Vitamin E reduces chromosomal damage and inhibits hepatic tumor formation in a transgenic mouse model

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Edited by Gerald N. Wogan, Massachusetts Institute of Technology, Cambridge, MA, and approved December 17, 1999 (received for review October 5, 1999)

We have previously shown that chronic activation of mitogenic signaling induced by over-expression of c-myc and transforming growth factor-α (TGFα) transgenes in mouse liver induces a state of oxidative stress. We therefore proposed that increased reactive oxygen species (ROS) generation might be responsible for the extensive chromosomal damage and acceleration of hepatocarcinogenesis characteristic for TGFα/c-myc mice. In this study, we show that vitamin E (VE), a potent free radical scavenging antioxidant, is able to protect liver tissue against oxidative stress and suppress tumorigenic potential of c-myc oncogene. Dietary supplementation with VE, starting from weaning, decreased ROS generation coincident with a marked inhibition of hepatocyte proliferation while increasing the chromosomal as well as mtDNA stability in the liver. Similarly dietary VE reduced liver dysplasia and increased viability of hepatocytes. At 6 mo of age, VE treatment decreased the incidence of adenomas by 65% and prevented malignant conversion. These results indicate that ROS generated by over-expression of c-myc and TGFα in the liver are the primary carcinogenic agents in this animal model. Furthermore, the data demonstrate that dietary supplementation of VE can effectively inhibit liver cancer development.

It has been postulated that overproduction of reactive oxygen species (ROS) and oxidative DNA damage play a major role in aging and cancer development (1, 2). Antioxidants, including vitamin E (VE), have been reported to initiate a wide range of physiological responses that lower cancer risk (3). One of the best characterized function of VE, an integral component of plasma lipoproteins, is scavenging of lipid peroxyl radicals in biological membranes thus preventing formation of highly reactive lipid peroxidation products (4), which have been shown to have both mutagenic and carcinogenic properties (5, 6). More recent work has demonstrated that VE can also directly reduce ROS production by interfering with the assembly of membrane-bound NADPH-oxidase complexes (7). New evidence indicates that VE, in addition to its well characterized antioxidant function, can protect against cancer formation by enhancing immunological surveillance as well as by affecting signal transduction pathways involved in regulation of cell proliferation and apoptosis (8–10). Consistent with these effects, VE has been found to negatively regulate protein kinase C (11) implicated as an endogenous tumor promoter (12, 13).

Oxidative stress is a feature of many chronic human liver diseases associated with a significant reduction in hepatic levels of VE, including chronic hepatitis virus infections, cirrhosis, and cancer, and might represent a common mechanism for liver injury by diverse etiologies (14–16). However, despite considerable research effort, the beneficial effects of VE in hepatocarcinogenesis, and, in particular, how it affects the early stages of the neoplastic process, remain largely unknown.

Recently, we have established a transgenic mouse model that provides a genetically relevant in vivo system to study the importance of ROS and oxidative tissue damage in the development of hepatic neoplasia as well as a means to test prevention strategies against liver cancer (17). In this model, constitutive co-expression of the c-myc proto-oncogene and transforming growth factor-α (TGFα), which are frequently up-regulated in human hepatocellular carcinomas (18), generates an oxidative stress environment prior to tumor development and predisposes mice to a 100% incidence of spontaneous liver cancer (19, 20). Notably, in these mice overproduction of ROS occurs concomitantly with liver hyperplasia, extreme genomic instability, and elevated apoptotic death (21, 22), thus modeling the preneoplastic condition. Moreover, we have found that the specific sites

Fig. 1. Effects of VE on liver weight in TGFα/c-myc transgenic mice. TGFα/c-myc mice fed with control diet exhibited greater liver mass than wt mice at 4 (∗∗∗P < 0.0004) and 10 (∗∗∗P < 0.0004) wk of age. VE dietary supplementation reversed TGFα/c-myc-induced increase in liver mass to the levels found in wt mice. After 1 wk of supplementation with VE starting at 3 wk of age, the liver/body weight ratios were only slightly reduced (ΔP = 0.18). By 10 wk of age, the difference in liver weights between TGFα/c-myc mice fed with control or VE supplemented diet reached a statistical significance (P < 0.004). Values are expressed as percentages of total body weights ± SE (n = 5 in each group of mice).

Abbreviations: TGFα, transforming growth factor-α; ROS, reactive oxygen species; VE, vitamin E; wt, wild type; PCNA, proliferating cell nuclear antigen; DCFH-DA, 2′,7′-dichlorofluorescein diacetate.

This paper was submitted directly (Track II) to the PNAS office.

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Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.040428797. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.040428797
of chromosomal breakage observed before an early onset of tumor development are associated with susceptibility to liver carcinogenesis and are later involved in stable rearrangement in solid liver tumors (23). Importantly, TGF\(\alpha\)\(\gamma\)c-myc-driven hepatocarcinogenesis occurs in a stage-specific and highly predictable manner, which models quite closely various histopathological, biochemical, and cytogenetic features characteristic of human liver oncogenesis. In this study, we have used this model to test the potential of VE to increase genetic stability and protect against development of liver cancer. To address these objectives, 3-wk-old TGF\(\alpha\)\(\gamma\)c-myc transgenic mice were divided into two groups, one receiving control diet with (2,000 units/kg diet) and one without VE supplementation. The effect of VE treatment on liver tumorigenesis was assessed both at the early (10 wk of age) and end stage (26 wk) of tumor development. At 10 wk of age, the extent of hepatic injury, ROS production, as well as chromosomal and mtDNA damage were determined. At 26 wk of age, a histopathological analysis was performed to estimate tumor incidence, multiplicity, and tumor size.

**Materials and Methods**

**Mice and Diet.** Male TGF\(\alpha\)\(\gamma\)c-myc double transgenic mice were produced by crossing homozygous MT/TGF\(\alpha\) (24) and Alb/c-myc (19) single transgenic mice in CD1\(\times\)B6CBA background and housed as described (17, 19). The animal study protocols were conducted according to the National Institutes of Health guidelines for animal care. VE (dl-\(\alpha\)-tocopherol acetate) supplemented (2,000 units/kg of diet) and control chow were prepared by Bioserv (Frenchtown, NJ) on the basis of AIN-93G amino acid-defined diet. The mice had free access to food and water. The diet was stored at 4°C and replaced every 1 or 2 days. As measured by food consumption (5 g/day), this supplementation corresponds to \(\approx\)10 units of VE/day/adult mouse. No overall differences in food consumption were noted between animals fed supplemented or control diet.

**Histology and Tumor Sampling.** The mice were killed by CO\(_2\) inhalation. The liver and body weights as well as the number and the size of all grossly visible tumors were recorded. Representative samples of liver taken from each lobe and all tumors were fixed in 10% neutral formalin at 4°C overnight. Paraffin sections (5 mm) were stained with hematoxylin-eosin, and hepatocellular lesions were graded as foci, adenomas, and carcinomas as defined previously (25).

**Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry.** After deparaffinization of the liver sections, endogenous peroxidase activity was inhibited by incubation in 0.01 mol/liter periodic acid in PBS (10 min), followed by treatment with 0.1 mol/liter sodium borohydride in PBS (10 min). Anti-PCNA

![Fig. 2. VE dietary supplementation reduced the rate of hepatocyte proliferation and apoptosis in TGF\(\alpha\)\(\gamma\)c-myc transgenic livers. (A) Labeling index of PCNA-positive hepatocytes. Histograms show a significantly higher labeling index in TGF\(\alpha\)\(\gamma\)c-myc mice fed with control diet than in TGF\(\alpha\)\(\gamma\)c-myc mice fed with VE supplemented diet (*P = 7.1E-05 and P = 0.01 at 4 and 10 wk of age, respectively). Values are expressed as mean percentage ± SE (n = 5 in each group of mice). (B) Apoptotic index of hepatocytes. Histograms show that the apoptotic index is significantly higher in TGF\(\alpha\)\(\gamma\)c-myc mice fed with control diet than in TGF\(\alpha\)\(\gamma\)c-myc mice fed with VE supplemented diet (*P < 0.05 both at 4 and 10 wk of age). (C) Representative PCNA immunoperoxidase staining of liver samples illustrating a decreased percentage of nuclei exhibiting PCNA-immunoreactivity in 10-wk-old TGF\(\alpha\)\(\gamma\)c-myc mice fed with VE supplemented diet. Note the high frequency of mitotic cells (arrows) and the presence of apoptotic cells (+) in control TGF\(\alpha\)\(\gamma\)c-myc livers. Paraffin-embedded sections were counterstained with hematoxylin (×150 original magnification).
mAb (Dako) was diluted 1:400 and incubated on slides overnight at 4°C followed by biotinylated secondary antibody (Vector Laboratories) and streptavidin peroxidase (Vector Laboratories) with diaminobenzidine as the chromogen. The PCNA-labeling index was determined by counting PCNA-positive cells after counterstaining with hematoxylin. Two thousand hepatocyte nuclei were counted per animal, and the index was expressed as percentage of the total number of counted nuclei.

**Incidences of Apoptosis.** The histological criteria of apoptosis described elsewhere were applied (21), and the apoptotic indices were calculated by counting the apoptotic figures per 2,000 hepatocytes on hematoxylin-eosin-stained liver sections and expressed as percentage of the total number of counted cells.

**Hepatocyte Isolation.** Hepatocytes were isolated by two-step collagenase perfusion of the liver followed by isodensity centrifugation in Percoll as described previously (26). Viability was determined by trypan blue exclusion and was 90% in both diet groups.

**Fluorescent Measurement of Intracellular Peroxides.** To assess levels of intracellular ROS, flow cytometric analysis was performed using the oxidative-sensitive probe (DCFH-DA) as described (17). Hepatocytes (0.5 × 10^6/ml) were incubated for 30 min at 37°C in the presence of 5 μM DCFH-DA. After incubation, the cells were transferred to ice bath, and formation of 2′,7′-dichlorofluorescein (DCF) was analyzed by flow cytometry using a Becton Dickinson Facscan with excitation and emission settings of 495 and 525 nm, respectively. The DNA of dead cells was stained with propidium iodine (5 μM) before the measurement of DCF fluorescence. Ten thousand viable cells from duplicate samples were analyzed.

**Chromosome Preparation.** Metaphase chromosomes were prepared from short-term primary hepatocyte cultures as described previously (22). The incidence of aberrations was determined on conventionally stained (5% Giemsa in distilled water) chromosomes. For each sample, 50–100 complete metaphase with minimal chromosome overlapping were coded, examined di-

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**Fig. 3.** VE dietary supplementation reduced liver pathology in TGFα/c-myc transgenic mice. Representative histology of liver samples for wt (A and B) and TGFα/c-myc transgenic mice fed with control (C and D), or VE supplemented (E and F) diet at 10 and 26 wk of age. Control TGFα/c-myc livers manifest an advanced nuclear atypia and marked cellular and nuclear enlargement as compared with wt healthy livers. VE dietary supplementation markedly decreased the severity of hepatic dysplasia and reduced tumor formation. Hematoxylin and eosin staining of paraffin-embedded sections (×125 original magnification).

**Fig. 4.** VE dietary supplementation reduced intracellular ROS levels in TGFα/c-myc hepatocytes. Hepatocytes were isolated by collagenase perfusion as described in Materials and Methods, incubated in the absence or presence of 5 μM of the oxidative-sensitive probe DCFH-DA for 30 min at 37°C and subjected to flow cytometry. The figure represents the overlay of three representative fluorescence-activated cell sorter analysis performed on TGFα/c-myc hepatocytes isolated from 10-wk-old mice fed with control (gray area) or VE-supplemented diet (black area). The white area shows the autofluorescence profile in unstained TGFα/c-myc hepatocytes. DCF fluorescence was measured as difference between DCF fluorescence and autofluorescence in unstained cell suspensions for each cell preparation analyzed. Representative result from three independent experiments is shown.
Percentage of cells with chromosomal alterations. The histogram shows that the cumulative frequency of aberrant metaphases as measured by the presence of chromatid and chromosome gaps, and/or breaks, and/or exchanges was significantly lower in TGFα-supplemented diet (\(P < 0.05\)). The cytogenetic analysis was performed on primary hepatocyte cultures established from 10-wk-old TGFα-c-myc mice fed with control or VE-supplemented diet as described in Materials and Methods. Values are expressed as the mean percentage ± SE. (B) Representative examples of chromatid breaks, and triradial and quadroradial exchanges.

Statistical Analysis. The data are presented as means ± SE. For statistical comparison the Student’s \(t\) test and nonparametric Wilcoxon test were used.

Results and Discussion

VE Normalizes Liver Mass and Reduces Liver Pathology. Chronic administration of VE (2,000 units/kg) induced no obvious toxicity as evident by clinical observations or gross pathology at necropsy. There were no significant differences in food intake, and at the end of the study, the body weights were nearly identical (37.9 ± 0.8 g in control transgenic mice receiving normal diet vs. 36.3 ± 0.6 g in VE-treated mice, \(P = 0.13\)). In contrast, VE treatment significantly reduced the transgene-induced hepatomegaly characteristic of fast growing TGFα/c-myc mice (19, 20). By 10 wk of age, VE treatment almost reversed both absolute and relative liver weights in transgenic mice to levels found in age- and strain-matched wild-type (wt) mice (2.4 ± 0.2 g in control mice vs. 1.39 ± 0.1 g in VE-treated mice, \(P = 0.004\) and 1.37 ± 0.04 in wt mice; Fig. 1). The normalization of liver mass correlated with a marked decrease in the rate of cell proliferation and associated apoptosis (Fig. 2). Although untreated TGFα/c-myc mice displayed characteristic high levels of mitogenesis and cell death between 4–10 wk of age, in VE-treated transgenic mice, the PCNA-labeling indices were reduced to levels observed in wt mice. More significantly, the VE-treated transgenic mice also showed improved liver histology compared with their aged-matched transgenic controls. At a microscopic level, VE-treated TGFα/c-myc livers exhibited only mild dysplasia with considerably less cellular and nuclear...
enlargement and did not display an advanced nuclear atypia commonly seen in livers of control littermates (Fig. 3). To analyze further the effects of VE, we performed a two-step collagenase perfusion and compared the yield of viable hepatocytes as a quantitative measure of membrane stability. Dietary supplementation with VE prevented excessive membrane damage and increased both the yield and viability of isolated hepatocytes. Specifically, the number of viable hepatocytes isolated from 10-wk-old VE-treated mice (17.7 ± 0.6 × 10^6 per gram of liver mass) was more than 3-fold greater than in untreated transgenic mice (5.3 ± 0.8 × 10^6/g; P = 2.5E-05) and closer to that in age- and strain-matched wt mice (22.4 ± 1.2 × 10^6/g; P = 0.028). However, there were no significant changes in plasma levels of cholesterol and triglyceride in both diet groups consistent with results reported previously (29). Likewise, additional liver function markers, such as alanine aminotransferase and aspartate aminotransferase, showed no consistent changes (data not shown). Taken together these findings suggest that the generation of ROS contributes to liver enlargement and development of dysplasia preceding an early onset of neoplastic growth in this model and demonstrate the capacity of VE to exert a hepatoprotective effect against oxidative stress.

**VE Lowers ROS Production.** In earlier studies, we have shown that TGFα/c-myc hepatocytes exhibit a greater level of oxidant production relative to either wt or c-myc single transgenic cells, as determined by the peroxidation-sensitive fluorescent dye DCFH-DA (17). In this study, we have used the same approach to measure the impact of VE supplementation on intracellular ROS generation. The relative intensity of DCF-staining in hepatocytes isolated from 10-wk-old VE-supplemented mice was 2.1 ± 0.4-fold (n = 3, P < 0.05) less than in their age-matched transgenic controls (Fig. 4). Thus, dietary VE was able to effectively reduce both the extent of ROS formation and diminish the associated free radical induced cytotoxicity.

**VE Reduces Chromosomal and mtDNA Damage.** Subsequently, we sought to determine whether the lowering of ROS production results in decreased DNA damage. Previous cytogenetic studies revealed that at 10 wk of age, the level of structural chromosomal aberrations was nearly 10-fold higher in TGFα/c-myc hepatocytes than in wt hepatocytes (22). To determine the effect of VE on chromosomal integrity, the cytogenetic analysis was conducted on primary hepatocyte cultures established from 10-wk-old mice maintained on control and VE-supplemented diet. Fig. 5 shows that VE supplementation markedly decreased the frequency of chromosomal damage at the early stage of hepatocarcinogenesis just before the development of morphologically defined preneoplastic and neoplastic lesions. The total number of aberrant metaphases exhibiting chromatid and chromosome gaps, and/or breaks, and/or exchanges was significantly decreased in the primary hepatocytes from VE-treated livers as compared with TGFα/c-myc untreated controls (3.1 ± 0.7-fold, P < 0.0001). Similarly, dietary VE significantly reduced the amount of age-related mtDNA deletion associated with direct sequence repeats (Fig. 6). In the latter case, the intensity of PCR signal was decreased to a level found in 4-wk-old untreated transgenic mice (data not shown). Together, these findings indicate that VE supplementation reduced both chromosomal as well as mtDNA damage in this transgenic mouse model.

**VE Reduces Tumor Formation.** We have reported previously that TGFα/c-myc mice exhibit high rates of hepatic tumor formation at a young age (19, 20). Transgenic mice maintained on control diet developed hepatocellular adenomas with 100% incidence and 27% of mice produced hepatocellular carcinomas by 6 mo of age (Table 1). VE dietary supplementation starting from weanling markedly suppressed the tumorigenic process in TGFα/c-myc mice. Moreover, all stages of the neoplastic development, including the early hyperplastic and dysplastic phases with the concomitant chromosomal lesions as well as multiplicity and size of preneoplastic lesions were significantly reduced (Fig. 3, Table 1). Of the 20 VE-treated mice, only seven mice produced hepatocellular adenomas and five mice were completely devoid of focus formation. Furthermore, no carcinomas were seen in the VE-supplemented group of mice indicating that VE effectively suppressed both the initiation stage and progression of early hepatic lesions into cancer. Fewer numbers and size of adenomas in VE-fed mice correlated with a smaller liver mass reflecting an apparent difference in the growth rate of preneoplastic and neoplastic lesions as compared with the dietary controls (Table 1). In addition, the chemopreventive efficacy of VE appears to be dose dependent. Thus, dietary administration of VE at a lower dose (500 units/kg of diet in an earlier pilot study) reduced the incidence of liver lesions by 27% vs. 65% at 2,000 units/kg.

Our data support an etiological role for ROS in the TGFα/c-myc mouse model of accelerated carcinogenesis and establish the importance of VE in suppressing the tumorigenic process in the liver. In this in vivo system, VE given at concentrations achievable via diet supplementation was able to modulate oncogenic activity of c-myc and TGFα and reverse the biochemical, cellular, and cytogenetic characteristics associated with both tumor initiation and progression (17, 20, 22). Most significantly, VE dietary supplementation restored the cellular redox balance before the development of preneoplastic lesions and decreased the rate of hepatocyte proliferation. Inhibition of mitogenesis occurred coincident with enhancing chromosomal stability suggesting “a cause and effect relationship”.

Evidence supporting the influence of diet on the level of chromosomal damage has been recently provided by human intervention studies. The epidemiological data show that increased consumption of a diet rich in antioxidant vitamins decreases the rate of chromosomal aberrations and lowers cancer risk (30). Given the striking difference in chromosome aberrations and tumor incidence between VE-treated and untreated mice, we hypothesize that VE exerts its antineoplastic effect through its capacity to restore the redox balance in the liver, thereby effectively preserving genomic stability. Together the findings strongly support the idea that accelerated cell proliferation and associated genetic instability are required for

<table>
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<th>Diet group</th>
<th>Incidence, %</th>
<th>Multiplicity</th>
<th>Maximum tumor size</th>
<th>LBW%</th>
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<td></td>
<td>Adenomas</td>
<td>Carcinomas</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
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<td>27 (4/15)</td>
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<td>0 (0/20)</td>
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*In parentheses, the number of mice with adenomas/carcinomas per total number of mice.
tumorigenesis (31). In conclusion, the results of the present studies provide in vivo evidence that long term dietary administration of VE at dose of 2,000 units/kg confers significant protection against both initiation and progression stages of hepatocarcinogenesis. The experimental model used in this study establishes that overproduction of ROS generated by endogenous metabolic processes has a direct impact on genomic stability and susceptibility to liver tumor development. Although considerably more work is needed to elucidate the precise mechanism(s) by which VE suppresses tumorigenesis, the promising results obtained with VE treatment in this potentially clinically relevant system suggests that dietary supplementation of VE and possibly other antioxidant agents might contribute to delaying/prevention of human liver cancer.

The authors thank Dr. L. M. Sargent for her invaluable support and advice in the early stages of the project and Dr. G. Merlino for providing the TGFβ transgenic mice.