Heterozygosity for the Mouse *Apex* Gene Results in Phenotypes Associated with Oxidative Stress


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**ABSTRACT**

Apurinic/apyrimidinic endonuclease is a key enzyme in the process of base excision repair, required for the repair of spontaneous base damage that arises as a result of oxidative damage to DNA. In mice, this endonuclease is coded by the *Apex* gene, disruption of which is incompatible with embryonic life. Here we confirm the embryonic lethality of *Apex*-null mice and report the phenotypic characterization of mice that are heterozygous mutants for the *Apex* gene (*Apex*+/–). We show that *Apex* heterozygous mutant cells and animals are abnormally sensitive to increased oxidative stress. Additionally, such animals manifest elevated levels of oxidative stress markers in serum, and we show that dietary supplementation with antioxidants restores these to normal levels. *Apex*+/– embryos and pups manifest reduced survival that can also be partially rescued by dietary supplementation with antioxidants. These results are consistent with a proposed role for this enzyme in protection against the deleterious effects of oxidative damage to DNA, allowing for the processing of free ends and subsequent repair synthesis and DNA ligation.

**INTRODUCTION**

ROS are highly reactive derivatives of molecular oxygen which are generated in aerobic cells by a variety of normal biochemical reactions, primarily mitochondrial oxidative metabolism (1–3). ROS can attack several cellular components, including lipids, proteins, and nucleic acids, and hence constitute a threat to cellular viability and to genomic stability. Eukaryotic cells have evolved specific defense mechanisms that counteract these potential problems, even in the face of continuous production of ROS. Principal among these are multiple classes of enzymes which convert ROS to reductive or unreactive metabolites. A second fundamental defense mechanism that mitigates against the deleterious effects of oxidative damage is the repair of DNA, allowing for the processing of free ends and subsequent repair synthesis and DNA ligation.

Mammalian cells contain a single major endonuclease encoded by a gene that has been variously designated as *Ref-1* or *APE* (12, 13). The gene that encodes the murine AP-endonuclease is called *Apex* (14). In addition to its absolute requirement for BER, the Apex protein has at least two other known functions. First, it is required for the redox activation of a number of spontaneously oxidized transcription factors (hence the designation *Ref-1*, for reducing factor), of which the Fos and Jun subunits are prime examples (15). Thus, oxidation of conserved cysteine residues in the DNA-binding domain of several transcription factors abolishes DNA binding. Apex/Ref-1 protein facilitates DNA binding of transcription factors by reducing such oxidized cysteine residues, thereby participating in a type of “protein repair.” In addition to this redox activity, recent studies have demonstrated that Apex protein is required for both the redox-dependent and independent activation of p53 in vitro (16). Consistent with this function, we reported previously that the predisposition to UV radiation-induced skin cancer in *Xpc* mutant mice, which are defective in nucleotide excision repair, is enhanced if these animals are heterozygous additionally for either *p53* or *Apex* (17, 18). The kinetics of cancer induction in *Xpc*−/− mice that are heterozygous additionally for both *p53* and *Apex* is indistinguishable from that in *Xpc*−/− animals heterozygous for just *p53*, suggesting that the enhanced predisposition to skin cancer in *Xpc*−/− *Apex*+/− animals results from loss of p53 activity, which in turn is dependent on normal Ref-1 activity.

Several attempts to generate a knockout mouse model for *Apex* have resulted in embryonic lethality (19, 20). In this study, we confirm this observation in independent experiments. However, we have observed distinct phenotypes in animals carrying a heterozygous mutation in the *Apex* gene. Specifically, we show that MEFs and specific cerebellar cells derived from *Apex*+/− animals are hypersensitive to redox-cycling drugs in vitro. We also observed a biased lethality of *Apex*+/− embryos in utero and in weaned pups and demonstrated that specific dietary manipulation of pregnant females with antioxidants rescues a fraction of this embryonic lethality. Finally, we observed significantly increased levels of serum...
markers of oxidative stress in Apex+/− animals compared with wild-type litter mates and showed that dietary supplementation with antioxidants restored these oxidative markers to normal levels.

MATERIALS AND METHODS

Generation of Apex Mutant Mice. A targeting vector was constructed that replaced the whole coding region of the Apex gene with the so-called β-geo selection cassette, which expresses a β-galactosidase-neo fusion protein (21). The promoter of the Apex gene was left intact and drives the expression of the positive selection marker. The replacement vector containing 3 kb of 5′- and 3.5 kb of 3′-flanking homology and a diphtheria toxin A gene cassette (for negative selection) was used to electroporate ES cells. After selection in G418-containing media, correctly targeted clones were extensively characterized by Southern hybridization and microinjected into C57Bl/6 blastocysts. Male germline chimeric mice representing four independently isolated ES cell clones were generated. Mice heterozygous for the mutant Apex allele were identified among the progeny of each of the chimeric founder mice crossed to both C57Bl/6 and 129.Sv females. All analyses were done with progeny resulting from two independent clones.

Table 1 Effect of vitamins E and C on genotypes of weaned pups from Apex heterozygote intercrosses

<table>
<thead>
<tr>
<th>Strain background</th>
<th>Diet</th>
<th>Inheritance</th>
<th>Genotype</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 % 129.Sv</td>
<td>Regular chow</td>
<td>Observed</td>
<td>47 (37%)</td>
<td>80 (63%)</td>
<td>0</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expected</td>
<td>(33.3%)</td>
<td>(66.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin-enriched diet</td>
<td>Observed</td>
<td>26 (33%)</td>
<td>52 (67%)</td>
<td>0</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expected</td>
<td>(33.3%)</td>
<td>(66.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 % 129.Sv/50% C57Bl/6</td>
<td>Regular chow</td>
<td>Observed</td>
<td>75 (40%)</td>
<td>114 (60%)</td>
<td>0</td>
<td>189</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expected</td>
<td>(33.3%)</td>
<td>(66.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin-enriched diet</td>
<td>Observed</td>
<td>11 (34%)</td>
<td>21 (66%)</td>
<td>0</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expected</td>
<td>(33.3%)</td>
<td>(66.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Apex heterozygote cells are hypersensitive to various redox-cycling drugs. The figure shows survival curves for Apex−/−(■) and Apex+/−(□) mouse embryo fibroblasts treated with menadione (A) or paraquat (B). C, the sensitivity of cerebellar granule cell cultures to treatment with menadione; photomicrograph of a representative field from wild-type and Apex−/−neuronal cultures treated with 50 μM menadione (×320). Bars, SE (n = 4 wells/treatment group). *P < 0.03 (ANOVA).
Diet. Vitamin E (dl-α-tocopherol acetate) supplemented (500 units/g of diet) and control chows were prepared by Harlan-Teklad (Madison, WI) on the basis of AIN-76A-purified diet. Vitamin E-enriched diet also contained trace amounts of selenium (as Na2SeO3 at 0.0005 g/kg of diet) to aid in the absorption of vitamin E. Vitamin C (Harlan-Teklad) was administered in the drinking water at a concentration of 1 g/liter. The mice had free access to food and water. The diet and vitamin C stock were stored at 4°C and replaced every 3–4 days. No overall differences in food consumption were noted between animals fed supplemented or control diet. No adverse effect of vitamin administration was noted in the treated animals.

Treatment of MEFs and Cerebellar Granule Cell Cultures with Genotoxic Agents. MEFs were obtained from E13.5 day embryos that were minced in tissue culture dishes containing DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were plated at a density of 5 × 104 cells/60-mm dish. Triplate plates were used for each dose. Paraquat dichloride (Sigma Chemical Co.) and menadione bisulfite (Sigma Chemical Co.) were dissolved in water and filter sterilized before use. Both drugs were prepared fresh before each experiment. Viability was scored by using the XTT Cell Proliferation kit (Roche), following the manufacturer’s instructions.

Cerebellar granule cell cultures (7 DIV) from wild-type and Apex heterozygote mice were treated with menadione for 20 h, the culture media removed and the cultures incubated for 10 min with fluorochrome (calcein-AM and propidium iodide) containing culture media. After incubation, fluorescently labeled cell cultures were photographed and cell viability determined by counting the total number of live (green) and dead (red) cells in fluorescent images taken from three random fields (approximately 200–300 cells/field) of each well.

Vitamin Rescue. Apex heterozygote female mice were either fed a specially formulated diet rich in vitamins E and C or normal chow (control diet). The vitamin-enriched diet was administered at least 2 weeks before mating. After these 2 weeks, Apex+/− females were crossed to Apex−/− male mice. The male mice used in the crosses were fed control diet. Females were either sacrificed between days 8.5 and 12.5 of gestation or allowed to carry gestation to term. No adverse effect of the diet was observed with respect to litter size. Total genomic DNA was prepared from dissected embryos or clipped tail from weaned pups for genotyping by PCR.

PCR Genotyping. A three-primer PCR strategy was developed to genotype animals or embryos generated by Apex+/− intercrosses. Genotyping of genomic DNA from embryos and tail clippings was performed using a common forward primer Apex401– 421 for 5′AGCGCGTTTCGCGAGCCCTGC, one reverse primer specific for the wild-type allele Apex652–651.rev 5′GGGTTCCTTCCCCGTCGTCGGC, and one reverse primer for the mutant allele ApexKO.rev 5′GCTGGCAGAAAGGGGATGTGC, located in the lacZ gene. The diagnostic fragment for the wild-type allele is 200 bp and for the mutant allele is 270 bp.

Measurement of Antioxidant and Oxidative Markers in Blood. Six- to 8-week-old Apex−/− animals, both males and females, were either fed the vitamin supplemented or the control diet. A minimum of 10 animals was used for each group. After 2 weeks of diet supplementation, animals were sacrificed by CO2 inhalation, and blood was collected in tubes containing EDTA as anticoagulant. Plasma levels of α-tocopherol were measured using reversed phase high-performance liquid chromatography as described previously (22). This was done to confirm that the vitamin E administered was also being absorbed by the body. Three markers of oxidative stress were also measured. These are plasma F2-isoprostanates, which are a direct in vivo measure of whole-body oxidative stress; protein carbonyls; and lipid peroxides, markers for protein and lipid oxidation, respectively. F2-isoprostane levels were measured in plasma samples that were frozen immediately after blood collection and separation. F2-isoprostanes were quantitated by ELISA, performed using reagents from Cayman Chemicals (Ann Arbor, MI), with 8-epi-PGF2α as standard. Plasma oxidation was measured at 0 and 4 h after incubation with 100 μM 2-2′ azo bis amido propane hydrochloride, an aqueous free radical initiator. The indices of oxidation include the measurement of protein carbon-
and lipid peroxides. The protein carbonyls were measured by ELISA developed in one of the laboratories involved in this study (23). Lipid peroxides were measured by the ferrous oxide xylenol orange method as described previously (24).

**Statistical Analyses.** All statistical analysis were carried out using Sigma Chemical Co. stat. Paired t tests were used to assess significance and Wilcoxon signed rank tests for nonparametric data, and the level of significance was set at P < 0.05.

**RESULTS AND DISCUSSION**

A targeting vector was constructed in which Apex sequences encompassing most of exon 2 and all of exons 3–5 were deleted. In essence, the entire coding region of the Apex gene was replaced by the β-geo cassette (21) representing a fusion between the β-galactosidase and neomycin resistance genes but containing no promoter sequences. The Apex promoter was left intact to drive expression of the selectable marker. The targeting vector was introduced into ES cells by electroporation, and cell clones resistant to G418 were screened by Southern analysis and injected into C57Bl/6 blastocysts. Four clones produced chimeric mice which transmitted the mutation to their offspring. The progeny were typed by PCR and genotyping E7.0 –E7.5 embryos derived from Apex heterozygote mutant animals. One of the animals had myocarditis, a second animals had pericarditis with atypical reactive elements, and a third displayed myocardial fibrosis. Reactive changes were found associated with inflammatory responses.

### Table 3 Histopathological analysis of deceased animals: evidence for an effect of the Apex heterozygous mutation on tumor incidence

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of animals</th>
<th>Normal (44%)</th>
<th>Hyperplasia (11%)</th>
<th>Reactive changes (22%)</th>
<th>Tumor (4 25%)</th>
<th>Heart affected (3 18.75%)</th>
<th>Other (2 12.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpc ^+/+</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apex ^+/+</td>
<td>16</td>
<td>7</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Xpc ^-/+</td>
<td>12</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apex ^-/+</td>
<td>21</td>
<td>11</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

* Reactive changes were found associated with inflammatory responses.
* One of the animals had myocarditis, a second animals had pericarditis with atypical reactive elements, and a third displayed myocardial fibrosis.
* In the wild-type animals, two cases of splenic hemosiderosis were observed; in Apex ^+/+ animals, one example of extramedullary hematopoiesis and one example of a hemangiomatous smooth muscle were noted; in Xpc ^-/+ animals, one example of splenic hemosiderosis and one example of extramedullary hematopoiesis were observed.

Fig. 3. Pathology of spontaneous tumors found in Apex heterozygote mutant animals. A, papillary adenocarcinoma found in the lung of an Apex ^+/− animal (×100). B, lymphoma in the lung of an Apex ^+/− mouse (×200).
and 2). This decrease was not statistically significant by the paired Student t test. However, we consider this to be an interesting trend and therefore investigated the possibility that might be reflective of increased oxidative stress in Apex+/− mice. In light of the relationship of the Apex protein to protection against the lethal effects of oxidative stress, we attempted to rescue embryonic lethality in Apex+/− mice by dietary modification in pregnant mothers. We fed an antioxidant-enriched diet to Apex+/− females 2 weeks before conception and throughout pregnancy. The antioxidant-enriched diet did not rescue lethality of Apex−/− animals and did not increase the life expectancy of mutant embryos, because at E7.5, we still could not detect Apex−/− animals (Table 2). However, as shown in Tables 1 and 2, dietary manipulation increased the number of viable heterozygous mutants in the pure 129.Sv genetic background and in mice of the mixed genetic background C57Bl/6 and 129.Sv (50% each), both for weaned pups and embryos. Regardless of the present statistical limitations, these observations suggest that a fraction of heterozygous Apex mutant embryos do indeed die as a result of oxidative stress.

In an effort to provide more direct evidence that the phenotypes associated with the Apex heterozygote state are the result of reduced levels of Apex protein, we measured the levels of several oxidative markers in the serum of wild-type and heterozygous mutants treated or not with vitamins E and C plus selenium. Levels of oxidative markers before and after dietary administration were essentially identical for males and females; hence, both data point sets were combined to increase statistical power. As shown in Fig. 2A, vitamin administration significantly increased the serum levels of α-tocopherol (the most potent isomer of vitamin E) in both Apex+/+ and Apex+/− animals. Apex+/+ animals showed significantly higher levels of lipid peroxidation (P < 0.01) and plasma F2 isoprostanes (P < 0.001) compared with wild-type controls (Fig. 2, B and C, respectively). Additionally, dietary manipulation lowered serum lipid peroxides and F2-isoprostanes to wild-type levels (Fig. 2, B and C, respectively). Dietary administration had a significant effect in lowering the levels of F2-isoprostanes (P < 0.05) and lipid peroxides (P < 0.01) in the serum of Apex+/− animals. The levels of a third measured oxidative marker, protein carbonyls, were not significantly different between Apex+/+ and Apex+/− animals with or without dietary treatment (data not shown).

Lipid peroxidation can be viewed as a degradative process arising as a consequence of the production and propagation of free radical reactions and has long been known to induce DNA damage (28, 29). It has been demonstrated previously that several agents that induce oxidative stress up-regulate the levels of DNA β-pol, another enzyme involved in BER (30). Ox-LDL is cytotoxic, and this toxicity has been related to DNA fragmentation and lipid peroxidation induced by Ox-LDL (31, 32). Recently, Chen et al. (33) demonstrated that treatment of mouse monocytes with Ox-LDL down-regulated BER activity and β-pol levels in whole-cell extracts and that treatment with the antioxidants ascorbate and α-tocopherol up-regulated BER and β-pol levels. Oxidative stress also results in up-regulation of the Apex gene (34). Hence, oxidative stress-mediated induction of Apex and β-pol can be considered an important mechanism for protection against genotoxic attack by ROS. Consistent with this model, the data presented here demonstrate that reduction in the levels of a BER enzyme can significantly increase oxidative stress and that antioxidant administration is effective in lowering levels of oxidation in vivo. Moreover, the role of the Apex protein as a redox-regulator of the activity of several transcription factors can also be an important determinant in the increased susceptibility of Apex+/− animals to oxidative stress. Limiting amounts of the Apex protein would be detrimental because less than optimal BER and protein repair activity would result in increased oxidative stress in cells. Indeed we have directly demonstrated increased levels of markers of oxidative stress in Apex+/− animals.

The increased susceptibility to oxidative stress does not seem to significantly decrease the life expectancy of Apex+/− animals kept under standard laboratory conditions (data not shown). Nor did we observe an obvious increase in cancers visible to the naked eye in such animals. However, detailed histopathological analysis of deceased Apex+/− animals and different control genotypes suggests that the Apex+/− state may indeed predispose to increased spontaneous carcinogenesis (Fig. 3 and Table 3). In wild-type animals and Xpc−/− animals, the frequency of spontaneous tumors varied between 0 and 5% (Table 3). In contrast, 25% of the Apex+/− animals examined developed microscopic tumors of one sort or another. The double mutant combination Xpc−/−/Apex+/− also showed an increased incidence of microscopic spontaneous tumors compared with the single Xpc mutant (16% versus 5%). The spontaneous tumors observed in Apex+/− animals were lymphomas (two cases), a single adenocarcinoma, and a sarcoma (Fig. 3). Interestingly, we also observed cardiac abnormalities in three Apex+/− mice but not in any other genotype (data not shown).

In conclusion, we report the phenotypic characterization of a mutant mouse strain which is a heterozygous mutant for the Apex gene. Our results indicate that decreased levels of the Apex protein associated with haploinsufficiency for the Apex gene leads to increased susceptibility to oxidative stress and suggests that Apex protein is important for protecting mammals from the deleterious effects of oxidative stress, including cancer. Future studies will examine phenotypes (including spontaneous cancer incidence) in Apex mutant mice treated with agents that promote oxidative stress and by breeding the Apex heterozygous state into mice that are mutant for other genes required for BER. The results of our present and proposed future studies may have important significance for cancer risk assessment in human populations carrying heterozygous mutations and/or polymorphisms in genes required for normal responses to oxidative stress in cells.

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REFERENCES

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