UV-induced DNA Damage and Mutations in Hupki (Human p53 Knock-in) Mice Recapitulate p53 Hotspot Alterations in Sun-exposed Human Skin

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ABSTRACT

The major etiological agent contributing to human nonmelanoma skin cancer is sunlight. The p53 tumor suppressor gene is usually mutated in these tumors, and the mutations are “UV signature” single or tandem transitions at dipyrimidine sequences in the DNA-binding domain (DBD). Cells that harbor these characteristic mutations are already present in sun-exposed skin areas of healthy individuals, and small epidermal patches that are immuno-reactive to anti-p53 antibody accrue as exposure increases. To explore carcinogen-specific human p53 mutation patterns experimentally, we generated a knock-in (Hupki) mouse in which the murine DBD of the p53 gene has been replaced by the homologous human p53 DBD segment; thus, the precise base sequence context frequently targeted by mutagens or endogenous mutagenic processes in human carcinogenesis is present in this strain (J. L. Luo et al., Oncogene, 20: 320–328, 2001). Here we show that when epidermal cells of Hupki mice (p53 KI/KI) are irradiated in vivo with a single acute dose of UVB light, they accumulate UV photoproducts at the same locations of the p53 gene as human cells. Chronic exposure of Hupki mice (4.5 kJ/m² 5×/week for 4 weeks) results in the appearance of cell patches that stain intensely with the anti-p53 antiserum CM1. DNA preparations from 2 cm² sections of chronically irradiated Hupki epidermis harbor C to T and CC to TT mutations at dipyrimidine sequence motifs identified in human skin cancer, one at codons 278–279, and one at codons 247–248; the latter is the most frequent UVB-association mutation site in humans but not in p53 wild-type mice. Thus, Hupki keratinocytes with these p53 mutations encode an aberrant DBD identical in amino acid sequence to the mutant p53 molecules in human UV-induced tumors. The Hupki mouse model offers a new experimental tool in molecular epidemiology and biomedical research.

INTRODUCTION

The p53 tumor suppressor protein is at the hub of multiple cellular control networks that regulate growth arrest, angiogenesis, DNA repair and apoptosis (1). This master-switch role is underscored by the fact that most human cancers have a malfunctioning p53 (2, 3). It is clear that the predominant mechanism by which p53 is inactivated during human carcinogenesis is acquisition of a single point mutation in the DBD that renders the mutant p53 protein unable to carry out its function in gene transcription regulation. Far less well understood are the causes of these mutations and the relative contributions of endogenous promutagenic factors and exogenous agents to the cellular mutation load.

Although a yeast-based system for examination of mutations in human p53 sequences on a plasmid is available (4, 5), there is currently no laboratory animal model to generate mutations in the human p53 gene experimentally in vivo. Such a test system would be useful not only for exploring hypotheses on the origins of mutations in cancer but also for in vivo screening of pharmaceuticals designed to restore DNA binding to human mutant p53 protein. We designed a transgenic animal model, the Hupki mouse to address this need. In this strain (p53 KI/KI), in which the endogenous murine p53 gene harbors the human instead of the murine DBD sequence, expression levels of the chimeric gene are normal, and various p53 functions are retained (6). The experiments we report here indicate that UVB-exposed Hupki mice show important molecular pathology features of sun-exposed human skin (7, 8): clusters of p53-immunoreactive cells with intensely staining nuclei develop; UV photoproducts map in Hupki mice to the same p53 gene dipyrimidine locations as in UVB-exposed human keratinocytes; and C to T transitions, including tandem mutations, arise at the p53 mutation hotspots identified in human basal and squamous cell carcinomas (9–11). Thus, the Hupki mouse allows for the first time experimental in vivo investigation of DNA damage sites and tissue hotspot mutation burden involving human p53 gene sequences.

MATERIALS AND METHODS

UV Treatment of Mice and Preparation of DNA and Tissue. For single acute exposure for DNA damage mapping by LM-PCR, the shaved dorsal areas (2 cm²) of four Hupki (p53 KI/KI) mice (6 weeks old; genetic background: 129/Sv) were exposed to a single acute dose of 50 kJ/m² UVB in a BioSpectra System (Vilber Lourmat, Marne-La Vallee, France) comprising an irradiation chamber equipped with 312-nm illumination lamps, a sensor/dosimeter, calibrator, and software. As chronic exposure for mutation analysis, shaved mice (five mice per exposure group) were exposed to 4.5 kJ/m² UVB radiation 5 × per week for 4 weeks with the Biospectra System. After sacrifice, epidermis was separated from dermis and divided into two segments. One segment was transferred to ABI lysis buffer [4M urea, 0.5% n-laurylsarcosine, 0.2 M NaCl, in 100 mM Tris (pH 8.0)/0.1 mM EDTA, Applied Biosystems International], digested for 2 h at 36°C with proteinase K, and extracted with phenol-chloroform. DNA was precipitated in an ethanol/sodium acetate solution and resuspended in 10 mM Tris (pH 8.0)/0.1 mM EDTA. The remaining portion was fixed in ethanol, embedded in paraffin, and prepared for histology. Epidermal sections (4 microns) were stained with H&E, and images were recorded with Leica Quantiimet 500 software (Leica, Bensheim, Germany). Immuno-reactivity to anti-p53 antibody was examined using polyclonal anti-human p53 antiserum CM1 (Novacasta; dilution 1:1000) and peroxidase-conjugated secondary antibody according to standard protocols.

Damage Mapping along the p53 Gene by LM-PCR. For mapping UV photoproduct distribution along the Hupki p53 gene after in vivo acute exposure of 6-week-old Hupki mice, DNA was prepared from epidermis as indicated above except that protein digestion was performed overnight at room temperature; DNA was cleaved with T4 endonuclease V and used as template for LM-PCR of exons 7 and 8 as described (12, 13). PCR products were resolved on 8% polyacylamide gels and hybridized to [32P]labeled single-stranded p53 gene exon 7- or 8-specific probes to visualize fragments.

Mutation Analysis (PCR, BAS-PCR, and DNA Sequencing). Detection of (unselected) p53 mutations in 2-cm² areas of chronically exposed Hupki epidermis was achieved with BAS-PCR, a biotin/restriction enzyme PCR method of mutation enrichment (Fig. 4A). Genomic DNA (200 ng) was digested with the restriction enzyme that cleaves the wild-type sequence of the

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3 The abbreviations used are: DBD, DNA-binding domain; BAS-PCR, biotin allele-specific PCR; LM-PCR, ligase-mediated PCR; Hupki, human p53 knock-in.
**RESULTS**

The human and mouse domains of the Hupki chimeric p53 are depicted in Fig. 1. The Hupki gene is under normal endogenous transcriptional regulation at the murine locus, and homozygous p53<sup>KI/KI</sup> mice show typical wild-type p53 responses to UV- and γ-irradiation-induced DNA damage: nuclear accumulation of Hupki p53 protein; enhanced expression of genes regulated by wild-type p53 including WAF1/p21, cyclin G, and noxa; and p53-dependent apoptosis of γ-irradiated thymocytes (6). Hupki mice develop normally; have no apparent physiological defects; and unlike p53-deficient or null mice, are not prone to spontaneous lymphomas, sarcomas, or other neoplasms.

**Immunoreactivity of Epidermal Cell Patches to Anti-p53 Antibody CM1 in Hupki Mice after Chronic UVB Exposure.** Sun-exposed individuals have clones of epidermal cells with nuclei strongly immunoreactive to anti-p53 antibody ("p53 patches") (8, 15). The staining from nuclear p53 protein accumulation is clearly distinguishable from the faint and diffuse reactivity to anti-p53 antibody observed shortly after a single exposure, which is attributed to transient stabilization of wild-type p53 (15). To determine whether Hupki mice show the characteristic precancerous p53 patch-response to chronic UVB irradiation, we exposed a 2-cm<sup>2</sup> dorsal area on each of five Hupki mice to UVB irradiation (4.5 kJ/m<sup>2</sup>/5 ×/week) for 4 weeks. At the end of treatment, patches of cells with enlarged nuclei that stained intensely with antihuman p53 CM1 antibody were detected in tissue sections prepared from ethanol-fixed, paraffin-embedded Hupki epidermis. In two of the five exposed Hupki mice we noted epidermal hyperplastic areas 3–8 cells deep that were strongly immunoreactive (Fig. 2). With antimouse p53 CM5 anti-
Fig. 2. p53 nuclear accumulation in mouse skin exposed to 4.5 kJ/m² UVB (5 × per week, for 4 weeks), visualized by immunohistochemistry with antihuman p53 antiserum CM1; sections were counterstained with hematoxylin. A, unexposed, wild-type; B, UVB-exposed, wild-type; C, unexposed, Hupki; D, UVB-exposed, Hupki. Cell patches with nuclear accumulation of p53 after chronic UVB exposure of wild-type mice also can be visualized using antimouse p53 antiserum CM5 (data not shown and Ref. 15).

Fig. 3. Mapping of UV-induced cyclobutane pyrimidine dimers in p53 exon 7 by ligation-mediated PCR. A, normal human keratinocytes grown in a Petri dish were irradiated with a UVB dose of 2000 J/m². Nonirradiated cells served as a control. B, shaved backs of Hupki mice were exposed to a single dose of 50 kJ/m² of UVB or were sham-treated (no UV). Mice were sacrificed directly after exposure, and cells were isolated immediately for DNA extraction. The lower strand of the p53 gene was analyzed by LM-PCR. Maxam-Gilbert sequencing lanes are shown as markers. The positions of two p53 codons are indicated.

For 4 weeks to UVB irradiation. To detect mutants among the predominantly normal epidermal cell population, a protocol was developed that used restriction enzyme digestion of wild-type p53 sequences at the two hotspot locations (HpaII/MspI for hotspot in exon 7). Unpublished observations.

Fig. 4. Distribution of UVB Photoproducts along the Human p53 Sequences of the Hupki Gene. LM-PCR has been used to investigate the distribution of UV-induced photoproducts along the p53 gene of human cells and to explore correlations between formation of UV-induced DNA adducts and sites of frequent mutation in human skin cancer (13, 16). In UVB-exposed human keratinocytes and Hupki mouse skin irradiated in situ with UVB, the UV photoproduct distributions are essentially superimposable (Fig. 3); signals from cyclobutane pyrimidine dimers are seen almost exclusively at adjacent pyrimidines. A particularly strong signal is seen at codon 248, which is a prominent mutation hotspot in human skin cancers but not in UV-induced murine skin tumors. The normal mouse p53 gene sequence lacks the transcribed strand dipyrimidine at the equivalent exon 7 codon (murine codon 245; numbering system as in Ref. 17). Photoproducts were also mapped in exon 8 of the p53 gene in Hupki mouse skin, and the distribution pattern again matched that of human cells (data not shown).

Detection of p53 Gene Transition and Tandem Mutations in UVB-exposed Hupki Epidermis at Two Dipyrimidine Nucleotide Sites That Are the Major Mutation Hotspots in Human Nonmelanoma Skin Cancers. Sun-exposed areas of skin from healthy individuals harbor p53 mutations that are typical signatures of UV irradiation mutagenesis: they are predominantly C to T transitions at sites of adjacent pyrimidines in the DBD of the p53 gene; they include CC to TT tandem mutations; and the mutations arise at sites that are frequently mutated in human skin cancers (7, 8). The two most prominent skin cancer mutation hotspots in the human p53 gene, codons 247–248 in exon 7 and at codons 278–279 in exon 8, together account for one-eighth of all of the p53 mutations identified to date in human skin cancers (IARC mutation database, version R4). Therefore, we tested for their presence in skin DNA of five Hupki mice exposed for 4 weeks to UVB irradiation. To detect mutants among the predominantly normal epidermal cell population, a protocol was developed that used restriction enzyme digestion of wild-type p53 sequences at the two hotspot locations (HpaII/MspI for hotspot in exon 7). Unpublished observations.

Unpublished observations.
then cloned the noncleaved DNA fragments into plasmid vectors and sequenced 59 clones, 39 harboring exon 7 fragments and 20 with exon 8 fragments. Fifty-eight of the 59 mutant clones harbored a transition mutation (Table 1), including 9 (15%) with tandem CC to TT mutations. At hotspot 278–279, all of the mutations (20/20) were at sites where the dipyrimidine resided on the nontranscribed strand, whereas at the exon 7 hotspot, a strand bias was less pronounced and shows the presence of transitions (28 of 39 clones) at locations where premutated dipyrimidine sequences are on the transcribed strand. The induction of mutations at the two human hotspots in UV-exposed Hupki mice and the very high preponderance of transition mutations over transversions (Table 1), as well as the presence of tandem CC to TT mutations and the more prominent nontranscribed strand orientation of the premutated pyrimidine seen for mutations at the exon 8 hotspot compared with the exon 7 site (codons 247–248; Table 1) are features that are characteristic of p53 mutations at these sequences in sun-exposed human skin tumors.

**DISCUSSION**

Individual mutagens generate characteristic changes at specific locations along a given DNA sequence. The murine p53 gene is not an optimal target sequence to explore origins and modulators of p53 mutations.

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**Fig. 4.** A, flow diagram illustrating the BAS-PCR method used to investigate mutations at human p53 mutation hotspots in exon 7 and exon 8. B, reconstruction experiment showing sensitivity of the method in detection of mutations. Top panel, exon 7 PCR product before final digestion with MspI. Bottom panel, DNA fragments after digestion.

**Fig. 5.** P53 hotspot mutations in epidermis of Hupki mice. A, ethidium bromide-stained agarose gel showing BAS-PCR exon 7 restriction enzyme-resistant fragment obtained with DNA from epidermis of five Hupki mice chronically exposed to UVB irradiation (Lanes 1–5); DNA of unexposed mice did not generate a visible enzyme-resistant fragment (Lanes 6–10). B, electropherograms of sequenced, restriction enzyme-resistant p53 exon 7 BAS-PCR products (i) and cloned enzyme-resistant PCR product (ii and iii) amplified from UVB-exposed Hupki mouse skin DNA. i shows multiple mutations at the investigated hot-spot position codon 247–248, whereas ii and iii show sequences from the mutant fragment pool of two exon 7 clones, one harboring a tandem CC to TT and the other a GG to AA mutation.
mutation patterns found in human tissues, because the mouse p53 gene differs from the human coding sequence at 15% of residues, and base sequence context is a primary determinant of a UV-irradiation or chemically induced mutation spectrum. For example, codons 248 and 249 of the human gene together account for ~10% of the mutations recorded in the IARC p53 mutation database, yet 3 of the 6 bases composing these two heavily mutated codons are dissimilar in the two species (human: CGG AGG and mouse: CGG CGA). Whereas UVBinduced and aflatoxin B1-associated human tumor p53 mutations accumulate at this sequence, not unexpectedly, UVB-induced mouse skin tumors and aflatoxin B1-induced murine lung tumors were not found to have p53 mutations at the equivalent location (18, 19). Particularly notable is the absence of UVB-induced murine p53 mutations in the mouse p53 gene position equivalent to human codon 247–248 (18), because this is the most frequently mutated site in human skin tumors.

Regarding applications of the Hupki model, tissue inflammatory responses involve a complexity of different cell and molecular interactions, generating free radicals such as nitric oxide or lipid peroxidation products that contribute to the mutation load in human carcinogenesis (20–22). The Hupki mouse offers an in vivo approach to such investigations, allowing comparison with human mutation data. It remains to be seen whether major differences in human and murine metabolism and metabolic rate will be reflected in mutations generated from oxidative damage. Some modulation in mutation patterns because of species-specific features of DNA repair may become apparent in mutagenesis studies with the model, because premutagenic lesions in the Hupki p53 gene are subject to the murine DNA repair system. Interbreeding of the Hupki strain with DNA repair-deficient transgenic mouse strains can be undertaken to investigate the role of different DNA repair pathways in shaping p53 mutation patterns observed in humans (23, 24). Measurement of p53 hotspot mutation load in clinical material (e.g., target tissue and plasma) from cancer patients or from individuals heavily exposed to carcinogens is a powerful new technique (22, 25) that can be explored experimentally in the Hupki mice at the identical p53 sequence. Given species and strain differences in carcinogen metabolism and tissue tumor response, carcinogenic agents that are direct-acting mutagens would be the most appropriate choice for tumor induction and p53 mutation profiling in Hupki mice to additionally validate the model. For some research questions Hupki epidermis can serve as surrogate tissue for induction of neoplastic lesions, an approach that has been used in mice with a wild-type murine p53 genotype to demonstrate G to T mutation specificity of the human carcinogen benzo[a]pyrene (26).

We have shown that UV-treated Hupki epidermis harbors specific p53 DBD mutants typical of sun-exposed human skin of healthy individuals and of sunlight-associated human tumors (Table 1). Although we have not addressed pharmacological applications of the Hupki mouse experimentally in the present study, our data imply that this strain will lend itself to in vivo preclinical testing of drugs designed to modulate the human p53 DBD and restore transcriptional and tumor suppressive functions to human p53 mutants (27–29). The remarkable vulnerability of p53 biological function to single amino acid residue substitutions has been appreciated for some time in view of the variety and abundance of single missense p53 mutations found in human tumors. More recent findings have broadened the implications of this sensitivity. Intragenic suppressors of common human tumor mutations have been discovered, and these second site amino acid substitutions restore DNA binding affinity to some mutants (30, 31). A common germ-line p53 variant in human populations at codon 72 also has been shown to be an intragenic modifier of tumor somatic DBD mutants: the presence of the arginine residue variant on an allele that in addition harbors a conformational mutation in the core domain invests certain of these mutant p53 molecules with the capacity to bind to proapoptotic p73, thereby compromising function of the latter (32). These observations underscore the potential value in testing p53-targeting drugs with an in vivo experimental model that presents the precise mutant p53 DBDs found in human tumors.

Table 1. P53 mutations in UVB exposed Hupki mouse skin detected by BAS-PCR at two major human skin hotspots

<table>
<thead>
<tr>
<th>Hotspot</th>
<th>Mutationa</th>
<th>Amino acid</th>
<th>Stranda</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>247–248 (ccgg)</td>
<td>ccAg</td>
<td>Arg/Gln</td>
<td>T</td>
<td>10 (26%)</td>
</tr>
<tr>
<td>247–248 (ccgg)</td>
<td>cggA</td>
<td>Arg/Arg</td>
<td>T</td>
<td>16 (41%)</td>
</tr>
<tr>
<td>247–248 (ccgg)</td>
<td>cAAA</td>
<td>Arg/Gln</td>
<td>T</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>Tgg</td>
<td>Arg/Trp</td>
<td>NT</td>
<td>5 (13%)</td>
<td></td>
</tr>
<tr>
<td>Tgg</td>
<td>Asn/Asn</td>
<td>NT</td>
<td>3 (8%)</td>
<td></td>
</tr>
<tr>
<td>TTgg</td>
<td>Arg/Trp</td>
<td>NT</td>
<td>3 (8%)</td>
<td></td>
</tr>
<tr>
<td>Tcgg</td>
<td>Pro/Ser</td>
<td>NT</td>
<td>2 (10%)</td>
<td></td>
</tr>
<tr>
<td>cTgg</td>
<td>Pro/Leu</td>
<td>NT</td>
<td>13 (65%)</td>
<td></td>
</tr>
<tr>
<td>TTgg</td>
<td>Pro/Phe</td>
<td>NT</td>
<td>4 (20%)</td>
<td></td>
</tr>
<tr>
<td>cAsg</td>
<td>Pro/His</td>
<td>NT</td>
<td>1 (5%)</td>
<td></td>
</tr>
</tbody>
</table>

a Mutated nucleotides are indicated in capital letters.
b The location of the dipyrimidine mutation site: transcribed (T) or non-transcribed (NT) strand.
c Percentage of mutant clones with this base change at this hotspot.

REFERENCES


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