Enhanced tumorigenesis in p53 knockout mice exposed in utero to high-dose vitamin E

Connie S. Chen1 and Peter G. Wells1,2,*

1Faculty of Pharmacy and 2Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada, MSS 2S2

*To whom correspondence should be addressed. Tel: +416 978 3221; Fax: +416 267 7797; Email: pg.wells@utoronto.ca

The limited antioxidative capacity of the embryo and fetus may increase their risk for cancer initiation and/or promotion by reactive oxygen species (ROS)-mediated oxidative DNA damage and/or signaling. To determine if cancer can originate in utero, a high dietary dose of the antioxidant vitamin E (VE) (10% dl-α-tocopherol-acetate) was given to cancer-prone p53 knockout mice throughout pregnancy. Although reducing fetal death (P < 0.05), in utero exposure to VE enhanced postnatal tumorigenesis in both +/+ (P < 0.04) and --/- (P < 0.0008) p53-deficient offspring. VE did not alter maternal weights, offspring p53 genotypic distribution or tumor spectrum. Constitutive embryonic DNA oxidation in untreated --/- p53 embryos [gestational day (GD) 13] was higher than in +/- and +/- p53 littersmates (P < 0.05). VE reduced DNA oxidation in --/- p53 embryos (P < 0.05) without affecting +/- and +/- p53 littersmates. VE had contrasting, tissue-dependent effects on fetal (GD 19) DNA oxidation, with reductions in +/- and +/- p53-deficient fetal brains (P < 0.01), increases in skin (P < 0.05) and no effect in liver and thymus. The 250-fold increase in dietetic VE levels produced only 1.6–6.3-fold, tissue-dependent increases in tissue concentrations. The greatest increase, in fetal skin, correlated with increased DNA oxidation in that tissue in +/- and +/- p53-deficient fetuses and enhanced tumorigenesis in these genotypes. These results show that some cancers may originate in utero and the risk can be enhanced by embryonic and fetal exposure to high dietary levels of VE. The elevated DNA oxidation in some tissues of untreated --/- p53 offspring suggests that ROS may contribute to their higher baseline tumor incidence. The limited and tissue-dependent disposition of VE indicates substantial conceptual regulation. The similarly selective and contrasting effects of VE on DNA oxidation may contribute to its controversial protective efficacy and suggest that its effects on tumorigenesis are cell-specific, possibly in high doses involving a pro-oxidative mechanism.

Introduction

Overproduction or insufficient scavenging of reactive oxygen species (ROS) can result in enhanced oxidative stress and DNA damage, which in turn have been implicated in cancer initiation and promotion (1,2), as well as in teratogenesis (3,4), aging and other neurological diseases (5,6). ROS are by-products of endogenous cellular metabolism and the reduction–oxidation (redox) status of a cell can directly alter cell growth and development, proliferation and survival via signal transduction (reviewed in refs 7 and 8). In excess, ROS also can oxidatively damage DNA and other cellular macromolecules. 8-Oxo-2′-deoxyguanosine (8-oxo-dG) is one form of oxidative DNA damage that may initiate cancer by causing mutagenic GC to TA transversions and this macromolecular lesion is commonly used as a biomarker for oxidative stress and cancer risk (9–11).

The developing embryo and fetus may be particularly susceptible to the signal transducing and oxidatively damaging effects of ROS due to their relatively low levels of antioxidants (12) and limited capacity of antioxidative enzymes (3,13–17). In utero endogenous and drug-enhanced oxidative stress have been implicated in embryonic death and teratogenesis (3,4,14,17). Consequently, if ROS contribute to carcinogenesis, then the embryo and/or fetus may be at increased risk for cancer initiation and/or promotion due to increased ROS signaling and/or oxidative DNA damage.

In this study, we investigated the hypothesis that some cancers originate in the developing embryo and/or fetus, due in part to reduced conceptual antioxidative capacity and enhanced exposure to endogenous oxidative stress. There is evidence indicating that in utero exposures contribute to human cancer risk, including childhood cancers (reviewed in ref.18) and a recent study highlighted the association between birth weight, a marker for in utero growth, and prenatal exposure and breast cancer (19). Fetal irradiation and diethylstilbestrol exposure are known agents that initiate and promote childhood cancers and animal data constitute an important basis for the estimation of human risk due to in utero exposures to chemicals and carcinogens. However, relatively little is known about in utero influences on the development of spontaneous postnatal cancers in genetically predisposed animals. We hypothesized that alterations in the in utero redox environment could alter embryonic and/or fetal ROS-related pathways that contribute to the development of postnatal cancer. Our approach was to determine whether postnatal tumorigenesis could be modified by changes in the in utero redox environment produced by maternal treatment with the lipid soluble antioxidant vitamin E. The p53 knockout mouse was selected as the model because it has a high and p53 gene dose-dependent incidence of spontaneous tumorigenesis (20,21). In addition, the p53 knockout mouse exhibits increased susceptibility to the teratogenic effects of ROS-initiating teratogens like benzo[a]pyrene (22) and phenytoin (23), suggesting that ROS may contribute to the tumorigenic process in this strain. Vitamin E was chosen as the antioxidant because it can reduce the embryopathic effects of ROS-initiating teratogens like phenytoin (24,25). Also, there is some evidence in adult animals (26,27),

Abbreviations: GD, gestational day; 8-oxo-dG, 8-Oxo-2′-deoxyguanosine; ROS, reactive oxygen species; VE, vitamin E (dl-α-tocopherol acetate).

© The Author 2006. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org.
Materials and methods

Mice and diet

Heterozygous (+/−) p53-deficient female and male mice (4 weeks old) were purchased from Taconic (Germantown, NY). The animal study protocols were approved by the University of Toronto Animal Care Committee in accordance with the guidelines established by the Canadian Council on Animal Care. Animals were kept in plastic cages with ground corn cob bedding (Bed-O’Cobs Laboratory Animal Bedding; The Andersons Industrial Products Group, Maumee, OH), in a temperature-controlled room with a 12-h light–dark cycle automatically maintained. Tap water was provided ad libitum, as was the feed for the male mice. Females were placed on either a normal or 10% (w/w) vitamin E (VE; dl-α-tocopherol acetate)-supplemented diet (prepared by Ziegler Brothers, PA, USA) for 4 weeks prior to breeding. Food consumption was measured to be 6 g/day, which corresponds to ~2.4 and 600 IU of VE/day/adult mouse for the control (standard diet) and VE-supplemented diets, respectively. For a pregnant 35-g-mouse, the daily VE dose is ~17 000 IU/kg, which is ~1000 times higher than a human daily intake of 1000 IU/day. To achieve comparable plasma xenobiotic concentrations in mice and humans, the mice usually must receive a 10-fold higher mg/kg dose, so our murine embryonic and fetal DNA oxidation were determined, respectively, during organogenesis and the last trimester. Our results show that maternal exposure to a high dietary dose of vitamin E during pregnancy, while reducing in utero fetal death, can enhance the onset of spontaneous postnatal tumorigenesis in the offspring, indicating the potential for in utero origins of some cancers and the hazard of excessive doses of vitamin E.

Breeding

Breeding entailed housing one (+/−) p53-deficient male mouse with three (+/−) p53-deficient females overnight between 17:00 and 09:00 h. This heterozygous breeding resulted in littersmates with the expected ratio of 1:2:1 for the +/+; +/− and −/− genotypes. Pregnancy was ascertained in the morning at 09:00 h by the presence of a vaginal plug and this time was designated as gestational day (GD) 1.

For studies of embryonic and fetal oxidative DNA damage, maternal VE supplementation was continued throughout breeding and pregnancy, until GD 13 or 19, when the dams were killed at 09:00 h and the embryos and fetuses were analyzed for DNA oxidation. For the studies assessing postnatal tumor development, maternal VE supplementation was continued until the day of birth. All offspring were p53-genotyped.

Chemicals

Chemicals were HPLC grade where available and otherwise were reagent grade. Protease K was from Roche Applied Science (Laval, QC). Chloroform/isoamyl alcohol phenol (CIP) (24:1:25, v/v/v) was purchased from Gibico Laboratories (Toronto, ON). Ribonuclease T1, ribonuclease A, nuclease P1 and Escherichia coli alkaline phosphatase were purchased from Sigma-Aldrich Canada (Oakville, ON). 8-OH-2′dG was purchased from Cayman Chemicals (ON, Canada) and DNeasy extractor kits and PCR reagents for genotyping were obtained from Qiagen (Mississauga, ON). A grunt was set as described above. The yolk sac was separated from each embryo, or a tail snip was taken from each fetus, to determine the genotype. Each tail or yolk sac was digested and DNA extracted following the manufacturer’s recommended protocol (DNeasy Tissue Extraction Kit, Qiagen). Extracted DNA was then genotyped by assaying for the wild-type p53 and the p53 knockout construct using PCR. The 3 primer sequences (provided by Taconic Biotechnology, Germantown, NY) were: p53-ESR (5′-AGC TCA CGA CCT CCG TC-3′) (reverse primer along Exon 5), p53-14P (5′-ACA CAC CCT TAG CTC CAG CAC –3′) (forward primer along Intron 4) and Opt-21 (5′-GTG TTC CGG TCT CTA CGA CT-3′) (aligns with Neomycin resistance deletion allele) (ACGT, Toronto, ON). Samples were amplified by adding ~200 ng of sample DNA template to a 50 μl PCR reaction mixture containing 1× PCR buffer (Qiagen), 2.0 mM MgCl2, 0.2 mM dNTP mix (PE Applied Biosystems, Foster City, CA), 1 μM of the p53-5′R primer, 0.5 μM of the p53-4P and Opt-21 primers each and ~1.75 U of HotStart Taq Polymerase (Qiagen). Cycling conditions using the Mastercycler (Eppendorf Canada, Mississauga, ON) were: 95 °C for 15 min; 35 cycles of 94 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min; and 72 °C for 5 min. Added to the program was a 4 °C ‘forever’ final step to ensure samples would be kept at 4 °C once the PCR was complete until samples could be examined. The reaction products were visualized using ethidium bromide fluorescence after electrophoresis in a 1% agarose gel in 1× TAE (Tris acetate EDTA buffer), which were run at constant 100 V for 1 h. PCR product fragment bands signifying the wild-type p53 gene were of 520 bp in size and the p53 knockout fragment/ band was of 730 bp. Digital images were captured using a digital camera (Kodak) and analyzed using commercial imaging software (Kodak).

DNA isolation for HPLC-EC analysis

Pregnant dams were killed on GD 13 or 19 at 09:00 h by cervical dislocation and embryos or fetuses, respectively were explanted. Embryos and/or fetuses were randomly selected from at least three separate litters, to account for random inter-litter differences. Embryos (GD 13) were exteriorized with their yolk sac, which was later separated for analysis of genotype. Fetuses (GD 19) were exteriorized separately from their yolk sac, the brain, liver and skin were removed for DNA oxidation analysis and a tail snip was taken for genotyping. Embryos or fetal brain or liver were snap-frozen in liquid nitrogen and stored at −80 °C for later analysis.

DNA extraction

DNA from embryonic and fetal tissue was isolated by a modification of the method of Gupta (40). Briefly, frozen embryonic or fetal tissues were homogenized using a handheld homogenizer (IKA, Wilmington, NC) in 500 μl of DNA digestion buffer [100 ml of 1 M Tris (pH 8.0), 10 ml of 0.5 M EDTA (pH 8.0), 2 g of sodium lauryl sulfate and 11.68 g of NaCl; volume adjusted to 1000 ml with water]. Homogenates were incubated overnight at 55 °C with proteinase K (500 μg/ml). DNA was extracted with 1 vol of CIP and micro-centrifugation at 14 000 g for 1 min (centrifuge model 5415 D, Eppendorf). The DNA was precipitated by addition of 1.5 vol (250 μl) of ammonium acetate 7.5 M and 2 vol (1 ml) of pre-cooled 100% ethanol and pelletted by micro-centrifugation for 1 min. The supernatant was discarded and the pellet was washed with 2 vol (1 ml) of pre-cooled 70% ethanol. The ethanol was discarded and the remaining pellet was dried under nitrogen gas (BOC Gases, Mississauga, ON), dissolved in 1 ml of ammonium acetate 7.5 M and 2 vol (1 ml) of pre-cooled 100% ethanol and pelletted by micro-centrifugation for 1 min. The supernatant was discarded and the pellet washed with 2 vol (1 ml) of water. DNA was quantified using a UV-visible spectrophotometer (model DU 640, Beckman Instruments, Mississauga, ON).

1359
DNA was then digested to nucleotides by a 30 min incubation at 37°C with nuclease P1 (67 mg/ml), followed by a 60 min incubation with E.coli alkaline phosphatase (0.37 U/ml) at 37°C. The resulting deoxynucleoside mixture was filtered (0.22 μm pore size, 13 mm diameter filter) and analyzed by high-performance liquid chromatography (HPLC) with electrochemical (EC) detection.

Analysis of DNA oxidation

The formation of 8-oxo-dG was measured using an HPLC system (Series, 200, PerkinElmer Canada, ON) including a data workstation with peak analysis software (Turbochrom software package, PerkinElmer), a reverse-phase (octadecyl silane) ODS 2 column (4.6 × 50 mm, 5 μ) (Exsil, Chromatographic Specialties, ON) and an electrochemical detector (model Coulchem II; ESA, Chelmsford, MA) with a guard cell (model 5020; ESA) operating at +450 mV and an analytical cell (model 5010; ESA). Samples were injected using a Peltier-cooled autosampler (Series 200, PerkinElmer) and eluted using a mobile phase consisting of 50 mM KH2PO4 buffer (pH 5.5) and methanol (95:5, v/v), at a flow rate of 0.8 ml/min and an oxidation potential window of +100 to +400 mV (41).

Histopathology

p53-deficient offspring and wild-type controls were followed for up to 19 months, or until they demonstrated signs of being moribund due to tumor growth and illness, when they were killed by cervical dislocation. Representative samples of tumors and abnormal tissue were taken and fixed in 10% neutral formalin (Sigma) at 4°C for at least one night. Paraffin sections (5 μm) were stained with hematoxylin-eosin and tumor lesions were graded in a blinded fashion by a veterinary pathologist.

Statistical analysis

The statistical significance of differences between treatment groups for each genotype was determined using a standard computerized statistical program (PRISM, GraphPad, CA). Survival curves were compared using PRISM and means for the level of DNA oxidation were compared using the Student’s t-test and ANOVA. The minimal level of significance used throughout was P < 0.05.

Results

Tumorigenicity

Offspring of p53 dams exposed in utero to dietary VE were observed postnatally for the onset of tumor development over 19 months, a measure of carcinogenesis commonly used with this model (20,21). Compared with offspring from dams fed with control diet, in utero exposure to 10% VE enhanced spontaneous tumor formation in both +/− p53 VE (35) and controls (42), +/− VE (65) and controls (62), +/− p53 VE (32) and controls (40). Inset: Median survival of p53-deficient offspring exposed in utero to dietary VE supplementation. VE exposure reduced the median survival age in −/− p53 offspring by 21% and in +/− p53 offspring by 9%, compared with their respective genotypically identical control littersmates ( Asterisks (*) indicate P < 0.05).

Histopathology

p53-deficient offspring and wild-type controls were followed for up to 19 months, or until they demonstrated signs of being moribund due to tumor growth and illness, when they were killed by cervical dislocation. Representative samples of tumors and abnormal tissue were taken and fixed in 10% neutral formalin (Sigma) at 4°C for at least one night. Paraffin sections (5 μm) were stained with hematoxylin-eosin and tumor lesions were graded in a blinded fashion by a veterinary pathologist.

Statistical analysis

The statistical significance of differences between treatment groups for each genotype was determined using a standard computerized statistical program (PRISM, GraphPad, CA). Survival curves were compared using PRISM and means for the level of DNA oxidation were compared using the Student’s t-test and ANOVA. The minimal level of significance used throughout was P < 0.05.
During organogenesis (GD 13), the endogenous level of embryonic oxidative DNA damage in the skin of /-/- p53 fetuses by 42% and 91%, respectively (P < 0.05), compared with their respective genotypically matched controls, without affecting +/- littermates (Figure 4C).

In the skin, VE exposed /-/- p53-deficient and +/- p53-deficient offspring also had respectively 38% and 41% higher levels of DNA oxidation compared with their +/- p53 littermates (P < 0.05), while there were no genotypic differences among fetuses exposed to the control diet (Figure 4C). In the thymus, among fetuses exposed to the control diet, null p53 fetuses had 1.6 and 2.1-fold higher baseline levels of DNA oxidation compared with their +/- and +/- p53 littermates (P < 0.01) (Figure 4D). Similarly, VE enhanced DNA oxidation by 30% in the thymus of null p53 fetuses compared with +/- p53-deficient littermates (P < 0.05).

VE concentrations in embryonic and fetal tissues

Embryos. During organogenesis (GD 13), the embryonic genotype did not influence the concentration of VE in embryonic tissue with either the control or VE-supplemented diets. However, in utero exposure to 10% VE elevated the embryonic tissue concentration of α-tocopherol 2.0- and 4.4-fold in +/- and +/- p53 littermates, respectively compared with genotypically matched embryos exposed to the control diet (P < 0.001) (Figure 5, upper panel). The apparent 51% increase in VE-supplemented p53 /-/- embryos was not statistically significant (0.05 < P < 0.1).

VE exposure reduced endogenous DNA oxidation in p53 null embryos by 36%, down to the level exhibited by the +/- and +/- littermates on control diet (P < 0.05). VE did not alter DNA oxidation in the +/- and +/- embryos.

Fetus. Fetuses at GD 19 exhibited a tissue-dependent, differential response to in utero VE exposure (Figure 4). VE exposure reduced DNA oxidation in the brain of /-/- and +/- p53-deficient fetuses by 42% and 47%, respectively, compared with their littermates with the same respective genotype on the control diet (P < 0.01), without significantly affecting +/- littermates (Figure 4A). VE had no effect in fetal liver (Figure 4B) or thymus (Figure 4D). In contrast, VE enhanced oxidative DNA damage in the skin of /-/- and +/- p53 fetuses by 42% and 91%, respectively (P < 0.05), compared with their respective genotypically matched controls, without affecting +/- littermates (Figure 4C).

With the control diet, the baseline concentration of VE in p53 /-/- embryos was significantly higher than that in all p53 /-/- fetal tissues, including 1.9-fold higher in fetal brain (P < 0.05) and 2.2-fold higher in fetal liver (P < 0.01). The baseline VE concentration in p53 /-/- embryonic tissue also was 3.2- and 2.7-fold higher than that in the skin of untreated /-/- and +/- p53 fetuses, respectively (P < 0.05).

Fetal tissues. In utero VE exposure elevated the concentration of VE in all fetal tissues examined on GD 19 (Figure 5, lower 3 panels), with moderate increases in the fetal brain and liver and substantial increases in the fetal skin. Increases in VE
concentrations in the fetal brain ranged from a 1.6-fold increase in \(+/−p53\) fetuses to a 2.4-fold increase in \(+/−p53\) littermates \((P < 0.01)\) (Figure 5A) and in fetal liver from 1.8- and 1.7-fold in \(−/−\) and \(+/−p53\) fetuses, respectively \((P < 0.05)\) (Figure 5B). The greatest elevation in VE concentration was observed in fetal skin, ranging from a 3.5-fold increase in \(+/−p53\) \((P < 0.01)\) fetuses up to as high as a 6.3-fold increase in the fetal skin of \(−/−p53\) littermates \((P < 0.01)\) (Figure 5C).

**Effects of gender**

Gender did not measurably affect tumor incidence or survival rate in \(null\) and heterozygous \(p53\) offspring in either control or VE-supplemented groups. However, among \(p53\) \(null\) offspring, the ratio of males to females was 2:1 in both control and VE-supplemented groups, suggesting a loss of \(−/−\)-fetuses due to in utero death.

**Resorption incidence**

VE dramatically reduced the resorption incidence assessed on GD 13 by 87% and on GD 19 by 59%, compared with dams fed the control diet \((P < 0.02)\) (Figure 6).

**Maternal weight**

There was no difference in maternal weight between the dams fed the control or VE-supplemented diet, whether measured at GD 13 or 19, or at the time of delivery (Figure 7).

**Offspring genotype**

VE had no effect on the number of pups within any \(p53\) genotype and an approximate Mendelian distribution was observed. The mean percentages of \(−/−p53\) pups per litter were 30.7 ± 18.9% \((± SD)\) versus 29.8 ± 21.2% in the control and VE groups, respectively. The mean percentages of pups in the control and VE groups for \(+/−p53\) and \(+/+/p53\) pups were 43.0 ± 18.4 versus 47.8 ± 20.6% and 26.8 ± 16.8 versus 22.4 ± 13.3%, respectively.

**Discussion**

It is thought that excessive oxidative damage to cellular macromolecules may play an important role in the initiation and promotion of carcinogenesis, based in part upon studies of endogenous and/or xenobiotic-enhanced levels of oxidative damage to lipids, proteins and DNA, on spontaneous mutation rates and cancer incidence \((1,2,5,10,42–44)\). Since the developing embryo and fetus have limited antioxidative protection against ROS-mediated signal transduction and oxidative DNA damage, which potentially are involved in carcinogenesis, we hypothesized that some spontaneous cancers may originate in utero. Antioxidants are widely believed to facilitate the maintenance of human health by decreasing or preventing oxidative damage to key cellular macromolecules and thereby may delay or prevent the onset of cancer. The enhancement in the onset of spontaneous postnatal tumorigenesis observed herein in both \(+/−\) and \(−/−\)-p53-deficient offspring by maternal treatment prior to and during pregnancy with a high dietary dose of the antioxidant VE shows that some cancers can originate in the conceptus prior to birth and that tumorigenic risk can be altered by modulating the in utero environment.

The high dietary dose of VE \((10\%)\) was chosen to explore the upper range of the potentially protective antioxidant dose–response curve, which mouse models of teratogenesis...
suggested might be considerably higher than the doses used in published carcinogenesis studies of non-pregnant animals. Furthermore, evidence is accumulating that the optimum recommended daily intake may need to be higher (1,45,46). In the study herein, the protective effect against ROS-mediated embryopathies was evidenced by the dramatic reduction in in utero fetal death (resorptions) in VE-treated dams. Similarly at the molecular level, the reduction by high-dose VE in oxidative DNA damage in the brains of p53-deficient fetuses suggests that VE may prove to be protective against ROS-mediated neurodevelopmental deficits. However, the contrasting observation that the onset of postnatal tumorigenesis was enhanced rather than inhibited by high-dose VE shows that antioxidative treatment at the upper range of the dose–response curve in pregnant animals has a modulatory effect on carcinogenesis opposite to that on teratogenesis and demonstrates the difficulty in rationalizing optimal recommended intakes during pregnancy.

Fig. 5. Concentration of VE in embryonic and fetal tissues of p53 offspring exposed in utero to all-rac-α-tocopherol-acetate. Pregnant dams treated with either control or 10% VE diet were killed on GDs 13 or 19 and the concentrations of VE were assessed in explanted embryos or fetal tissues. (n) = number of embryos or fetuses analyzed. GD 13 embryos: treated pregnant dams were killed on GD 13 and VE concentrations were assessed in explanted embryos. Alpha symbols significantly different than control littermates of the same genotype (P < 0.05). GD 19 fetal tissues: treated pregnant dams were killed on GD 19 and VE concentrations were assessed in fetal tissues. (A), in fetal brain; (B), in fetal liver, (C), in fetal skin. Alpha symbols significantly different from control littermates of the same genotype (P < 0.05).

Fig. 6. Effect of dietary VE on in utero death (resorptions) during the embryonic (GD 13) and fetal (GD 19) periods of development. Dams received control or VE-supplemented diets as described in Figure 1. The alpha and beta symbols indicate differences in VE-exposed animals on GDs 13 (P < 0.002) and 19 (P < 0.02) compared with their respective gestationally matched controls.

Fig. 7. Effect of dietary VE on maternal weight during the embryonic (GD 13) and fetal (GD 19) periods of in utero development. Dams received control or VE-supplemented diets as described in Figure 1. The number of dams is given in parentheses.
Dietary content and quantity and in particular the amount of polyunsaturated fat intake, exhibits a close correlation with tumorigenicity (47,48). Conversely, caloric restriction in the diet typically reduces cancer in animal models (49–51) and similar trends have been noted in humans (52,53). In our studies, the maternal weight and fetal birth weight did not differ between the control and VE-supplemented groups, which indicates that the exacerbation of tumorigenesis by VE cannot be attributed to differences in caloric intake or fat content between the two treatment groups. Interestingly, with higher polyunsaturated fatty diets, a higher intake of VE is recommended to prevent the oxidative stress resulting from lipid peroxidation (54). Similarly, the observed lower incidence of female offspring was similar in both controls and VE-supplemented offspring, indicating that the effect may be due in part to the gender- and gene dose-dependent exencephaly and in utero death observed for p53 null females (22,55). However, the latter effect appears to have been negligible, since VE did not alter the Mendelian distribution of p53 genotypes in the offspring.

The level of oxidative stress in the conceptus was examined at the embryonic and fetal stages to investigate whether endogenous levels of DNA oxidation could explain the differences in tumorigenicity among p53 genotypes and if the enhanced tumorigenic effects seen with in utero dietary VE exposure could be explained by changes in oxidative DNA damage. Two key time-points were investigated, since the earlier time-point, GD 13, falls within the critical embryonic period of organogenesis, while the later GD 19 falls within the fetal period when the conceptus is most susceptible to common transplacental carcinogens that chemically induce postnatal tumorigenesis (reviewed in ref. 18). The higher embryonic level of endogenous DNA oxidation in untreated −/− p53-deficient embryos on GD 13 is consistent with their enhanced postnatal tumorigenic risk compared with heterozygote and wild-type littersmates. At this gestational time, embryos lacking p53 appear to be more susceptible to oxidative damage at least to DNA and probably to other cellular macromolecules, potentially due in part to deficient DNA repair, which would be consistent with studies showing an embryoprotective role for p53 against DNA-damaging teratogens (22,23,57,58). This deleterious effect may be further enhanced because embryos at this early developmental stage lack most of the antioxidant enzymes and antioxidants that increase near the end of gestation and protect the fetus (13,59). However, since the endogenous level of oxidative DNA damage did not differ between the heterozygotes and their wild-type littersmates, this mechanism cannot entirely explain the postnatal susceptibility of untreated p53-deficient mice to tumorigenesis, suggesting that redox effects later in the last trimester of gestation may contribute to the carcinogenic process. Nevertheless, the expression of p53 declines in most tissues after organogenesis (60–62), so an embryonic role for the p53-dependent nature of postnatal tumorigenesis observed in this study cannot be entirely excluded.

In the embryo, although the VE-supplemented diet elevated α-tocopherol levels in embryonic tissues, the associated reduction by VE in DNA oxidation in −/− p53-deficient embryos on GD 13 is not consistent with the enhancement in tumorigenicity in this genotype, suggesting that embryonic redox alterations do not contribute to this effect, unless a reduction in ROS-mediated signal transduction is involved. The latter possibility would not be consistent with the absence of a similar VE-dependent reduction in DNA oxidation in p53 +/− littersmates, since this genotype also exhibited VE-enhanced tumorigenesis. Conversely, the VE-dependent reduction in embryonic oxidative DNA damage suggests that the resorption incidence and its substantial reduction by VE on GD 13 may involve ROS-mediated oxidative stress, consistent with other spontaneous and drug-enhanced embryopathies (3,14).

In the fetus, the contrasting, tissue-dependent levels of DNA oxidation and their alteration by VE precluded a straightforward interpretation of the potential contribution of in utero ROS to the enhanced postnatal tumorigenesis. On one hand, the consistent elevation of VE tissue levels by dietary supplementation with high-dose VE and the subsequent enhancement of oxidative DNA damage in the skin of −/− and +/+ p53-deficient fetuses is consistent with a tissue-dependent paradoxical pro-oxidant mechanism, suggesting that enhanced DNA oxidation contributes to tumorigenicity. On the other hand, while VE supplementation also raised α-tocopherol levels in the fetal brain and liver, the absence of any VE-dependent effect on DNA oxidation in fetal liver or thymus, or the contrasting VE-dependent reduction in DNA oxidation in the brain of −/− and +/+ p53-deficient fetuses, is not consistent with a ROS-mediated mechanism contributing to a fetal origin of tumorigenicity. The decrease in resorptions caused by high-dose VE also may have resulted in more fetuses with increased tumor susceptibility due to primary determinants such as oncogene activation and/or secondary determinants such as compromised DNA repair and decreased immune surveillance.

One potential mechanism for the paradoxical enhancement in tumorigenicity by high-dose VE involves a pro-oxidant effect, suggested by in vitro studies where, in the presence of metals such as copper (II), VE increased oxidative effects (34,63). This is similar to the pro-oxidant effect postulated for the water soluble antioxidant vitamin C based on in vitro studies (64). In utero exposure to high-dose dietary VE did not enhance DNA oxidation in null or +/+ p53-deficient embryos on GD 13, nor in fetal brain, thymus or liver on GD 19, indicating that the tumorigenic enhancement by VE cannot be fully explained by a pro-oxidant effect as postulated for high doses of antioxidants like vitamin C. However, the increased oxidative DNA damage in −/− and +/+ p53-deficient fetal skin tissue following high-dose VE exposure, which constitutes the first in vivo evidence for this phenomenon, may reflect a tissue- and/or cell-specific pro-oxidant effect that contributed to enhanced tumorigenicity.

In contrast to fetal liver and skin, the reduction of oxidative DNA damage in the brain of p53 −/− and +/+ fetuses does suggest a potential benefit of high-dose VE in protecting against ROS-mediated neurodevelopmental deficits, as was observed herein for VE protection against in utero fetal death (resorptions). If similarly selective molecular effects are observed in adults, it may be that higher VE doses are necessary for optimal antioxidative protection in the brain, which might explain in part the conflicting results reported for VE efficacy in reducing ROS-mediated neurodegeneration. The contrasting results for oxidative DNA damage in the fetus reveal a tissue and possibly cellular specificity in the ontogeny and molecular consequences of oxidative stress. This tissue specificity may also be due in part to differential gestational development of antioxidative enzymes, with relatively higher hepatic levels. In the case of fetal skin, the similarity in baseline levels of DNA oxidation compared with constitutive levels...
in other fetal tissues may reflect a marginally sufficient antioxidative capacity in skin that is readily overwhelmed in p53-deficient fetuses by high-dose VE, resulting in substantially enhanced oxidative DNA damage. Different organs exhibit highly variable levels of endogenous DNA oxidation and hence likely respond differently to VE. Since p53 knockout mice are not prone to developing brain tumors (20,21), this model may not be appropriate for assessing the possible relationship of in utero oxidative stress and brain tumors despite the protective effects of VE in reducing DNA oxidation in the brain of p53-deficient fetuses. However, this model might prove relevant for evaluating ROS-mediated neurodevelopmental deficits. In any event, higher levels of oxidative stress in a particular tissue, or cell type, may be necessary but not sufficient for tissue-specific tumorigenicity and may even be required (65) to maintain normal growth and development in that tissue.

In addition to oxidatively damaging cellular macromolecules, ROS may participate in the initiation and execution of apoptosis (66,67), as well as regulating transcriptional activity (68,69). Pro-apoptotic activity has been observed in transformed fibroblast cells after depletion of the cellular antioxidant glutathione and antioxidative enzyme catalase (70) and tumor cells may upregulate antioxidative enzymes like superoxide dismutase to provide resistance from intracellular induction of apoptosis (71). Similarly, excessively high levels of VE might scavenge lipid peroxyl radicals, prevent the formation of hydrogen peroxide and superoxide and block apoptotic activity in initiated cells, or conversely permit excessive transcriptional activity. However, our results showed that ROS-mediated DNA oxidation was not decreased in all tissues by VE in all the p53-deficient genotypes that exhibited enhanced tumorigenesis, indicating that excessive ROS scavenging by VE with a resultant decreased apoptosis and/or increased transcription does not provide a consistent explanation for the enhanced tumorigenicity.

Different organs may metabolize, accumulate and thus respond to VE differently. The higher baseline levels of VE seen in embryos compared with fetal tissues seen in p53-deficient littersmates exposed in utero to high VE may reflect increasing capacity for the metabolism of VE in the fetal period and possibly developmental expression of VE membrane transporters and transport proteins. The mechanisms that regulate tissue concentrations and relative proportions of tocopherols are not well understood, but the relatively modest increases in embryonic and fetal tissue concentrations of VE in this study following dietary supplementation with over a 250-fold excess of VE suggests a remarkably efficient regulatory system. This appears to be relatively selective, since VE did not accumulate in the liver to the same extent as in other tissues, possibly in part due to hepatic metabolism by cytochromes P450. Overall regulation of VE disposition also is relatively selective among vitamins, since VE did not accumulate to an acutely toxic concentration in any embryonic or fetal tissue, as has been reported for other fat-soluble vitamins (vitamins A and D). Two types of α-tocopherol metabolites have been described; (i) those that arise from the oxidative action of its antioxidant action; and, (ii) those that have recently been discovered to arise from P450-catalyzed omega-oxidation and subsequent β-oxidative degradation, which produce carboxyethyl hydrochroman (CEHC) metabolites (72,73) that can be conjugated and excreted in the urine or bile (74,75). The latter arise independently of α-tocopherol antioxidative functions. In particular, it appears CYP4F2 (73) and possibly CYP3A4 (72,76,77) are involved in the oxidative metabolism of α-tocopherols. Notably, VE can act as a ligand for the pregnane X receptor (PXR) (78), which regulates a constellation of genes involved in xenobiotic detoxification (79), especially CYP3As that metabolize over 50% of drugs (80). Expression of both CYP4F and CYP3A are very low in early gestation and low fetal hepatic levels of CYP4F are not expressed until late gestation in humans (81). Similarly, levels of CYP3A start to increase during mid-late gestation, particularly in fetal liver (81,82). Conversely, the lower levels of VE in fetal tissues compared with that in embryonic tissues may also involve elevated utilization of VE by the fetus.

In general, epidemiological studies suggest that low intake or a low serum concentration of antioxidants is associated with elevated risk of some cancers (83–85), (reviewed in ref. 86) and several observational surveys have linked a large intake of VE with reduced incidence of heart disease (reviewed in ref. 31). Further, supplementation with antioxidants, including VE, have been linked to reduced risks of cancers such as those of the prostate and stomach (87,88). However, the revelation that high β-carotene supplementation increased the incidence of lung cancer in smokers (33,89) has resulted in a closer examination of antioxidants and their usage. α-Tocopherol supplementation in humans has provided ambiguous results. The human intervention Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention trial in Finland assessed the effect of supplemental α-tocopherol and/or β-carotene in various combinations and found that α-tocopherol had no effect on the incidences of lung, urothelial, stomach, kidney or colorectal cancers, but did reduce the incidence of prostate cancer by 34% (33). While the amount of VE used in this study far surpasses that which would be consumed on a daily basis in humans, these results may be relevant to some recent epidemiological observations. For example, in the Selenium and Vitamin E Chemoprevention Trial (SELECT) and four recent prospective studies, while VE reduced the risk of prostate cancer in past/current and current smokers and those with low levels of this vitamin, VE supplements in higher doses (>100 IU) were associated with a higher risk of aggressive or fatal prostate cancer in non-smokers.

Several human studies also suggest an in utero origin for some cancers. In a case-control study in Australia, a protective association was observed between folate and iron supplementation together in pregnancy and the risk of common acute lymphoblastic leukemia (ALL), the most common childhood cancer in more-developed countries (90). Other epidemiological evidence, obtained primarily from neonatal twin studies, supports the suggestion that some pediatric leukemias may be initiated in utero (91). High hyperdiploidy is the most common genetic abnormality in ALL and limited but significant data suggest that the chromosomal abnormalities defining ALL reflect early initiating events in utero that are associated with postnatal ALL development (92,93). In a recent study investigating the associations between growth patterns and the risk of breast cancer, a correlation was found between birth weight, a marker for in utero growth, and prenatal exposure and breast cancer (19). Furthermore, birth weight was found to be independent of the effect of subsequent growth patterns, suggesting that fetal factors are important risk factors for breast cancer in adulthood. Thus, while it has been established that carcinogenic exposures
within critical windows in the prenatal period result in enhanced postnatal carcinogenic risk (56), the epidemiological studies are consistent with our evidence that some cancers are initiated in utero.

In conclusion, our results in p53 knockout mice indicate that susceptibility to some postnatal cancers can be modulated in utero and the postnatal risk of cancer can be altered by modulating the embryonic and fetal environments, which may have diagnostic and therapeutic implications. The mechanisms underlying the enhanced tumorigenic onset in p53-deficient mice by high-dose VE remain to be established, but may not be fully explained by the pro-oxidant effects of increased oxidative stress or oxidative DNA damage. Our premise is that the developing conceptus may be at increased risk for the initiation and/or promotion of cancer due to its lower antioxidative defenses, and this hypothesis is consistent with preliminary results showing protection against the onset of postnatal tumorigenesis in p53 knockout mice exposed in utero to low-dose (0.1%) dietary VE supplementation (94). Given that large numbers of cell division occur during development, wherein more than half of all cell divisions occur prior to birth, there is a substantial opportunity for tissue- and possibly cell-specific mutations that can eventually lead to cancer. Finally, the effects of dietary VE are outcome-, tissue- and dose-dependent and, unlike in teratogenesis, biphasic. The case for embryoprotection is reflected in the substantial reduction in resorptions by high-dose VE and in the related decrease in DNA oxidation in fetal brain, suggesting that higher doses of VE may be similarly protective against ROS-mediated neurodevelopmental deficits. For tumorigenesis, however, high-dose dietary VE can exacerbate rather than block the onset of spontaneous tumorigenesis, while low-dose VE in contrast may offer some protection. The contrasting tissue-dependent effects of VE on oxidative DNA damage also may in part explain the conflicting reports of protective, ineffective and pathological effects of VE in various diseases and drug toxicities. These results suggest novel carcinogenic mechanisms and potential therapeutic strategies, as well as some caution in the use of very high doses of VE, particularly during pregnancy.

Acknowledgments

Preliminary reports of this research were presented at the annual meetings of the Society of Toxicology [Toxicol. Sci. (Supplement: The Toxicologist) 61: No.1502, 2002] and the American Association for Cancer Research (Proceedings of the Annual Meeting of the American Association for Cancer Research, Vol. 43: No. 4303, 2002). The authors are grateful to Dr Colin McKerlie and Lily Morikawa of the Research Institute of the Hospital for Sick Children, Toronto, for preparation and analysis of tumor sections and to Carol Wang of the Faculty of Pharmacy, University of Toronto, for assistance in analyzing α-tocopherol levels. The study was supported by grants from the National Cancer Institute of Canada and the Canadian Institutes of Health Research.

Conflict of Interest Statement: None declared.

References


Received April 16, 2005; revised May 31, 2005; accepted December 20, 2005