in cell proliferation for p53. This contradiction would weaken the grounds for suggesting a contribution of the p53-dependent G1 arrest to the p53-dependent suppression of UVB-induced mutation observed in the present study. UV is known to induce replicational block, thereby arresting cells at the S phase (18). The present study suggests that p53 would facilitate the recovery of cell proliferation from the S arrest, which has already been mentioned before in another study (21).
p53 is also involved in the promotion of GGR efficiency (9,10) by inducing the damage recognition factors of GGR, DDB2 and XPC, after UV exposure (12,13). Nevertheless, in the present study, little GGR defect by p53 deficiency was evident as shown in the results of the photosensone lesion removal assay (Figure 3). Delays in the damage removal were certainly detected in the epidermis (Figure 3, upper), but most of the delayed differences were ascribable to the delay in the apoptotic replacement of the epidermis in p53+/− mice as discussed above. No remarkable differences in the damage removal were obvious in the dermis. However, this may not be surprising because the Ddb2 gene is insensitive to the UV-inducible, p53-mediated transcriptional activation in mouse cells (35). Accordingly, the observation shown in Figure 3 suggests that p53 deficiency does not largely change the GGR activity in the mouse skin.

We found that p53 suppresses the mutation induction in the UVB-exposed mouse skin through an NER-dependent mechanism, which seems rather paradoxical since the damage removal assay indicated that GGR does not depend on the p53 status. The relevance of TCR may be conceivable since the dependence of TCR on p53 has previously been reported in the wavelength range of UVB (44), but the gene used in the present study for the mutation detection was an unexpressed transgene, which would not be subject to TCR. Wang et al. (45) observed in human cells that p53 facilitates the recruitment of NER factors such as XPC and transcription factor IIH to damaged sites in DNA after UV irradiation and expedites CPD, but not 64PP, removal by GGR. This observation of a p53-dependent GGR subpathway specific to CPD could clear the apparent discrepancy in the present study between the damage removal and mutation induction assays. We have to postulate the existence of some mutagenic minor DNA photolesions that are excised more efficiently by the p53-dependent GGR than CPDs. If such minor mutagenic photolesions were to exist, p53 deficiency would lead to an insufficient exclusion of such mutagenic lesions from DNA, resulting in enhanced mutation induction after UV irradiation, although the MF increase should not be so prominent because the photolesions occupy only a minor fraction of the total amount of DNA damage. On the other hand, regardless of the p53 status, removal of CPDs by GGR is extremely inefficient in mouse cells compared to human cells (35,46), and removal of 64PPs by GGR does not depend as largely on p53 as CPDs (10,45). Thus, postulation of the minor mutagenic photolesions repairable by the p53-dependent GGR could explain well the relatively small enhancement of UVB-induced mutation by p53 deficiency (Figure 4A) and the small differences in the overall damage removal by GGR between p53+/− and p53−/− mice (Figure 3).

Some unidentified novel UV damage could be presumed for such minor mutagenic photolesions or some specifically modified types of well-known UV lesions such as CPD and 64PP might be the ones.

Mechanism of mutation induction suppression

In the mutation induction study, we noticed that the mutation induction was suppressed and levelled off at higher UVB doses specifically in the epidermis regardless of the p53 genotype (Figure 4). This mutation induction suppression (MIS) in the epidermis after UVB irradiation has been already reported (33), and similar epidermis-specific MIS responses were also observed after irradiation of sunlight and UVA1 (47,48). The mechanism of MIS had been unknown, but now could be accounted for by a tissue-specific response found in the present study: the total renewal of damaged epidermis by apoptosis and hyperplasia after exposure to a relatively high dose of UVB, which was clearly depicted above (Figures 1 and 2). In a low-dose range, apoptosis should happen in a sporadic and scattered manner over the epidermis because the amount of DNA damage is not enough to induce the apoptotic response in all of the epidermal cells. At those low doses, MIS does not occur and only a dose-dependent linear increase in MF is observed. Above a certain threshold dose, UV should produce sufficient DNA damage to induce massive cell apoptosis all over the epidermis, which would result in the elimination or, at least, a large loss of mutations in the epidermis because massive cell death excludes the heavily damaged cells which would otherwise acquire a large number of mutations and could transform malignantly in the future. The mutations appearing after the massive apoptosis should occur in the epidermal layer newly grown by post-irradiation hyperplasia. The origin of the hyperplastic epidermal cells is not clear but should include the epidermal layer that was directly exposed to UV and excluded by apoptosis. Those cells residing deeper in the skin would experience insufficient DNA damage to induce apoptosis and would survive and proliferate, thereby acquiring a number of mutations depending on the amount of DNA damage, which should be lower than the damage amount in the apoptotic cells of the upper epidermis. Consequently, MFs in those newly proliferated cells could not go over the MF that would be observed in the cells with the apoptosis-inducing amount of DNA damage. Thus, the dose-dependent increase of the MF should be suppressed above the UV dose that induces massive apoptosis, thereby resulting in the appearance of MIS. If this mechanical model for MIS is correct, the threshold dose for massive apoptosis induction should be equal to the minimum dose for MIS induction, which was 0.5 kJ/m² of UVB for the wild-type mice (Figure 4, upper). Based on this model, the appearance of MIS regardless of the p53 status (Figure 4) would also disprove the essential involvement of p53 in the UVB-induced apoptosis observed in the epidermis.

Supplementary data

Supplementary Tables S1 and S2 are available at Mutagenesis Online.

Funding

Grant-in-Aid for Scientific Research (C) of Japan Society for the Promotion of Science (15510040) to H. I.

Acknowledgements

We thank Dr Shinichi Aizawa (RIKEN Center for Developmental Biology) for generously providing p53-knockout mice; A. Miura, Y. Itoh, A. Otake, Y. Hasegawa, Y. Ikeda, Y. Takahashi and Y. Shono for experimental assistance; S. Kikuchi for helping with manuscript preparation and B. Bell for helping in editing the manuscript. This study utilized the Biomedical Research Core of Tohoku University Graduate School of Medicine.

Conflict of interest statement: None declared.

References


