Ultraviolet Radiation Induction of Squamous Cell Carcinomas in p53 Transgenic Mice

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ABSTRACT

Mutations of the p53 gene have been implicated in the pathogenesis of cutaneous squamous cell carcinoma (SCC). To examine the role of p53 in skin carcinogenesis, we observed the development of skin cancers in p53 transgenic mice which carry multiple copies of a mutant allele of the p53 gene with or without chronic UVB radiation. Thirty-one tumors developed in 19 UV-irradiated p53 transgenic mice versus 14 of 19 in the control group; 9 p53 transgenic mice but none of the control mice developed multiple tumors. Historologically, 14 of 14 tumors in the CD-1 mice were SCCs. In the p53 transgenic mice, 25 of 31 tumors were SCCs, and 6 were benign tumors. The mean time to appearance of tumors did not differ between CD-1 mice (26.3 weeks) and the p53 transgenic mice (25.7 weeks; P = 0.512). The p53 protein, which was undetectable by immunohistochemistry in the keratinocytes of CD-1 mice, was elevated in 93% (13 of 14) of tumors from CD-1 mice. These data indicate that mutation of the p53 gene is an important step in the development of SCC. p53 mutations do not alter the latent period of UV-induced SCC but significantly increase the number of tumors and the propensity for multiple tumor development.

INTRODUCTION

SCC is a common skin cancer. Epidemiological and experimental data strongly implicate UV light as an important factor in the development of SCC (1). It is known that UVR induces the formation of pyrimidine dimers and other photoproducts which can lead to mutations (2), but it remains unclear what genes are involved in cutaneous carcinogenesis. Recent experimental data suggest that mutations of the p53 gene may be an important event in UVR-induced SCC (3).

The p53 gene is the most frequently mutated gene in human cancers (4). Mutations of the p53 gene have been observed in carcinomas of the colon, breast, lung, bladder, brain, bone, and chronic myelogenous leukemia (5), as well as SCC (3). It is believed that the p53 gene acts as a tumor suppressor by regulating the normal cell cycle (6). Wild-type p53 has been shown to suppress the transformation of cells in culture and the development of tumors in animals (7). The introduction of an expression vector of wild-type p53 gene suppresses the growth of human lung cancer cells (8), human breast cancer cells (9), and human colorectal carcinoma cells (10). Conversely, loss of wild-type p53 function through mutation or inactivation leads to cell transformation and tumor formation. The mutant p53 gene acting in concert with the ras oncogene can cause malignant transformation of primary rat cells in culture (11). Li-Fraumeni syndrome patients who carry a germine p53 mutation are predisposed to multiple primary tumors, including breast cancer and diverse types of soft tissue sarcomas and carcinomas (12).

Several lines of evidence suggest that mutation of the p53 tumor suppressor gene is involved in the development of SCC. Mutations of the p53 gene have been observed in UV-induced SCC in humans (3) and animals (13). Most of the mutations occurred at dipyrimidine sequences, with C→T or CC→TT transitions predominating, suggesting that these mutations are direct results of UV radiation (3). Other workers have demonstrated that the amount of p53 protein is elevated in SCC compared to normal epithelial cells (14). Altered expression of the p53 gene was also found during murine epithelial cell transformation (15).

To study the role of p53 gene mutation in the development of SCC, we have used a p53 transgenic mouse model (16) in skin photocarcinogenesis experiments. The p53 transgenic mice, which was generated by introducing mutant p53 gene fragments from a tumor cell line into fertilized eggs, carry 10–20 copies of the mutant p53 gene. Our results indicate that the p53 transgenic mice have a higher incidence of SCC and are prone to multiple tumors, but the latent period for the development of SCC is similar to control mice.

MATERIALS AND METHODS

Mice. p53 transgenic mice were kindly provided by Dr. A. Bernstein (16). These mice contain a mutant p53 genomic fragment which was originally cloned from the Friend cell line CB7 (17). The mutant p53 gene has a mutation at residue 193 (Arg to Pro; Ref. 18) and encodes a protein that is not recognized by the anti-p53 monoclonal antibody PAb246 (16). This mutant protein, unlike the wild-type p53 protein, complements ras in the transformation of rat primary cells (16). The transgenic p53 mice were mated with normal CD-1 mice (Charles River Breeding Laboratories, Inc.). Fifty % of the offspring were transgenic, and 50% were normal mice. To identify the p53 transgenic mice, DNA extracted from mouse tail biopsies by the proteinase K/SDS method (19) were subjected to slot blot analysis using an EcoRI-HindIII fragment as a probe (16). The normal CD-1 littermates were used as control. The animals were 6 weeks old at the start of the photocarcinogenesis experiment.

Exposure to UBV Radiation. The animals were divided into four groups: p53 transgenic mice exposed to UBV, p53 transgenic mice without UBV exposure, CD-1 mice exposed to UBV, and CD-1 mice without UBV exposure. Prior to treatment, hair on the dorsal surface of all the mice including control mice which were not exposed to UVR was shaved using an Oster electric clipper with a No. 40 blade, and this procedure was performed two or three times a week when necessary. For UBV irradiation, a bank of four FS40 sunlamps (Westinghouse, Bloomfield, NJ) was used. The intensity of the UV light was measured by an IL 700 radiometer fitted with a WN 320 filter and an A127 quartz diffuser (International Light, Inc., Newburyport, MA). The mice were exposed to 4.5 J/m²/s for 30 min three times a week (Monday, Wednesday, and Friday). This dose is an adjustment of the photocarcinogenesis protocol (2.8 J/m²/s for 1 h, 3 times a week) by Kripep (20) and did not cause skin tissue damage, e.g., ulceration.

Tumor Observation and Analysis. All animals were examined weekly for tumor development. The location and size of each tumor were recorded. For the purpose of this study, a tumor is defined as a visible and palpable nodule with a minimum diameter of 5 mm and persistence for at least 2 weeks. Tumors ≥10 mm in largest diameter were removed for histopathological examination. The Kaplan and Meier analysis (21) was used to describe the patterns of tumor development in the carcinogenesis study. This is a life table analysis and thus takes into account animals that die before developing a tumor. The results are expressed as the probability that an animal may have a
tumor as a function of the duration of UVR. Differences in tumor incidence between groups were analyzed by χ² test, and differences in tumor latency were analyzed by Student’s t test.

Light Microscopic Examination. Tumors were excised and fixed in 10% formalin for at least 24 h. Tissue was then processed and embedded in paraffin by routine methods. Six-μm sections were cut and stained with hematoxylin and eosin for light microscopic examination.

Immunohistochemistry. All tumor specimens were formalin fixed and paraffin embedded. Six-μm sections were prepared and mounted onto silane-coated slides. Sections were then slowly boiled for 12.5 min in the microwave for 10% formalin. Sections were then stained for p53 using a polyclonal antibody CM1 (Dimension Laboratories, Inc.), which reacts with both wild-type and mutant forms of p53. A sensitive streptavidin peroxidase method was used as described previously (22). For positive controls, sections from a breast carcinoma known to overexpress p53 was used. For negative controls, sections prepared without primary antibodies were used. Samples with less than 5% of tumor cell nuclei staining were considered negative for p53 overexpression (23).

Southern Blot Hybridization. Tumor samples were lysed and digested with SET [100 mM NaCl, 20 mM EDTA, and 50 mM Tris-HCl (pH 8.0)] buffer containing 1 mg/ml proteinase K (GIBCO-BRL) and 0.5% SDS at 37°C overnight. DNA was extracted successively with phenol, phenol:chloroform (1:1), and by ethanol precipitation. RNA was removed by digestion with pancreatic RNase (Sigma Chemical Co.). The DNA concentration was measured with a Lambda 3 UV/VIS spectrophotometer (Perkin-Elmer). DNA samples were digested with restriction enzymes and subjected to electrophoresis in 0.8% agarose gel. Gels were soaked twice in 1.5 M NaCl-0.5 M NaOH for 20 min each to denature the DNA and twice in 1.5 M NaCl-0.5 M Tris-HCl (pH 7.4) for 20 min each to neutralize. After capillary transfer of DNA onto nitrocellulose filters (Schleicher & Schuell, Inc.) in 20X SSC buffer [3 M NaCl-0.3 M sodium citrate (pH 7.0)] overnight, the filters were dried in vacuo for 1 h at 80°C. A 0.7-kilobase EcoRI-HindIII fragment of the p53 gene (16) was labeled with [3²P]dCTP to high specific activity by random primer labeling. The filters were prehybridized in 6X SSC, 5X Denhardt’s, 100 μg/ml yeast tRNA, and 0.1% SDS for 1 h and then hybridized in the above solution containing 2 X 10⁶ dpm/ml of probe for 24 h. The filters were washed with 0.2X SSC and 0.1% SDS at 65°C and exposed to X-ray film (Kodak X-Omat AR5) with an intensifying screen.

RESULTS

Tumor Induction. The probability of tumor development for UV-exposed CD-1 mice and p53 transgenic mice is shown in Fig. 1. Tumors started to appear after 22 weeks of UV radiation in both CD-1 and p53 transgenic mice. The experiment was terminated when all 19 p53 transgenic mice developed tumors at week 34. The number and latency of tumors are summarized in Table 1. Thirty-one tumors in the p53 transgenic mice appeared histologically benign. While 25 of 31 tumors from the p53 transgenic mice were SCC. Six of 31 tumors in the p53 transgenic mice appeared histologically benign. Statistical analysis indicates that p53 transgenic mice have a significantly higher rate of SCC than CD-1 mice (χ² = 5.13, P = 0.023). The difference of benign tumor development between these two groups was not significant (χ² = 3.45, P = 0.063). Most of the SCCs demonstrated a poorly differentiated spindle cell morphology. These tumors were located in the superficial dermis and originated from dysplastic epidermis. There did not appear to be a difference in the morphology between the CD-1 and p53 groups. There was no evidence of metastasis in either group. Nine of 19 p53 transgenic mice developed multiple tumors: 8 mice with multiple SCCs and 1 mouse with a benign and a malignant tumor, while none of the CD-1 mice developed multiple SCCs (χ² = 10.89, P = 0.001).

Twenty CD-1 mice and 18 p53 transgenic mice which did not receive chronic UV radiation were used as controls. No tumor developed in non-UV-irradiated CD-1 mice. Two non-UV-irradiated p53 transgenic mice developed tumors on the posterior back and ear; one was a sarcoma, and the other, a benign fibroma. There is no significant difference of benign tumor incidence between the p53 transgenic mice which received UVR (6/19) and the p53 transgenic mice which did not receive UVR (1 of 18; χ² = 2.83, P = 0.10).

The latent period for tumor development defined as the time between the first UV exposure and the appearance of a visible and palpable skin nodule ≥5 mm in diameter was 26.5 weeks on average for the UV-irradiated CD-1 mice and 25.7 weeks for the UV-irradiated p53 transgenic mice. There is no significant difference between these two groups (Student’s t test, P = 0.512).

p53 Expression in Tumors. To further define the role of the p53 gene in UV-induced skin carcinogenesis, we compared the expression of the p53 gene in keratinocytes of non-UV-irradiated skin, in UVB-induced skin tumors, and normal UVB-irradiated skin adjacent to tumors by immunohistochemistry (Table 3). p53 is expressed in basal keratinocytes of non-UV-irradiated skin of p53 transgenic mice but was undetectable in keratinocytes of non-UV-irradiated skin of CD-1 mice (Fig. 2, A and B). Thirteen of 14 (93%) SCCs in CD-1 mice (Table 3; Fig. 2C), and 19 of 22 (86%) SCCs in p53 transgenic mice (Table 3; Fig. 2D) showed overexpression of the p53 protein. All the

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Table 1: Incidence and latency of tumors induced by UVB radiation

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of mice</th>
<th>CD-1 + UV</th>
<th>P53 + UV</th>
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<tr>
<td>CD-1</td>
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<td>19</td>
<td>0.046</td>
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<tr>
<td>P53</td>
<td>19</td>
<td>19</td>
<td>0.001</td>
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Table 2: Histology and distribution of tumors induced by chronic UV radiation

<table>
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<th>Strain</th>
<th>Site</th>
<th>No.</th>
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<th>Benign</th>
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<td>Eye</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>20</td>
<td>16</td>
<td>4</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>25</td>
<td>6</td>
</tr>
</tbody>
</table>

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Example graph and figure captions:

Fig. 1. Rate of tumor appearance (time to first tumor (0.5 cm)) in CD-1 and p53 transgenic mice after UV radiation. The CD-1 group consisted of 9 males and 10 females. The p53 group consisted of 10 males and 9 females.
UV-INDUCED SCC IN p53 TRANSGENIC MICE

Table 3 p53 expression in tumors, overlying epidermis, and adjacent normal skin

<table>
<thead>
<tr>
<th></th>
<th>Tumor</th>
<th>Overlying epidermis</th>
<th>Adjacent normal skin</th>
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</thead>
<tbody>
<tr>
<td>p53 expression</td>
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<td></td>
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<tr>
<td>CD-1*</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>p53 malignant</td>
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<td>3</td>
</tr>
<tr>
<td>p53 benign</td>
<td>0</td>
<td>3</td>
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</table>

*Two tumor biopsies from CD-1 mice did not contain overlying epidermis, and four tumor biopsies did not contain adjacent normal skin. ND, not done.

**DISCUSSION**

We have elected to use p53 transgenic mice (16) as an in vivo model to examine the hypothesis that mutations of the p53 gene predispose keratinocytes to develop squamous cell carcinoma following exposure to UVR. Our p53 transgenic mice contain both wild-type and mutant alleles of the p53 gene, thus they closely mimic the "real life" situation where mutations of the p53 gene originate in one of the alleles. We chose this transgenic model over p53 knockout mice (24) for photocarcinogenesis experiments because the knockout mice have a very short latency for developing multiple internal tumors and have a short life span and, therefore, may not be suitable for photocarcinogenesis experiments.

Our results indicate that the p53 transgenic mice develop twice as many tumors as CD-1 control mice after UVR, but the latent period for tumor development is the same. The p53 transgenic mice were prone to multiple tumors, while none of the control CD-1 mice developed multiple tumors. Our data correlate well with other observations in human and in animal models. Toguchida et al. (25) reported that Li-Fraumeni patients had increased susceptibility to osteosarcoma, but the latent period for tumor development was similar to those who did not have a p53 germline mutation. Kemp et al. (26) found that the latent period for chemical-induced skin tumors was similar in wild-type, heterozygous, and homozygous p53 knockout mice. However, there seems to exist differences in UV- and chemical-induced skin tumors. We observed an increased number of UV-induced SCCs (Fig. 3, Lanes 6 and 15) which showed negative p53 nuclear staining.

Southern Analysis of the p53 Gene in Tumors. All of the benign tumors and 3 of 22 malignant SCCs in p53 transgenic mice showed faint or negative p53 staining. Seventy-five % (9 of 12) of the SCCs in the CD-1 mice and 91% (20 of 22) of the SCCs in the p53 transgenic mice showed positive p53 staining in the overlying epidermis. The normal UV-irradiated skin adjacent to SCCs from CD-1 mice showed negative p53 staining (100%; 10 of 10). All of the normal UV-irradiated skin adjacent to benign tumors from p53 mice showed positive staining of p53 mutant protein. These results suggest that the p53 gene is mutated in UVB-induced SCC, and the mutations may occur early during the development of SCC.

Fig. 2. Immunohistochemical detection of p53 expression in normal skin and SCC. A, normal skin of CD-1 mice. B, normal skin of p53 transgenic mice. C, epidermis overlying a SCC from CD-1 mice. D, SCC from CD-1 mice. Arrows, positive p53 nuclear staining. The dashed line in (A) outlines the epidermis. X 250.
induced skin tumors in p53 transgenic mice, while the number of chemical-induced skin tumors was similar in wild-type and heterozygous and decreased in p53 null mice (26). We observed no historical difference in UV-induced tumors from control mice and p53 transgenic mice, while Kemp et al. (26) found that the tumors in p53 knockout mice were more malignant, and the loss of p53 alleles enhanced the rate of malignant progression. These differences may reflect differential molecular pathways of UV- and chemical-induced skin carcinogenesis.

The exact molecular mechanism of the p53 gene in UV-induced skin carcinogenesis is not known. Recent studies indicate that p53 suppresses tumor development via cell cycle control and apoptosis. It has been suggested that wild-type p53 acts as a G1-S checkpoint control for DNA damage (27–33). It has been shown that G1 arrest associated with rapidly increased levels of p53 protein after exposure to DNA-damaging agents, such as UV radiation (29), nonlethal doses of gamma irradiation (28, 29), and actinomycin D (33), occurred in cells containing wild-type p53 genes but not in cells containing mutant p53 genes. Cell cycle regulation of p53 protein may contribute in maintaining genetic stability in the circumstance of a DNA-damaging event. The p53 gene has also been shown to play an important role in inducing apoptosis, a mechanism to prevent the replication of damaged DNA. Wild-type p53 protein appears to be necessary for apoptosis (34–36). Wild-type p53 induced apoptosis in myeloid cells (36), whereas thymocytes that lack p53 do not undergo radiation-induced apoptosis (34, 35). Bone marrow-derived progenitor cells from p53 transgenic animals are also resistant to ionizing radiation-induced apoptosis (37), as are fibroblasts from patients with the Li-Fraumeni syndrome (29). Recently, it has been demonstrated that nuclear p53 function is required to trigger UV-induced apoptosis (38). In addition, p53 has been shown to down-regulate the Bcl-2 gene (39, 40), which is thought to prevent most types of apoptotic cell death (41, 42).

Mutations of the p53 gene may be required in the development of UV-induced SCC, p53 mutation has been found in sun-related SCC in humans (3) and in animals (13, 43). Kanjilal et al. (43) found that the p53 tumor suppressor gene is mutated at 100% incidence rate in 11 UV-induced murine skin cancers. The mutations were predominantly UV specific C→T or CC→TT transitions (3, 13, 43). Mutations of the p53 gene appear to be an early event in the development of SCC, since overexpression of the p53 protein was observed in human actinic keratosis, a precursor lesion of SCC (44–46). Recently, Ziegler et al. (47) have demonstrated that actinic keratoses contain UV-specific p53 mutations. We observed p53 overexpression in 13 of 14 tumors in CD-1 mice (Table 3), suggesting that the p53 gene is mutated in these tumors. The overexpression of the p53 gene in the tumors were not due to the physiological induction by UV light, since the UV-irradiated skin adjacent to the tumors in CD-1 mice showed negative p53 staining. It is not clear why p53 protein was not overexpressed in chronic UV-exposed skin of CD-1 mice, whereas it has been shown that p53 protein was induced by acute UV exposure in human skins (48, 49). It is interesting that all of the benign tumors in p53 transgenic mice showed negative p53 staining (Table 3). Since the p53 mutant protein was expressed in the normal UV-radiated skin adjacent to the tumors (Table 3) and the mutant p53 transgene was still present in the tumors (Fig. 3, Lanes 3, 7, 8, and 9), these benign tumors may originate from cells which did not express the mutant p53 protein. This is supported by the observation that different cells of the transgenic mice express different levels of mutant p53 protein, and some tissues do not express the mutant p53 protein (16). A small percentage (14%; 3 of 22) of the malignant SCC showed negative p53 staining. Since the mutant p53 transgene is present in these tumors (Fig. 3, Lanes 6 and 15), the transcriptional regulation of the transgenes may have been altered during the process of cell transformation.

An alternate explanation for the higher tumor incidence in the p53 transgenic mice is that UV may induce a systemic change (such as decreased immunity) which would promote tumor growth. If the immune system was altered in the p53 mice, tumors might be expected in other organ sites. However, the UV-exposed p53 transgenic mice did not develop such tumors in different organs other than the skin.

The reason for the similar latent period between CD-1 and p53 transgenic mice is not completely understood. p53 mutation may be a prerequisite but not the only factor in the development of UV-induced SCC. That is, mutations of other genes or other factors are necessary for the development of UV-induced SCC. Suarez et al. (50) have shown that skin tumors from xeroderma pigmentosum patients contained amplified H-ras, N-ras, and c-myc mRNA transcripts, in addition to a mutated N-ras and a rearranged H-ras oncogene. Furthermore, high mutation frequency in ras genes has also been found in the skin cancers of xeroderma pigmentosum patients (51). The involvement of other genes may account for the similar latent period of developing SCC for the p53 transgenic mice and CD-1 control mice.

In summary, our data indicate that mutations of the p53 gene is an important step in the development of SCC. p53 mutations do not alter the latent period of UV-induced SCC but significantly increase the number of tumors and the propensity for multiple tumor development.

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REFERENCES


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