p53 Heterozygosity Results in an Increased 2-Acetylaminofluorene-Induced Urinary Bladder but not Liver Tumor Response in DNA Repair-Deficient Xpa Mice

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ABSTRACT

Both nucleotide excision repair (NER) and the p53 tumor suppressor protein play crucial roles in the prevention of cells becoming cancerous. This is clearly demonstrated by the fact that NER-deficient xeroderma pigmentosum patients and Li-Fraumeni patients who carry a germ-line p53 mutation are highly tumor prone. The NER-deficient Xpa and the p53+/−/− mouse models clearly mimic their human counterparts, because they are both tumor prone as well. The aim of the study presented here was to analyze the relative contribution of these two pathways in tumor suppression and to analyze a possible link between NER and p53 activation. For this, we exposed Xpa, p53+/−/−, and Xpa/p53+/−/− mice to 2-acetylaminofluorene (2-AAF). We show that 2-AAF-induced urinary bladder tumor suppression is dependent on p53 status, because p53+/−/− mice were highly tumor prone. Xpa/p53+/−/− mice were even more tumor prone, whereas no increased tumor response was found in Xpa mice. Short-term assays revealed a decreased apoptotic response in Xpa/p53+/−/− mice, pointing in vivo toward a link between NER and p53-mediated apoptosis. In contrast, liver tumor response was primarily dependent on appropriate DNA repair, because Xpa-deficient mice were liver tumor prone. p53 heterozygosity had no influence on liver tumor incidences, in line with the results obtained from the short-term 2-AAF studies revealing no altered cellular response in p53+/−/− or Xpa/p53+/−/− mice. Interestingly, however, mice completely deficient in both NER and p53 (Xpa/p53+/−/− mice) showed a dramatic increase of hepatocellular proliferation accompanied by lacZ reporter gene mutations.

INTRODUCTION

The integrity of genetic material of living organisms is constantly threatened by exposure to DNA-damaging agents. When DNA damage remains unrepaired, it may lead to gene mutations that can, depending on the target gene(s) that become affected, influence the proper functioning of the cell. An important outcome of sustained DNA damage is the development of cancer as a consequence of the accumulation of mutations in genes involved in cell cycle regulation. Fortunately, the cell can activate several mechanisms to prevent mutation fixation and malignant transformation. One crucial step is the repair of acquired DNA damage before mutations can take place. Probably the most complicated and intensively studied DNA repair process, harboring a broad specificity, is nucleotide excision repair (NER). NER is able to recognize lesions that disturb the double helix conformation, such as those induced by UV light and chemicals that give rise to bulky DNA adducts and DNA cross-links. A stretch of nucleotides, containing the damaged nucleotide, is excised and replaced by a newly synthesized DNA strand. NER is a complex process, consisting of two distinct pathways, i.e., transcription coupled repair and excision repair. The idea that appropriate DNA repair is important for the prevention of mutation fixation and, consequently, cancer, is supported by the fact that xeroderma pigmentosum (XP) patients, who are NER deficient, have a clear cancer-prone phenotype (>1000-fold increased risk to develop skin tumors primarily at sun-exposed sites of their body; Refs. 1, 2).

Activation of the tumor suppressor protein p53 is another crucial mechanism in the prevention of cancer, demonstrated by the fact that Li-Fraumeni syndrome patients, who carry a germ-line mutation in p53, are highly cancer prone (3). Furthermore, it is one of the most frequently mutated genes found in human somatic cancer (4, 5). Through the activation of numerous target genes, p53 induces several cellular processes, including inhibition of the cell cycle (6–10). This idea is based on the in vitro finding that stalled RNA polymerase II at UV-induced lesions in actively transcribed genes constitutes a strong signal for the apoptotic response as well as for p53 activation (21). This suggests that the transcription coupled repair subpathway of NER is involved in activation of p53-dependent apoptosis when DNA damage in the transcribed strand is encountered (22–26).

To gain more insight into the specific roles of NER and p53 in tumor suppression, we and others have generated several mouse models mimicking the human cancer syndromes. These include Xpa−/− knockout mice (27–29) that are completely deficient in NER activity and p53+/−/− mice (30–32). Xpa−/− (further annotated as Xpa) mice develop, like their human XPA counterpart, skin cancer at UV-exposed areas (28, 29, 33). Furthermore, they appear to be susceptible to develop internal tumors upon exposure to several chemical carcinogens, including benzo(a)pyrene (34–36), 7,12-dimethylbenzanthracene (28, 29), aflatoxin B (37), and 4-nitroquinoline 1-oxide (38).
MATERIALS AND METHODS

Effect of p53 heterozygosity and/or NER deficiency on tumor development

In line with the results obtained from the short-term 2-AAF studies, we hypothesized that p53 heterozygosity had no influence on liver tumor incidences (frequency studies) or 4 weeks (immunohistochemical studies). To perform cell proliferation analyses (5 males and 5 females per genotype, 4, 8, and 12 weeks 2-AAF) or 300 ppm 2-AAF. The mice were used either for determination of cell proliferation and p53 protein accumulation. Because we were interested in the relative contribution of the NER and p53 pathways in tumor suppression and the suggested link between NER and p53 activation and p53 pathways in tumor suppression and the suggested link between NER and p53 mediation of apoptosis. In contrast, the liver tumor response was primarily dependent on appropriate DNA repair, because Xpa-deficient mice were clearly liver tumor prone. p53 heterozygosity had no influence on liver tumor incidences in line with the results obtained from the short-term 2-AAF studies, which revealed no altered cellular response in p53+/− or Xpa/p53+/− mice as compared with wild-type (WT) or Xpa mice. Interestingly, however, we showed that when mice are completely deficient in both NER and p53 (Xpa/p53−/− mice), an enormous increase in hepatocellular proliferation and lacZ gene mutations was observed.

Taken together, these studies clearly demonstrate a relative distinct effect of p53 heterozygosity and/or NER deficiency on tumor development in different tissues of mice.

2-AFF treatment

For the carcinogenicity study, WT, Xpa, p53+/−, and Xpa/p53+/− mice (6–9 weeks old) were treated for 39 weeks with 300 ppm 2-AAF in the diet and were subsequently fed normal diet for 2 weeks. All of the treatment groups consisted of 25 males and 25 females per genotype. The control groups consisted of 10 males and 10 females per genotype and were fed normal diet for 41 weeks.

For the subchronic studies, WT, Xpa, p53+/−, Xpa/p53−/−, and Xpa/p53+/− mice (6–9 weeks old), heterozygous for the lacZ reporter gene, were treated for different time periods (ranging from 1 to 12 weeks) with 300 ppm 2-AAF. The mice were used either for lacZ mutant frequency analyses (5 males and 5 females per genotype, 4, 8, and 12 weeks 2-AAF) or immunohistochemical studies to determine levels of cell proliferation and apoptosis (3 males and 3 females per genotype; 1, 2, 4, and 12 weeks). Control mice (aged 6–9 weeks) were fed normal diet for 6 weeks (lacZ mutant frequency studies) or 4 weeks (immunohistochemical studies). To perform cell proliferation studies, mice were injected intraperitoneally with 25 mg/kg body weight bromodeoxyuridine (Brdu; Sigma, St. Louis, MO) 1 h before necropsy.

The 2-AFF-containing feed (Altromin, Lage, Germany), normal feed, and water were available ad libitum throughout the course of all of the studies.

Negro and Pathology

All of the mice were monitored daily for clinical abnormalities and body weights were determined every 2 weeks. Mice that became moribund or lost >20% of their body weight were killed intercurrently. All of the surviving mice were killed by cervical dislocation at the end of the study.

Gall bladder, intestines, kidneys, liver, lung, reproductive organs, spleen, stomach, thymus, urinary bladder, and tissues showing abnormalities were collected and fixed in 3.8% neutral buffered formaldehyde. Subsequently, the samples were embedded in paraffin wax, cut into 5-μm sections, and finally stained with H&E or immunohistochemically for cell proliferation, apoptosis, or p53 accumulation (see below).

For the lacZ gene mutant frequency analyses liver, spleen, and urinary bladder were isolated and snap frozen in liquid N2.

LacZ Gene Mutant Frequency Analyses

Total genomic DNA was isolated using a procedure described earlier by de Vries et al. (34). LacZ mutant frequencies were determined in liver, urinary bladder, and spleen (negative control tissue) using a procedure described extensively by Döll et al. (44).

Briefly, pUR288 plasmids were rescued from total genomic DNA (20–50 μg) with magnetic beads coated with the lacZIIac fusion protein. These plasmids were subsequently transfected into electrocompetent Escherichia coli strain C. A fraction (2 μl of the 2 ml total) of the bacterial sample was plated on nonselective X-galactosidase plates to determine the rescue efficiency. The remainder was plated onto selective P-galactosidase plates to select for mutants. The lacZ mutant frequencies were calculated by dividing the number of mutants by the total number of rescued colonies × 1000.

Immunohistochemical Detection of p53 Protein

p53 protein accumulation was detected in paraffin-embedded, formalin-fixed tumor sections of urinary bladder and liver of WT, Xpa, p53+/−, and Xpa/p53−/− mice. Polyclonal CM5 antibody, which recognizes several epitopes within the N- and C-terminal regions of p53 protein (Novacast, Newcastle, United Kingdom), and Pab240 antibody, specific for mutant p53 protein only (Labvision, Fremont, CA), were used. Deparaffinized tissue sections were placed in 0.01 M citrate buffer (pH 6.0) and heated at 95°C for 30 min. Subsequently, endogenous peroxidase was deactivated with 0.3% hydrogen peroxide (Merck, Darmstadt, Germany) in 50% methanol (Merck). The slides were incubated overnight with p53 antibody at 4°C. CM5 immunohistochemical staining was performed with a secondary goat antirabbit/biotin antibody (Vector Laboratories, Burlingame, CA), followed by the avidin-biotin complex procedure with streptavidin-HRP (NEN Life Science, Boston, MA) followed by streptavidin-HRP (NEN Life Science). Peroxidase was visualized with 3,3′-diaminobenzidine (Sigma) and ammonium-nickel sulfate hexahydrate (Fluka, Buchs, Germany). Counterstaining was performed with nuclear fast red.

Sections were scored by dividing the area of p53-positive fields by the total area of the tumor × 100%. The CM5 staining was scored either negative (−, <5%) or positive (+, 5–24%, ++, 25–75%, +++ >75%) positive. The fields that were positive for the CM5 antibody were scored either negative (−) or positive (+) for Pab240 immunoreactivity.

Immunohistochemical Detection of Cell Proliferation

Cell proliferation was analyzed in paraffin-embedded, formalin-fixed urinary bladder, liver, and small intestine (positive control) tissue sections by the detection of BrdUrd incorporation. The immunohistochemical staining procedure was performed as described by Hoogervorst et al. (35). In short, the tissue slides were treated with 20% hydrogen peroxide (H2O2) to inactivate endogenous peroxidase. The slides were incubated with biotinyl tyramide followed by streptavidin-HRP. 3,3′-Diaminobenzidine with ammonium-nickel sulfate hexahydrate were used as substrate for HRP. Counterstaining was performed with nuclear fast red.

Cell proliferation was assessed in the epithelial tissue lining the urinary bladder and hepatocytes of the liver. The labeling index was calculated by dividing the number of labeled cells by the total number of cells counted × 100. Per tissue, a total of 1200–1500 cells, recorded in three different tissue sections, were analyzed.


**References**

(38). In addition, p53+/− mice are also highly tumor prone, both spontaneously as well as upon exposure to numerous chemical carcinogens (39). With the notable exception of the liver, p53+/− mice are generally predisposed to develop carcinogen-induced tumors in a wide variety of tissues, including skin, esophagus, stomach, lung, breast, urinary bladder, uterus, and hematopoietic system (reviewed elsewhere).
Immunohistochemical Detection of Apoptosis. Apoptotic cells were visualized in paraffin-embedded, formalin-fixed urinary bladder and liver sections with antiactive caspase-3 antibody (Asp175; Cell Signaling, Beverly, MA). De-paraffinized sections were boiled in 10 mM citrate buffer (pH 6.0). After deactivation of endogenous peroxidase with 0.3% hydrogen peroxidase in 50% methyl alcohol, the slides were incubated overnight with the antiactive caspase-3 antibody. The next day, slides were incubated with the secondary goat antirabbit/biotin antibody followed by the avidin-biotin complex procedure. 3,3’-Diaminobenzidine substrate with ammonium-nickel sulfate hexahydrate were used as HRP substrate, and counterstaining was performed with nuclear fast red.

In the urinary bladder, the number of apoptotic cells was assessed by counting the antiactive caspase-3-positive cells per mm epithelial tissue (average of 15 mm total length of epithelial tissue per mouse). In the liver, the amount of apoptotic hepatocytes was counted per mm² liver tissue (average of 90 mm² per liver slide).

Statistical Analysis. For the 2-AAF carcinogenicity study, the statistical significance of tumor response of the treated mice compared with the controls was calculated with the one-tailed Fisher exact test. The two-tailed Student t-test with a two-sample unequal variance was used to analyze the significance of the lacZ mutant frequency, cell proliferation, and apoptosis assays. The level of statistical significance was taken as P ≤ 0.05.

RESULTS

2-AAF-Induced Tumor Response in WT, p53+/−, Xpa, and Xpa/p53+/− Mice. To analyze 2-AAF-induced tumor responses, WT, p53+/−, Xpa, and Xpa/p53+/− mice were treated for 39 weeks with 300 ppm 2-AAF (in feed, ad libitum) and put on normal diet for an additional 2 weeks. The control groups were fed normal diet for the entire experimental period.

A summary of the histopathological changes found in bladder and liver tissue of mice that died intercurrently or were killed at the end of the study is given in Table 1. With regard to the urinary bladder, none of the untreated mice (all genotypes) showed any pathological abnormalities. Upon 2-AAF treatment, however, mice of the various genotypes analyzed developed pathological lesions including preneoplastic atypical foci and transitional cell tumors. These tumors were predominantly transitional cell carcinomas and to a lesser extent transitional cell papillomas and carcinomas in situ. A relatively low incidence of urinary bladder tumors was found in WT mice (2 of 49 mice examined) and NER-deficient Xpa mice (5 of 50; Table 1). Interestingly, however, 2-AAF-treated p53+/− heterozygous mice developed a significantly increased frequency of bladder tumors as compared with untreated p53+/− mice (13 of 45 mice examined; P = 0.005). Furthermore, mice with both a NER and p53 deficiency (Xpa/p53+/−) showed an even higher and more significantly increased urinary bladder tumor incidence (25 of 40 mice; P < 0.005). As compared with 2-AAF-exposed WT mice, the urinary bladder tumor responses of both p53+/− mice and Xpa/p53+/− were significantly increased upon 2-AAF treatment (both P < 0.005).

In the liver, some untreated mice developed hepatocellular hypertrophy/pleomorphism and oval cell hyperplasia (Table 1). Furthermore, we found a hepatocellular adenoma in 1 untreated WT and p53+/− mouse at time of necropsy (Table 1). Upon 2-AAF treatment, an increased number of hepatic lesions were found in mice of all of the genotypes tested. In WT and p53+/− mice, these lesions were predominantly at the level of oval cell hyperplasia, without a dramatic increase in tumor incidence. This is in contrast to Xpa mice, which showed high frequencies of hypertrophy/pleomorphism and oval cell hyperplasia and above that a significantly increased incidence of hepatocellular tumors as compared with untreated Xpa mice (P = 0.007). Interestingly, this increase in 2-AAF-induced liver tumor incidence of Xpa mice was not found in mice additionally heterozygous for p53 (Xpa/p53+/− mice), as the incidence was similar to that found in DNA-proficient p53+/− mice. However, the incidences of hypertrophy/pleomorphism and to a lesser extent oval cell hyperplasia are comparable with those observed in Xpa mice as such indicative for increased sensitivity to 2-AAF of Xpa/p53+/− mice compared with WT or p53+/− mice.

Detection of p53 Protein in 2-AAF-Induced Urinary Bladder and Liver Tumors. On the basis of the 9-month 2-AAF study, we concluded that p53 heterozygosity primarily results in the development of 2-AAF-driven urinary bladder tumors and that a deficiency in both NER and p53 results in an even higher tumor response. This in contrast to the liver, where we found an increased incidence of 2-AAF-induced liver tumors in NER-deficient mice, whereas p53+/− mice are not susceptible at all to develop 2-AAF-induced liver tumors, not even in a NER-deficient background. To analyze whether the tumors found in p53+/− and Xpa/p53+/− mice are accompanied by p53 mutations, we assessed p53 protein accumulation by immunohistochemistry. Tumor tissues were analyzed for CM5 immunoreactivity (recognizing both stabilized WT as well as mutated p53 protein) and Pab240 immunoreactivity (specific for conformational-changed, mu-

<table>
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<tr>
<td>Tumor incidence (%)</td>
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<td>6</td>
<td>13</td>
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Table 1 Pathological changes found in urinary bladder and liver tissue of WT, Xpa, p53+/−, and Xpa/p53+/− mice

Wt, wild-type; 2-AAF, 2-acetylaminofluorene.

a P < 0.05 as compared with untreated control mice.

b P < 0.05 as compared with 2-AAF-treated WT mice.
tated p53 protein). These results are shown in Table 2A for the urinary bladder and in Table 2B for the liver.

As shown in Table 2A, the number of CM5-positive cells found in urinary bladder tumors varied between completely negative (-) and up to 75% positive cells (+ + +). Remarkably, none of the urinary bladder tumors found in WT or Xpa mice showed any p53 accumulation (2 and 5 tumors tested, respectively). With regard to the p53<sup>+/–</sup> heterozygous mice, the one benign transitional cell papilloma was negative, whereas ~50% of the malignant transitional cell carcinomas stained positive with the CM5 antibody (5 of 11). In Xpa/p53<sup>+/–</sup> mice, the one transitional cell papilloma found stained positive for the CM5 antibody, and again ~50% of the carcinomas analyzed (both carcinomas in situ and transitional cell carcinomas) showed CM5 positive fields (1 of 2 examined carcinomas in situ and 7 of 17 examined transitional cell carcinomas), similar to the staining patterns observed in bladder tumors found in p53<sup>+/–</sup> mice. As expected, all of the urinary bladder tumors negative for the CM5 antibody also showed no immunoreactivity with the Pab240 antibody (incidences are shown between parentheses in Table 2). In contrast, 12 of 14 CM5-positive urinary bladder tumors were also found positive for the Pab240 antibody (both in p53<sup>+/–</sup> and Xpa/p53<sup>+/–</sup> mice), indicating that these tumors contained conformational-changed up-regulated p53 protein.

As shown in Table 2B, none of the 2-AAF-induced liver tumors found in WT and Xpa mice (3 and 13 tumors, respectively) were positive for CM5 or Pab240 antibody staining. Furthermore, 5 of 6 tumors of the p53<sup>+/–</sup> mice and also 5 of 6 tumors of the Xpa/p53<sup>+/–</sup> mice showed no immunoreactivity for the CM5 antibody. The single CM5-positive liver tumor of a p53<sup>+/–</sup> mouse most likely contained up-regulated WT p53 protein, because this tumor was negative for Pab240 antibody staining. In contrast, the liver tumor derived from the Xpa/p53<sup>+/–</sup> mouse contained Pab240-positive p53 protein. The two hepatocellular adenomas found in control mice (1 WT and 1 p53<sup>+/–</sup> mouse) showed no reactivity with either CM5 or Pab240 antibody (data not shown).

**Early Responses of Liver and Bladder Tissue to 2-AAF.** On the basis of both the carcinogenicity and p53 immunohistochemistry study, we conclude that the p53 protein plays an important role in 2-AAF-induced urinary bladder tumor suppression. In contrast, the liver tumor response was not altered in p53 heterozygous mice, and furthermore we found only one liver tumor containing up-regulated conformationally changed p53 protein. To gain more knowledge about the apparent differences in tumor responses, we examined acute effects of 2-AAF treatment in liver and urinary bladder. Because p53 can regulate both cell cycle arrest and apoptosis upon presence of DNA damage, we analyzed potential differences of these processes in bladder and liver tissue upon short-term 2-AAF treatment. For this, WT, p53<sup>+/–</sup>, Xpa, and Xpa/p53<sup>+/–</sup> mice were treated with 2-AAF for 1, 2, 4, or 12 weeks.

**Effect of Short-Term 2-AAF Treatment on Cell Proliferation.** Cell proliferation was measured by immunohistochemical detection of BrdUrd incorporation (injected i.p. 1 h before necropsy). A summary of BrdUrd labeling indices found in bladder and liver is shown in Fig. 1.

With respect to the urinary bladder (Fig. 1A), the amount of background cell proliferation of all of the genotypes tested was comparable (labeling indices between 0.2 and 0.4). Upon 2-AAF treatment, a clear and significant induction of cell proliferation in the urinary bladder was observed in all of the genotypes tested. After 1 or 2 weeks of 2-AAF treatment, the highest labeling indices were found in all of the genotypes analyzed, but still after 4 and 12 weeks of 2-AAF exposure significantly increased labeling indices as compared with untreated mice of the same genotype were found. When focusing on the differences in cell proliferation between the genotypes, the absence of NER or p53 heterozygosity had no clear effect on 2-AAF-induced cell proliferation in the urinary bladder, because the labeling indices were comparable with those found in WT mice. However, double-knockout Xpa/p53<sup>+/–</sup> mice showed, as compared with treated WT mice, significantly increased urinary bladder cell proliferation after 1 week of 2-AAF treatment (P = 0.05).

In the liver, treatment with 2-AAF in general resulted in a very small increase in cell proliferation, clearly distinct from the high proliferative response detected in the bladder (see Fig. 1, A and B). Still, however, 1 and/or 2 weeks of 2-AAF treatment resulted in a significant induction of hepatocellular proliferation. With respect to the different genotypes tested, 2-AAF-treated p53<sup>+/–</sup> and Xpa/ p53<sup>+/–</sup> mice showed the lowest proliferation induction as compared with WT mice. This difference was very small but still significant for 2 weeks-treated p53<sup>+/–</sup> mice (P = 0.006) and 1 and 2 weeks-treated Xpa/p53<sup>+/–</sup> mice (P = 0.01 for both 1 and 2 weeks of treatment).

**Effect of Short-Term 2-AAF Treatment on Apoptosis.** Next, we analyzed the apoptotic responses in urinary bladder and liver tissue of 2-AAF-treated mice by immunohistochemical detection of active caspase-3. The results of these studies are shown in Fig. 2. In the urinary bladder, no significant increase in apoptosis was found in 2-AAF-treated WT or p53<sup>+/–</sup> mice. This in contrast to Xpa and Xpa/p53<sup>+/–</sup> mice, which both showed significantly increased numbers of apoptotic cells upon 2-AAF treatment. When focusing on the differences in apoptosis between the genotypes, the absence of NER had a clear effect on 2-AAF-induced apoptosis, because 1 and 2 weeks of 2-AAF exposure resulted in significantly increased numbers of apoptotic cells in Xpa mice (P = 0.05 and P = 0.04, respectively). In

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Table 2 Detection of p53 protein in urinary bladder (A) and liver (B) tumors with CM5 and Pab240 antibody

Mice were treated with 2-AAF for 9 months. Urinary bladder and liver tumors were analyzed for p53 reactivity with the CM5 antibody as described in ‘Materials and Methods.’ The CM5 staining was scored either negative (-, <5%) or positive (+, 5-24%, + +, 25-75%, + + +, >75%). The fields that were positive for the CM5 antibody were scored either negative (-) or positive (+) for Pab240 antibody, which is indicated between parentheses.

### A. Urinary bladder tumors

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<td></td>
<td>−</td>
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<tr>
<td>WT</td>
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### B. Liver tumors

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* 2-AAF, 2-acrylamidophluorone; WT, wild-type; TCP, transitional cell papilloma; CS, carcinoma in situ; TCC, transitional cell carcinoma; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma.
general, the 2-AAF-induced response in Xpa/p53−/− mice was lower as compared with the response found in Xpa mice, but still, however, we observed a significantly increased apoptotic response in 12-weeks-treated double-knockout Xpa/p53−/− mice as compared with equally treated WT mice (P = 0.04).

In the liver, the number of apoptotic cells remained low upon 2-AAF treatment. In fact, the WT, p53+/−, and Xpa/p53+/− mice did not show any enhancement in the number of apoptotic cells after 1, 2, 4, or 12 weeks of 2-AAF treatment. In the NER-deficient Xpa mice, a slight increase in apoptotic cells was found; however, this increased response was not statistically significant.

LacZ Mutant Frequency Analyses. On the basis of the short-term studies, we conclude that the acute response of bladder and liver tissue to 2-AAF is clearly different as a high induction of cell proliferation and, especially in Xpa mice, p53-dependent apoptosis upon DNA damage was found in the urinary bladder, whereas these responses were not apparent in the liver. To examine whether the differences in acute cellular responses also result in differences in the frequency of gene mutations and to compare mutation frequency with eventual tumor outcome, we measured lacZ mutant levels in urinary bladder, liver, and spleen (negative control tissue) of WT, p53+/−, Xpa, and Xpa/p53−/− mice. These results are shown in Fig. 3A for the urinary bladder and Fig. 3B for the liver.

In the spleen, lacZ mutant frequencies were all low (between 5.4 and 14.3 × 10−5; data not shown). In both the urinary bladder and liver, the mean lacZ mutant frequency of the untreated mice was comparable in all of the genotypes tested (between 5.2 and 8.6 × 10−5). This indicates that the DNA repair and/or p53 status of the mice had no influence on spontaneous mutant frequencies occurring in these two tissues, at least not in mice of 3–4 months of age. 2-AAF treatment, however, resulted in a clear increase of lacZ mutants in both the urinary bladder and liver. The increase of 2-AAF-induced mutant frequencies was already significant after 4 weeks of treatment in all of the genotypes tested. Sustained treatment with 2-AAF resulted in higher lacZ mutant frequencies in both urinary bladder and liver.

Comparing the mutant frequency in the urinary bladder of 2-AAF-treated WT mice with the equally treated Xpa and/or p53+/−-deficient
mice revealed no significantly increased levels of lacZ mutants in the genotypes tested, with the exception of 12-weeks-treated Xpa/p53+/− mice (P = 0.03).

The lacZ mutant levels in liver tissue of 2-AAF treated mice (all genotypes) were significantly lower as compared with those found in the urinary bladder. Nevertheless, the repair status of the mice clearly influenced the level of lacZ mutants in the liver. All of the NER-deficient mice showed significantly increased numbers of lacZ mutants as compared with equally treated WT mice already after 4 weeks of treatment (P = 0.02 for Xpa and P = 0.03 for Xpa/p53+/− mice). This effect was even more pronounced in mice exposed to 2-AAF for a longer period of time (P < 0.005 for 8- and 12-week 2-AAF-treated Xpa mice and P = 0.007 and P < 0.005 for 8- and 12-week 2-AAF-treated Xpa/p53+/− mice, respectively).

Effect of Short-Term 2-AAF Treatment on p53+/− and Xpa/p53+/− Mice. In the previous section, we showed that 2-AAF-induced lacZ gene mutant frequencies in the urinary bladder are very high in all of the genotypes analyzed and, thus, do not clearly predict the tumor response found in the various mouse models upon prolonged 2-AAF exposure. Still, however, the highest levels were found in Xpa/p53+/− mice, also showing the highest tumor incidence. On the other hand, lacZ gene mutation induction in the liver reliably reflected the tumor susceptibility of Xpa mice upon 2-AAF treatment. Interestingly, as also found at the level of cell proliferation and apoptosis induction, no effect of p53 heterozygosity was found on the lacZ mutant frequency in the liver, in line with the low tumor response in p53+/− and Xpa/p53+/− mice. This could mean that the p53 protein does not play an important role in liver tumor suppression or that the remaining p53 allele in p53+/− or Xpa/p53+/− mice is still sufficient to do so. To discriminate between these two possibilities, we generated complete p53 knockout mice both in a NER-proficient (p53+/−) and -deficient (Xpa/p53−/−) background. Because we were not able to perform a carcinogenicity study due to the high spontaneous tumor susceptibility of these mice (as soon as 6 months), we only analyzed the acute effects of 2-AAF treatment.

First, as described in the former section, we determined acute 2-AAF-induced cellular responses in livers of p53+/− and Xpa/p53+/− mice. As expected, we did not observe any effect of complete absence of the p53 gene on apoptotic response (data not shown). Interestingly, however, analyses of cell proliferation revealed an increased labeling index in double-knockout Xpa/p53−/− mice, whereas p53+/− mice showed cell proliferation levels comparable with p53+/− or WT mice (Fig. 4A and Fig. 5 for a typical example). The lacZ mutant frequencies in the liver of Xpa/p53−/−...
mice increased dramatically upon 2-AAF exposure, exceeding levels of $200 \times 10^{-5}$ (Fig. 4B). This in clear contrast with $p53^{-/-}$ mice, showing a mutant frequency similar to those found in $p53^{+/+}$ and WT mice (Fig. 3). Apparently, in the liver NER is relatively efficient in the removal of 2-AAF-induced lesions in $p53^{-/-}$ mice. In contrast, when mice are both NER as well as $p53$ deficient, mutant frequencies increase dramatically, indicating that the $p53$ protein might also have tumor suppressive activity in the liver.

**DISCUSSION**

In the present study we exposed WT, $p53^{+/+}$, $Xpa$, and $Xpa/p53^{+/+}$ mice to 300 ppm 2-AAF, a bladder and liver carcinogen, to compare the relative contribution of a deficiency in NER, $p53$, or both on tumor development in two different tumor target tissues. Under the conditions used in this study, WT mice showed no clear tumor response in the liver or the urinary bladder. This finding is in line with previous reports showing that the development of 2-AAF-induced bladder and/or liver tumors takes longer than 39 weeks (40). In fact, sustained 2-AAF exposure would most likely have resulted in the development of both urinary bladder and liver tumors, because a clear induction of preneoplastic lesions was found in 2-AAF-treated WT mice (Table 1). Interestingly, NER-deficient $Xpa$ mice were clearly susceptible to develop hepatic tumors upon 2-AAF treatment, because a significant increased number of hepatocellular adenomas and carcinomas were found (Table 1). Surprisingly, these mice showed no significantly increased incidence of urinary bladder tumors. In contrast, $p53^{-/-}$ and $Xpa/p53^{+/+}$ mice were highly susceptible to develop urinary bladder tumors upon 2-AAF exposure (Fig. 1A), whereas the tumor response in the liver was similar to that observed in WT mice.

Thus, $p53$ heterozygosity results in a high predisposition to develop 2-AAF-induced urinary bladder tumor response in mice. Moreover, because $50\%$ of the urinary bladder tumors of both $p53^{-/-}$ and $Xpa/p53^{+/+}$ mice showed immunoreactivity with the PAb240 antibody (Table 2A), it is reasonable to assume that mutations in the remaining $p53$ allele are frequent events in bladder tumor development. In fact, mutant $p53$-positive fields were already found in atypical preneoplastic lesions, indicating that these presumptive $p53$ mutations occur at early stages of tumor development (data not shown).

Sequence analyses confirmed the $p53$ mutations detected by immunohistochemistry and revealed additional $p53$ gene mutations not detected by the antibodies used in this study (data to be published elsewhere). The conclusion that $p53$ plays an important role in urinary bladder tumor suppression has also been made by others (45–48), but in the present study we show for the first time in vivo that this is most likely because of the induction of $p53$-mediated death of cells with DNA damage by triggering apoptosis. We found a relatively high number of apoptotic cells in $Xpa$ mice, which was clearly reduced in $Xpa$ mice carrying only one functional $p53$ allele. This observation is in line with the in vitro finding that UV-induced lesions on actively transcribed genes constitute a strong signal for the apoptotic response as well as for $p53$ activation (21), suggesting that NER is involved in activation of apoptosis when DNA damage in the transcribed strand is encountered (22–26). Whether the signal for the apoptotic response is indeed given by stalled RNA polymerases at DNA lesions on the transcribed strand remains to be elucidated.

2-AAF treatment clearly induced cell proliferation in the urinary bladder. However, in contrast to apoptosis, $p53$-mediated cell cycle regulation seems to play a minor role in urinary bladder tumor suppression, because levels of cell proliferation were comparable in all of the genotypes tested and were not different from those found in WT mice. The high proliferative response might explain the relatively high levels of gene mutations found in the $lacZ$ marker gene. Interestingly, mutant frequencies of WT, $p53^{+/-}$, and $Xpa$ mice were within the same range, whereas the frequency in $Xpa/p53^{+/-}$ mice was significantly increased as compared with WT mice. This again indicates that $p53$-mediated apoptosis upon the presence of DNA damage might be the critical cellular response determining the levels of $(lacZ)$ gene mutations in the urinary bladder and ultimately tumor outcome after 2-AAF exposure.

As outlined before, NER-deficient $Xpa$ mice are clearly susceptible to develop 2-AAF-induced liver tumors preceded by increased frequency of $lacZ$ gene mutants, whereas $p53^{+/-}$ mice show no increased liver tumor response nor mutant frequencies. This is in line with the data obtained by Cheo et al. (49), who reported that NER-deficient $Xpc^{-/-}$ mice are also highly predisposed to develop liver cancer after i.p. treatment with either 2-AAF or its activated derivate NOH-AAF, whereas $p53$ heterozygosity did not influence the fre-
quency of liver tumors. In addition, no significant difference in the rate of hepatocellular adenoma or hepatocellular carcinoma development was found between WT and \( p53^{+/+} \) mice after exposure to numerous other chemical carcinogens (50, 51), indicating that \( p53^{+/+} \) mice in general are not liver tumor prone. We here show that this low tumor susceptibility of \( p53^{+/+} \) and \( Xpa/p53^{+/+} \) mice as compared with WT or \( Xpa \) mice, respectively, can be explained by the fact that the acute hepatocellular response (cell proliferation and apoptosis) to 2-AAF is not affected in \( p53 \) heterozygous mice. Moreover, hepatocytes completely deficient for \( p53 \) still show no increased cell proliferation nor lacZ gene mutations upon exposure to 2-AAF, indicating that 2-AAF-induced removal of DNA damage and cell cycle control in liver cells is very efficient and not dependent on \( p53 \). Interestingly however, mice completely deficient for \( p53 \) as well as NER (\( Xpa/p53^{-/-} \)) showed an enormously increased number of dividing hepatocytes and induction of lacZ gene mutations, in contrast to \( Xpa/p53^{+/+} \) mice. On the basis of these data, we hypothesize that the remaining \( p53 \) allele in \( Xpa/p53^{+/+} \) mice is sufficient to suppress 2-AAF-induced mutation induction and cell proliferation resulting in a phenotype comparable with \( Xpa \) mice, whereas complete absence of \( p53 \) in combination with a NER deficiency results in a high frequency of lacZ gene mutations and cell proliferation, presumably also resulting in a high cancer predisposition. One possible explanation for this finding could be that hepatocytes become polyploid, resulting in a higher copy number of \( p53 \) alleles per cell. This polyploidy might enhance the tumor suppressive capacity of \( p53 \) in liver cells as compared with diploid cells (such as found in the urinary bladder). Beyond the studies described here, this hypothesis could explain the generally low tumor response of \( p53^{+/+} \) mice when they are exposed to a wide variety of genotoxic carcinogens.\(^3\) In conclusion, the absence of 1 functional \( p53 \) allele in (NER-deficient) mice results in an increased sensitivity to develop 2-AAF-induced urinary bladder tumors, most likely due to decreased levels of \( p53 \)-dependent apoptosis. In addition, 2-AAF treatment results in mutations in the remaining \( p53 \) WT allele.

In line with these findings in mice, the \( p53 \) gene is one of the most frequently deleted genes in human urinary bladder tumors, with a frequency up to 70% in high-grade and/or invasive cases (52). Furthermore, \( p53 \) mutations have been found in up to 60% of urinary bladder tumors analyzed (53–55) indicating that \( p53 \) plays an important role in urinary bladder tumor suppression in humans as well. In contrast, human liver tumor development is not clearly correlated with \( p53 \) mutations, because only 20% of these tumors carry a \( p53 \) mutation (56). Above this, the mutations that have been identified (at codon 249) are specifically found in patients living in restricted areas. This is explained by the fact that the diet in these countries is often contaminated with aflatoxin B1, a compound that specifically hits codon 249 of the human \( p53 \) gene (4). Comparably, our studies with mice show that absence of one \( p53 \) allele did not result in altered acute cellular responses or in increased tumor susceptibility in the liver of 2-AAF-exposed mice. However, we clearly show for the first time \textit{in vivo} that the liver can become sensitive to a genotoxic compound in a \( p53 \)-dependent manner, but this effect becomes apparent when hepatocytes are completely devoid of both NER capacity and \( p53 \) protein activity. With the mouse models currently used, it is not possible to study the importance of our findings in terms of 2-AAF-induced liver tumor development, because \( p53 \) knockout mice are highly susceptible to develop spontaneous tumors (osteosarcomas, soft tissue sarcomas, and to a lesser extend lymphomas) at a very early age. Therefore, to enable the analysis of the effect of complete absence of both NER and \( p53 \) function on tumor development, it will be interesting to perform 2-AAF carcinogenicity studies in recently generated \( Xpa \) and \( p53 \) (57) conditional knock-out mice. These mice will be NER as well as \( p53 \) deficient exclusively in liver tissue upon crosses with liver-specific \( \alpha \)-AT-Cre-ER mice (58). If the increased mutant frequency in \( Xpa/p53^{-/-} \) mice is predictive for tumor development, one would expect an increased incidence of liver tumors in these mice.

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2-AAF-INDUCED CARCINOGENESIS IN p53+/− AND Xpa−/− MICE


p53 Heterozygosity Results in an Increased 2-Acetylaminofluorene-Induced Urinary Bladder but not Liver Tumor Response in DNA Repair-Deficient Xpa Mice

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