ABSTRACT

Mutations in nucleotide excision repair (NER) genes in humans result in the UV-induced skin cancer-prone disease xeroderma pigmentosum (XP). Mouse models that mimic XP have provided an informative experimental system with which to study DNA repair, as well as the molecular pathology of UV radiation-induced skin cancer. We reported previously that mice defective in the Xpc gene (Xpc+/–) are highly predisposed to UVB radiation-induced skin cancer and that the appearance of skin cancer is more rapid in Xpc Trp53 double mutants. Extended studies now demonstrate an increased predisposition to UVB radiation-induced skin cancers in Xpc heterozygous mice compared with normal mice. We also show that Xpc Trp53 double heterozygous mutants are more predisposed to skin cancer than Trp53 single heterozygous mice. No mutations were detected in the cDNA of the remaining Xpc allele, suggesting that haploinsufficiency of the Xpc gene may be operating and is a risk factor for UVB radiation-induced skin cancer in mice. Skin tumors from Xpc+/− mice were exclusively well or moderately well-differentiated squamous cell carcinomas. In Xpc+/− and Xpc+/+ mice, many of the squamous cell carcinomas were less well differentiated. We also documented previously increased predisposition to UV radiation-induced skin cancers in Xpc−/− Apex−/− mice. Here we show the absence of mutations in the cDNA of the remaining Apex allele, a further suggestive indication of haploinsufficiency and its resulting predisposition to skin cancer. The Trp53 and Apex heterozygous conditions altered the skin tumor spectrum to more poorly differentiated forms in all Xpc genotypes.

INTRODUCTION

Hereditary disorders that compromise cellular DNA repair pathways have severe consequences for human health (1). XP is a classic example of such a disease, with a primary defect in a DNA repair pathway called NER. The hallmark features of XP are dermatological and ophthalmic photosensitivity and a high incidence of sunlight-induced skin cancers of various types (2–4). Seven genetic complementation groups (XP-A→XP-G) and a variant form (XP-V) of the disease have been identified (1–4). In recent years, several mouse models of human XP gene defects have been generated by targeted germline mutation to study DNA repair, as well as the molecular pathology of cancer predisposition associated with defects in this DNA repair process.

In previous studies, we generated Xpc mutant mice and demonstrated that cells from such mice are deficient for NER (6). These mice are highly predisposed to UVB radiation-induced skin cancer (7) and to tumors of the liver and lungs after administration of the chemical carcinogen acetylaminofluorene (8). In both humans and mice, XPC protein is specifically required for NER of base damage in transcriptionally silent regions of the genome, as well as the nontranscribed strand of transcriptionally active genes (9, 10). The protein is not required for NER of the transcribed strand of transcriptionally active genes (9, 10). Recent studies (11) suggest that XPC protein complexed to HHR23B protein may play a specific role in the recognition of base damage during NER, but that when this process occurs in transcriptionally active regions of the genome, this role is subserved by some other moiety, perhaps the arrested transcription machinery.

The p53 (mouse Trp53) gene is one of the most commonly mutated genes in human cancers (12) and is a frequent target for mutation in UVB radiation-induced skin cancer (13). The p53 protein is integral to several cellular responses to DNA damage (12, 14, 15). After insult by chemical or physical agents that damage DNA, p53-dependent mechanisms are invoked that inhibit cell cycle progression and activate apoptosis (12, 14, 15). Additionally, several studies have suggested that p53 protein may directly modulate the activity of DNA repair pathways (16–18). To examine the consequences of a combined deficiency in NER and Trp53 gene function, we made genetic crosses between Xpc and Trp53 mutant strains and reported previously that the time of appearance of skin cancer in Xpc−/− mice is significantly reduced if they are additionally heterozygous or homozygous mutant for Trp53 (7, 19).

We also extended our investigations to include the role of defects in the BER pathway in UV radiation-induced carcinogenesis. Inactivation of the mouse AP-endonuclease (Apex; human HAPI = Ref-1) gene in embryonic stem cells results in embryonic lethality (20, 21). However, Apex−/− mice are viable. When crossed to Xpc−/− mice, the loss of a single Apex allele increased the predisposition of these animals to UV radiation-induced skin cancer (19). This effect apparently operates genetically through Trp53 function, because the Apex−/− state did not further increase the predisposition to skin cancer in either Xpc−/− Trp53−/− or Xpc−/− Trp53+/− mutant mice, i.e., the Apex−/− and Trp53−/− states are epistatic (19). Independent studies have demonstrated that human HAP-1 (Ref-1) protein is required for activation of p53 protein in vitro (22). Hence, in addition to its indispensable role in BER, HAP-1 (Ref-1) protein also influences p53-dependent processes. HAP-1 (Ref-1) protein is additionally essential for the redox-dependent activation of various transcription factors (23), possibly including p53 (22, 24, 25). In view of these multiple functions of the HAPI (Ref-1) protein, it is not surprising that ablation of the gene in mice results in early embryonic lethality (20, 21).

In the present studies, we document a comprehensive analysis of UVB radiation-induced skin cancer in Xpc−/− and Xpc+− mice and in various Xpc, Trp53, and Apex mutant combinations using larger cohorts of animals and longer periods of observation. We demonstrate the predisposition of Xpc−/− animals to UVB radiation-induced skin cancer more rapidly than previously reported (7).

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5 The abbreviations used are: XP, xeroderma pigmentosum; Xpc, xeroderma pigmentosum group C gene; NER, nucleotide excision repair; BER, base excision repair.

6 L. B. Meira and E. C. Friedberg, unpublished observations.
MATERIALS AND METHODS

Mice. Xpc and Apex mutant mice were generated previously (6, 19), and Trp53 mutant mice were purchased from The Jackson Induced Mutant Resource (Bar Harbor, ME). Mice heterozygous for both Xpc and Trp53 were bred as described (7), generating progeny with all nine possible combinations of normal and mutant alleles of these two genes. All mice were of identical strain background, comprising 75% 129/Sv and 25% C57Bl/6. Mice heterozygous for the Apex gene were bred to Xpc Trp53 mutant animals, generating progeny consisting of all possible 18 genotypic combinations of normal and mutant alleles of these three genes. These animals were also of identical strain background, comprising ~70% 129/Sv and ~30% C57Bl/6. Results obtained with animals originating from the two different crosses (Xpc × Trp53 or Xpc, Trp53 × Apex) were analyzed independently. All skin tumor incidence curves represent study and control animals from identical genetic backgrounds. Results were only pooled when background differences were found to have no effect on cancer incidence curves.

UV-induced Skin Cancer. The dorsal skin of mice, 8–12 weeks of age, was shaved and irradiated for 5 days/week at a dose rate of 120 J/m²/min for 14 min, using two FS20 erythemal (UVB) lamps (Phillips) filtered by Kodacel sheeting (Kodak, Rochester, NY). Mice were irradiated until either skin tumors were visible to the naked eye or for a maximum of 18 weeks. Skin tumors were biopsied, and a portion of each tumor was fixed in 10% neutral-buffered formalin and prepared for routine and special histology. Skin tumors were graded and classified by standard histopathological criteria.

RESULTS

Skin Cancer Predisposition in Xpc Heterozygous Mice. Consistent with previous studies using smaller cohorts of animals (7, 19), we observed that Xpc heterozygous mice are highly predisposed to UVB-induced skin cancer compared with normal mice (Fig. 1). In our earlier studies, we observed no significant differences in skin cancer predisposition in Xpc wild-type and heterozygous mutants monitored for 30 weeks after the onset of daily doses of UVB radiation (7, 19). We now show that when animals were monitored for longer periods, the latency time for the appearance of skin cancer was reduced in Xpc heterozygous mice compared with Xpc heterozygous litters. Xpc heterozygous mice manifested a 50% incidence of skin cancer by ~92 weeks after the onset of 18 weeks of daily UVB radiation (Fig. 1). In contrast, Xpc heterozygous mice suffered a 50% incidence at ~50 weeks after irradiation (Fig. 1).

We reported previously that the predisposition to skin cancer in Xpc heterozygous mice is further enhanced in both Trp53 and Trp53 animals (7, 19). We have now extended these studies to include Xpc heterozygous mice. As shown in Fig. 2, Xpc heterozygous mice are more cancer prone than wild-type or Xpc heterozygous litters. However, double heterozygous mutants (Xpc heterozygous mice) are even more cancer prone and manifest a 50% skin cancer incidence about 3 weeks earlier than Xpc heterozygous mice (Fig. 2), despite the fact that (as indicated above) increased cancer predisposition in Xpc heterozygous mice does not manifest until ~50 weeks after the onset of irradiation. One obvious explanation for the predisposition to skin cancer in Xpc heterozygous mice compared with appropriate controls is that the single remaining Xpc allele is frequently mutated as a result of exposure to UVB radiation. However, direct examination of Xpc cDNA identified mutations in the coding region of the Xpc gene in only 2 of 16 skin tumors examined. One of these, from an Xpc heterozygous animal, was a C→T transition at a dipyrimidine site in codon 724, which did not alter the encoded amino acid (leucine). The second mutation, from an Xpc heterozygous animal, was also a C→T transition at a dipyrimidine site. This mutation is expected to substitute leucine with proline at codon 319. Because proline is the amino acid encoded by the corresponding codon in the human XPC gene, we predict that this mutation is functionally silent in the mouse genome. Although we cannot formally exclude the (unlikely) possibility of mutations in the promoter regions of the single XPC gene in all these tumors or that epigenetic effects may have altered the expression of the gene, our results suggest that the increased predisposition to UVB radiation-induced skin cancer in Xpc heterozygous mice results from haploinsufficiency of the Xpc gene.

Effect of the Apex Genotype on UV Radiation-induced Skin Cancer in Xpc Mutant Mice. We reported previously (19) that as is the case with Xpc mice that are additionally heterozygous mutant
for Trp53, Xpc<sup>−/−</sup> mice that are heterozygous mutant for Apex are more skin cancer prone than Xpc<sup>−/−</sup> Apex<sup>+/+</sup> animals. This result is Trp53 dependent, because the effects of the Trp53 and Apex heterozygous states are epistatic, i.e., Xpc<sup>−/−</sup> mice that are double heterozygous (Trp53<sup>+/−</sup> Apex<sup>+/−</sup>) have the identical cancer predisposition as Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> Apex<sup>+/−</sup> mice (19). These results are now confirmed with larger cohorts of animals (Fig. 3). Sequence analysis of Apex cDNA from 12 skin tumors derived from Xpc<sup>−/−</sup> Apex<sup>+/−</sup> mice failed to reveal mutations in a single case. In contrast, we detected mutations in Trp53 in all cases (26). These observations lead us to the suggestion that, as is the case for the Apex heterozygous state, the effect of heterozygous deletion of the Apex gene reflects haploinsufficiency with respect to predisposition to UVB radiation-induced skin cancer.

Extended periods of observation revealed that mice that are heterozygous mutant for the Apex gene are not more cancer prone than wild-type animals (Fig. 4). The same is true for the comparison between Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> Apex<sup>+/−</sup> and Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> Apex<sup>+/−</sup> animals, and between Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> Apex<sup>+/+</sup> and Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> Apex<sup>+/−</sup> animals (Fig. 4). Indeed, in the latter case, the Apex<sup>+/−</sup> state actually protected older Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> animals from skin cancer. Hence, the skin cancer predisposition associated with the Apex<sup>+/−</sup> state only manifested when NER was defective (Xpc<sup>−/−</sup> state; Fig. 3). One possible explanation for this observation is that in the absence of NER, there is a critical requirement for BER of damaged pyrimidine mononucleotides of the type generated by exposure to UV radiation or to reactive oxygen species (e.g., thymine glycols; Ref. 1), and that haploinsufficiency of the Apex gene impairs BER of these photoproducts. This explanation is consistent with independent studies showing enhanced sensitivity to killing of cells from Apex<sup>+/−</sup> mice after exposure to agents that are known to promote oxidative damage to DNA. However, in light of the fact that the Apex protein also modulates the activity of a number of transcription factors and activates Trp53 protein by both redox-dependent and redox-independent mechanisms (22–25), other explanations are possible for the observation that the skin cancer predisposition associated with the Apex<sup>+/−</sup> state only manifests when NER is defective. The complex regulatory functions of Apex protein may also account for the curious paradox noted above that older Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> Apex<sup>+/−</sup> animals are less skin cancer prone than Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> Apex<sup>+/−</sup> controls (Fig. 4).

**Influence of Various Genotypes on Skin Tumor Pathology.** Skin tumors from Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> mice were exclusively well differentiated or moderately well differentiated (grade 1 or 2) squamous cell carcinomas (Fig. 5). In contrast, a significant number of the skin tumors from Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> and Xpc<sup>−/−</sup> Trp53<sup>+/−</sup> mice were poorly differentiated (grade 3–4) squamous cell carcinomas (Fig. 5). The observation that Xpc<sup>−/−</sup> mice are more predisposed to skin cancer but suffer a less aggressive form of the disease may relate to the observation that when these mice are exposed to an acute dose of UVB radiation, the epithelium of the skin rapidly undergoes marked thickening because of hyperplasia and hyperkeratosis (7). UVB radiation only penetrates ~70 μm of the skin (27). Hence, the increased epidermal thickening and limited penetration of the radiation may increase the fraction of well-differentiated target cells that are progenitors for skin cancer. In contrast, the absence of epidermal hyperplasia in Xpc<sup>+/+</sup> and Xpc<sup>−/−</sup> mice (7) may allow deeper penetration of the UVB radiation, resulting in skin cancers that derive from less...
well-differentiated progenitor cells of the basal layer of epidermis and/or from deeper skin appendage structures.

Heterozygous deletion of the \( Trp53 \) gene resulted in the appearance of more malignant tumors, including undifferentiated spindle cell tumors, sarcomas, and lymphomas (Fig. 6). Once again, in general carcinomas in \( Xpc^{-/-} \) mice were better differentiated than in \( Xpc^{+/+} \) and \( Xpc^{+/-} \) animals. However, in \( Xpc^{-/-} \) mice that were also \( Trp53^{-/-} \), a significant fraction of the tumors was poorly differentiated squamous cell carcinomas, undifferentiated spindle cell tumors, sarcomas, and lymphomas (Fig. 6). These observations are consistent with the increased epidermal dysplasia observed in \( Trp53 \) mutant mice after exposure to acute doses of UVB radiation (7). We observed several unusual skin tumors in \( Xpc^{-/-} \), \( Trp53^{-/-} \) mice, including acantholytic granular cell variants and tumors of dermal appendage structures. Skin cancer could not be examined in \( Trp53^{+/-} \) mice that were additionally \( Xpc^{-/-} \) or \( Xpc^{+/-} \) because the animals died of other \( Trp53^{-/-} \)-related tumors before they had time to manifest skin cancer.

Consistent with the notion that normal \( Trp53 \) function is dependent on the normal expression of the \( Apex \) gene (see above), histological analysis of tumors from \( Xpc^{-/-} \), \( Apex^{+/-} \) animals revealed the presence of less well-differentiated states compared with \( Xpc^{-/-} \), \( Apex^{+/-} \) controls (Fig. 7).

**DISCUSSION**

Mice defective in the genes \( Xpa \) and \( Xpc \) required for NER of DNA have proven to be reliable models for the human hereditary cancer-prone disease XP (28, 29). In our hands and those of others (30), \( Xpc \) mutant mice reflect the extreme predisposition of humans to UV radiation-induced skin cancer. Additionally, such mice are more prone to cancer of internal organs after exposure to chemical carcinogens such as acetylaminofluorene (8). The latter observation suggests that involvement of p53 in such repair has been provided by other studies (16, 17). The observation that \( Xpc^{-/-} \), \( Apex^{+/-} \) mice reveal the presence of less well-differentiated states compared with \( Xpc^{-/-} \), \( Apex^{+/-} \) controls (Fig. 7).

The present studies provide the first documented evidence in mammals of phenotypes associated with the \( Xpc \)-heterozygous state. \( Xpc^{+/-} \) mice are at a greater risk for UVB radiation-induced skin cancer than wild-type litter mate controls. Additionally, such mice show an increased risk of skin cancer when one allele of the \( Trp53 \) gene is inactivated compared with \( Trp53 \) heterozygous mutants alone. In both of these genotypic states, we found no evidence of mutations in the coding region of the remaining \( Xpc \) allele in skin tumors. Although we cannot exclude the possibility of mutations or epigenetic effects that altered expression of this allele, we are led to the notion that loss of one \( Xpc \) allele results in haploinsufficiency. On the basis of this conclusion, we suggest that it may be prudent to alert obligate XP heterozygous human individuals (of whom there may be as many as 2 to 3/1000 in the general population) of a possible increased risk for cancer associated with exposure to sunlight and to other known carcinogens. We are presently screening skin cancers from humans, especially older individuals, for heterozygosity in one or more \( XP \) genes.

Our previously published and present studies demonstrate additive effects with respect to skin cancer predisposition in mice defective in both NER and \( Trp53 \) functions. Conceivably, both parameters operate independently in promoting genomic instability in the presence of DNA damage. Alternatively, or additionally, inactivation of the \( Trp53 \) gene may compromise residual repair of the transcribed strand of transcriptionally active genes in \( Xpc \) mice. Direct evidence for the involvement of p53 in such repair has been provided by other studies (16, 17). The observation that \( Xpc^{-/-} \), \( Trp53^{-/-} \) mice are more cancer prone than \( Xpc^{-/-} \), \( Trp53^{+/-} \) animals suggests that in the latter group the remaining \( Trp53 \) allele is frequently inactivated in the tumors. Evidence that this is indeed the case is presented in an accompanying report, which also details the mutational spectrum in the \( Trp53 \) gene (26).

Mice that are defective in one \( Apex \) allele also manifest haploinsufficiency with respect to skin cancer predisposition. However, this is only observed in the presence of defective NER. On the basis of the results of studies in human and mouse cells, normal levels of \( Apex \) protein are apparently required for optimal activation of \( Trp53 \) protein (19, 22). Hence, the increased cancer predisposition observed in \( Xpc^{-/-} \), \( Apex^{+/-} \) mice may derive from reduced activity of \( Trp53 \) protein. Alternatively, defective NER associated with the \( Xpc^{-/-} \) state may require optimal levels of Apex protein for BER of specific photoproducts. Both brain cells and fibroblasts from \( Apex^{-/-} \) mice are abnormally sensitive to agents that cause oxidative damage to DNA.

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