Dietary antioxidant depletion: enhancement of tumor apoptosis and inhibition of brain tumor growth in transgenic mice

Rudolf I. Salganik1,3, Craig D. Albright1, Jerilyn Rodgers2, John Kim2, Steven H. Zeisel3, Mikhail S. Sivashinsky1 and Terry A. Van Dyke2

1Department of Nutrition, School of Public Health, School of Medicine, University of North Carolina, Chapel Hill, NC 27599 and 2Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA

3To whom correspondence should be addressed
Email: rsalganik@unc.edu

Apoptosis, or regulated cell suicide, eliminates unwanted and damaged cells, including precancerous and cancerous cells. Since reactive oxygen species (ROS) act as essential apoptotic mediators, we reasoned that increasing the ROS level might enhance apoptosis and thereby slow down tumor growth. Here, using a defined transgenic brain tumor model with known tumor apoptosis rates, we test the impact of antioxidant-depleted diet, capable of increasing ROS levels, or antioxidant-enriched diets on tumor growth. Dramatically increased apoptosis occurs within tumors, but not in normal tissues of antioxidant-depleted mice. The presence of detectable increased oxidant stress within tumors indicates that the likely mechanism of enhanced tumor apoptosis is via ROS and DNA oxidative impairment. Importantly, due to the ROS-enhanced apoptosis, tumor growth is inhibited in mice fed an antioxidant-depleted diet. In clear contrast, an antioxidant-rich diet had no impact on tumor growth.

Introduction

Apoptosis, a regulated non-necrotic form of cell death, is triggered by specific intra- or extracellular stimuli encoded intrinsically by a genetic 'suicide' program (1–3). Apoptosis occurs through a highly conserved central death pathway that involves the activation of a cysteine protease (caspase) cascade (4,5), with cleavage of the ultimate substrates resulting in orderly cellular demise. Apoptotic cells undergo characteristic morphological changes resulting in fragmentation into several membrane-bound compartments, and subsequent engulfment by neighboring cells and macrophages. The result is cellular elimination with minimal tissue damage and without inflammation. Apoptosis is widely recognized as the major mode of cell death during development and in normal tissue turnover, facilitating the precise regulation of cell numbers. It also serves as a defense mechanism, eliminating potentially dangerous cells such as those infected with virus or exposed to toxins or other adverse environmental conditions (3,6). Recently, primarily through the study of animal tumorigenesis models, it has become clear that the modulation of apoptosis can play a direct critical role in cancer (7,8). In some de novo mouse tumor models in which specific genetic alterations are used to induce multi-step tumorigenesis, the level of tumor apoptosis is inversely related to tumor growth (9,10). Thus factors that will increase the apoptotic rate specifically within tumors should slow tumor growth and provide therapeutic possibilities.

It is well known that general nutrition can affect the outcome of many disease states, including cancer (11,12). Because antioxidants can protect cells from oxidative DNA damage, these agents may prevent the accumulation of genetic defects that can lead to cancer. However, antioxidants can also inhibit apoptosis via the inhibition of reactive oxygen species (ROS). Such inhibition within a developing tumor could accelerate its growth. These potentially opposing effects may explain why the results of intervention trials evaluating the effects of vitamin supplementation on cancer rates are mixed (13,14).

Most forms of aerobic apoptosis involve utilization of ROS as essential intermediate messengers (15–18), though the mechanisms are not clear. Oxidative stress results in the production of several ROS (including O2–, H2O2 and OH·) that induce a variety of intracellular changes such as DNA oxidative modification, increased calcium release, energy depletion and oxidation of glutathione, NADPH, proteins and lipids (19). In addition, the oxidative regulation of proteins that directly modulate apoptosis, such as caspases (20), NF-κB and AP1 (22) has been observed. Whatever the mechanism, the fact that antioxidants can inhibit apoptosis induced by a variety of stimuli indicates a central role for ROS in this process (23,24).

Since increased ROS can induce apoptosis and antioxidants can inhibit apoptosis in vitro, it is possible that modulation of antioxidant levels within the intact organism will affect tumor cell apoptosis and, thus, tumor growth rates. We tested this hypothesis here using a well characterized de novo tumor model in which the level of tumor cell apoptosis inversely correlates with the tumor growth rate (9). In the transgenic TgT121 brain tumor model, tumors of the choroid plexus (CP) are initiated by tissue-specific inactivation of the pRb tumor suppression pathway (9). Inactivation of this pathway is achieved by cell-specific expression of the T121 oncprotein, a protein that binds to and inactivates the pRb proteins (25,26). This event induces the normally non-dividing brain epithelial cells to proliferate. Widespread tumor growth occurs during the course of several months and the mice die of their tumors at an average of 26 weeks. p53-dependent apoptosis is present within these tumors such that inactivation of p53 (9) or its downstream effector, bax (10) accelerates tumor growth. Since the correlation between apoptosis and tumor growth rate is well established in this model and tumor growth is predictable in all mice within the lineage, we utilized these mice to test the effects of manipulating antioxidant levels on tumor growth in vivo.

Materials and methods

Transgenic mice and diets

The TgT121 transgenic line was previously referred to as LST1137-5 (25). These mice harbor the T121 mutant T antigen gene driven by the lymphotropic...
papovavirus (LPV) promoter. Under LPV control expression of T antigen is targeted uniformly to the CP epithelium, a tissue that is located in the ventricles of the brain. T121 (previously referred to as dl1137) contains the first 121 amino acids of SV40 T antigen followed by 11 missense residues resulting in a 31 bp out-of-frame deletion (26). The standard AIN-93-M diet contained an AIN-93-VX vitamin mix providing antioxidants: 75 IU/kg diet of vitamin E (all-rac-alpha-tocopheryl acetate) and 4000 IU/kg diet of vitamin A (all-trans-retinyl palmitate) (27). Antioxidant-poor diet was devoid of vitamins E and A while in the antioxidant-rich diet their concentrations were doubled.

Analysis of tumorgenesis

After four months, the mice in all groups were killed and brain, liver, small intestine, and spleen were harvested and prepared for histological studies and tumor analysis. During the course of the experiment there was one spontaneous death among the experimental animals and two among the control group. Due to tissue autolysis, these animals were excluded from the analysis. Otherwise all animals remained relatively healthy throughout the course of the experiment.

Brains were collected from animals at the time of death, fixed in neutral buffered 10% formalin and embedded in paraffin. Random 5 μm step-sections were collected in triplicate at 25 μm intervals and stained with hematoxylin and eosin (H&E). The size of the tumor was measured using the formula: mean diameter = (A + B)/2, where A and B = maximum orthogonal diameters of the tumor measured using a calibrated ocular scale (J.R.Crane, Westmont, IL) and a 40X objective. Image analysis of brain histologic sections was performed on a Nikon FXA microscope (Nikon Inc., Garden City, NY) equipped with an Optronics TEC-470 CCD Video Camera System (Optronics Engineering, Goleta, CA). Images viewed at a final magnification of 40X (4X objective, N.A. 0.75, 10X ocular) and corrected for coverglass thickness, were captured on an Apple Macintosh 840AV computer (Apple Computer, Cupertino, CA) using a Scion LG-3 capture card (Scion Corp., Frederick, MD). For each section of individual brains, the profile area of tumor per area of brain was measured and the volume fraction of tumor per volume of brain was determined based on the stereological identity of volume fraction (Vv) to area fraction (Aa) (Vv = Aa). Image processing, measurement and analysis were performed using the public domain NIH Image program version 1.61 (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image) (28).

Apoptosis analyses

Apoptotic cells were detected using the terminal dUTP nucleotide DNA end-labeling (TUNEL) assay, a direct immunoperoxidase method (ApoTag, Oncor, Gaithersburg, MD). After four months, the mice in all groups were killed and brain, liver, small intestine, and spleen were harvested and prepared for histological studies and tumor analysis. Analysis of tumorigenesis

To determine whether increased intracellular ROS levels can increase tumor growth, we altered antioxidant levels in the diets of TgT121 transgenic mice and measured the impact on tumor apoptosis and growth. Under normal conditions, CP tumors in these mice grow slowly for 3–4 months with an average tumor apoptotic index of 7.3% (9). Subsequently, most tumors naturally progress to lower apoptotic states and more aggressive growth (our unpublished observations), leading to death at an average of 6–7 months (9). To measure the impact of antioxidant-altered diets on the growth and progression of these tumors, TgT121 and control non-transgenic mice were fed either a standard control diet, or diets enriched in or depleted of antioxidants. The standard AIN-93-M diet contained an AIN-93-VX vitamin mix providing vitamins E and A (27). The antioxidant properties of these vitamins are well established (33,34). Vitamins E and A were depleted from the antioxidant-poor diet, and their concentrations were doubled in the antioxidant-rich diet (see Materials and methods). Mice were monitored daily for food consumption, health status and gross signs of tumor development. No differences were observed among the groups in the amounts of diet ingested (mean of 2.7–3.0 g daily per animal), in body weight or in general behavior. After four months, the mice in all groups were killed and brain, liver, small intestine, and spleen were harvested and prepared for histological studies and brain tumor analysis.

In all mice fed a control or antioxidant-rich diet, highly vascularized advanced CP tumors developed that completely displaced the volume of the ventricular space (Figure 2A and B). In contrast, feeding an antioxidant-poor diet for 4 months significantly reduced the mean tumor diameter by 65% (Table I; Figure 1C). To determine the effects of antioxidant levels on total tumor burden, step sections of tumors were measured using an unbiased stereological method (28). Consistent with mean tumor diameter measurements, this analysis showed that feeding an antioxidant-poor diet, but not an antioxidant-rich diet, significantly decreased the total tumor burden (by ~50%) in the brains of TgT121 mice compared with those fed a standard diet (Figure 2A).

Increased tumor apoptosis and oxidative stress in antioxidant-depleted mice

Reduced tumor burden correlated with an increased rate of tumor cell apoptosis. Significantly higher numbers of morphologically apoptotic cells were visibly present in the tumors of animals fed the antioxidant-poor diet compared with controls or to mice fed an antioxidant-rich diet (Table I). Quantification of apoptotic indices using the TUNEL assay (29) showed a 5-fold increase in the percentage of apoptotic cells in tumors of antioxidant-poor animals compared with controls (19.5 ± 3.4 versus 3.4 ± 0.5%, respectively; Figures 2B, and 3A and B). Regardless of diet, no differences in apoptosis rates were observed in the liver, spleen or small intestine (not shown). For example, in H&E-stained liver sections no apoptotic nuclei were observed by morphological inspection of 1400–2600 cells per animal regardless of the diet consumed.

We originally hypothesized that an antioxidant-depleted diet would increase apoptosis by a mechanism in which intracellular ROS levels were increased. Given that clear enhancement of tumor cell apoptosis indeed occurred in response to such a diet, we tested whether increased apoptosis was linked to an increased intracellular ROS concentration. Two in situ methods were utilized to detect oxidized guanine residues 8-oxo-dGua and 8-oxo-Gua, major reaction products resulting from the
Antioxidant depletion inhibits brain tumor growth

Fig. 1. Tumors of mice fed an antioxidant-devoid diet are reduced in size. Compared with TgT121 CP tumors in controls (A and B), the CP tumors in mice fed an antioxidant-devoid diet were significantly smaller (C). Feeding an antioxidant-rich diet did not reduce the tumor size (Table I). Magnifications: A = 60×; B and C = 120×.

generation of OH-radicals and sensitive biomarkers for the intensity of ROS generation and the induction of oxidative DNA damage (19,20,32,35). Recent studies show that biotin is structurally homologous to 8-oxo-dGua, and that cellular binding of FITC-conjugated avidin is a highly sensitive method for the detection of oxidatively modified DNA in fixed cells and tissues (32). Using this method, we detected increased oxidative stress in brain tumors of animals fed an antioxidant-poor diet relative to controls (Figure 3, compare panels E and F). To confirm this result, immunohistochemistry was applied using a monoclonal antibody specific for oxidized guanine residues (30). By this method, intracellular guanine oxidation in epithelial tumor cells of animals fed an antioxidant-depleted diet was nearly twice that of mice fed standard or antioxidant-rich diets (Figures 2C, and 3, compare C and D). Taken together, these data indicate that feeding an antioxidant-poor diet during tumor growth results in increased intracellular ROS within the tumor, an enhancement of tumor cell apoptosis, and inhibition of tumor growth. In contrast, feeding an antioxidant-rich diet does not modulate ROS, tumor apoptosis or growth.

Discussion

Numerous previous studies established that ROS function as intermediary signals for the activation of apoptosis (15–18). The studies presented here show that feeding an antioxidant-poor diet causes a significant increase in ROS and apoptosis in de novo CP brain tumors resulting in a substantial reduction in tumor burden while sparing normal tissues. The observed increase in apoptotic index reflects a direct increase in apoptosis rather than an indirect reduction in apoptotic cell phagocytosis because the overall tumor volume is decreased. The specificity of this response for tumor cells is striking. One possible reason is the reportedly higher than normal concentration of free oxygen radicals in many malignant cells (36–38), where high superoxide dismutase activity enhances H_2O_2 formation from

Table I. Morphological tumor alterations induced by an antioxidant-depleted diet

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<thead>
<tr>
<th>Diet antioxidant level</th>
<th>Brain tumor characteristics&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Tumor diameter (mm)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Standard</td>
<td>0.939 ± 0.229</td>
</tr>
<tr>
<td>Rich</td>
<td>0.815 ± 0.289</td>
</tr>
<tr>
<td>Poor</td>
<td>0.327 ± 0.053&lt;sup&gt;**&lt;/sup&gt;</td>
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<sup>a</sup>Representative results from one data set are shown. The full experiment was replicated with similar results (~50% reduction in tumor diameter and 2-fold increase in tumor apoptosis in antioxidant-poor mice). Overall, tumors were somewhat smaller in the second experiment.

<sup>b</sup>Data are expressed as means ± SE; bars identified with different letters differ significantly from one another (P < 0.01). Number of tumors analyzed: control n = 6; rich n = 7; poor n = 12. Number of mice per group: control = 4; rich = 7; poor = 7 (note that some mice had more than one tumor since choroid plexus is located in all four ventricles). This analysis was performed on the animals in the second study (Table I) due to the availability of complete paraffin blocks for stereological analysis.

Fig. 2. Reduced tumor burden (A) is linked to increased tumor apoptosis (B) and increased oxidative stress (C). CP tumors were identified morphologically, and the volume fraction (V_v – total brain tumor burden) of brain occupied by tumor was measured in triplicate, random step sections through each brain for a total of 75 step sections using a computer-based stereology method (28). Apoptotic cells were identified using the TUNEL assay. Oxidized guanine residues, a measure of oxidative stress, were analyzed using a monoclonal antibody and an image analysis method (30). Data are expressed as means ± SE; bars identified with different letters differ significantly from one another (P < 0.01). Number of tumors analyzed: control n = 6; rich n = 7; poor n = 12. Number of mice per group: control = 4; rich = 7; poor = 7 (note that some mice had more than one tumor since choroid plexus is located in all four ventricles).
superoxides, and low catalase activity leads to excessive H$_2$O$_2$ accumulation (39). Subsequent reaction of H$_2$O$_2$ with transition metal ions produces high levels of the highly reactive hydroxyl radical (19,20). Thus, TgT121 tumor cells could have an elevated baseline of ROS compared with normal tissues, such that even a moderate ROS increase could trigger higher, and possibly sustained, apoptosis. In addition, a recent study in cultured cells indicated an important role for ROS in p53-dependent apoptosis (40). Since p53-dependent apoptosis is activated in TgT121 CP (9), this tissue may be ‘primed’ for increased apoptotic effects in response to ROS. Further experiments in TgT121 mice that are also deficient in p53 will determine whether p53 is required for the increased tumor cell apoptosis induced by an antioxidant-poor diet.

The study described here establishes a striking correlation between a vitamin E- and A-depleted diet, increased tumor cell ROS and apoptosis, and a reduction in tumor burden. The antioxidant properties of both vitamins A and E have been documented in animal models. In addition to protecting against lipid peroxidation in mitochondria in brain (33), previous reports also show strong synergistic interaction between vitamins A and E against lipid peroxidation in phosphatidyl choline liposomes (41). Further studies will be required to determine whether depletion of one particular vitamin or of both is required for optimal effects on tumor growth. Furthermore, although the correlation between ROS and increased apoptosis is clear, in vivo studies such as these cannot rule out other mechanisms for enhanced apoptosis. Although previous data in cultured cells indicate that increased ROS is the most likely mechanism, direct proof will require in vitro analyses of tumor cells derived from these mice and/or the use of mice with specific genetic deficiencies in oxidative metabolism. Regardless of the mechanism, however, the dramatic effect of antioxidant depletion from the diet on apoptosis and tumor growth is clear.

A previous study on the growth of cultured tumor cells in nude mice indicated that high doses of vitamin E could inhibit tumor growth (42). While this result may seem contradictory to the current report, significant differences between these exist. First, of major importance, the current study analyzes the natural development of de novo tumors induced by events similar to those that occur in human tumors. Thus, the effects observed here cannot be caused by an anomaly of tumor cells in culture. Second, in the previous study (42), non-physiological doses of vitamin E were used (10-fold higher than that in the standard mouse diet). Thus, the possibility of toxic pro-oxidant effects could not be ruled out.

Significantly, in the studies presented here there was no
obvious protective effect of antioxidant supplementation on tumor burden. This observation indicates that the effects of antioxidants on cancer development should be carefully considered. It is possible that antioxidants, such as beta-carotene, vitamins E and A, ascorbic acid, flavonoids, and others, could have different effects depending on the health status of the individual. Antioxidants, by preventing oxidant-mediated damage to diverse targets (DNA, RNA, proteins and lipids), may play a protective role in healthy individuals prior to the appearance of cancerous cells. However, by inhibiting apoptosis, these same antioxidants could exert a cancer-promoting effect in patients with pre-existing tumors or in individuals exposed to environmental carcinogens. The present study did not address overall survival times, although no obvious acceleration of tumor growth was observed in TgT121 mice fed an antioxidant-enriched diet. Antioxidant inhibition of apoptosis may explain why two large human studies observed that administering a beta-carotene supplement significantly increased lung cancer incidence in heavy smokers (13, 14). α-Tocopherol supplementation did not reduce the incidence of lung cancer, but did result in fewer cases of prostate cancer. As shown here in a preclinical transgenic brain tumor model, reduced rather than increased antioxidant intake could actually slow tumor growth. Since many anticancer therapeutic agents function by apoptotic mechanisms which involve ROS as essential mediators (43), it is possible that feeding an antioxidant-poor diet could even enhance the therapeutic effects of standard anticancer agents. Although the studies presented here are in a single tumor type, there is no a priori reason to believe that this model will not be representative of other epithelial cancer models. In fact, brain tumors are often the most resistant to therapies given their selective isolation from the bloodstream. The CP tumors studied here are initiated by events common to most, if not all, human tumors, and are developed de novo from normal precursor cells in their natural physiological environment. The current study provides an intriguing foundation for further preclinical assessment of this approach.

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