Dietary Depletion of Vitamin E and Vitamin A Inhibits Mammary Tumor Growth and Metastasis in Transgenic Mice1,2

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ABSTRACT We showed previously that dietary antioxidant depletion enhances tumor reactive oxygen species (ROS) and apoptosis, resulting in a reduction in brain tumor size in the TgT121 transgenic mouse model, a nonmetastatic tumor model. Here, in a transgenic mouse model of mammary tumorigenesis with defined rates of tumor growth and lung-targeted metastasis, we determined the ability of dietary antioxidant depletion to inhibit tumor growth and metastasis. Compared with control mice fed a standard diet, antioxidant-depleted mice exhibited tumor-targeted generation of ROS manifested by increased levels of oxidatively modified DNA/RNA (8-hydroxy-2′-deoxyguanine, 8-hydroxyguanine) and lipid peroxidation (4-hydroxy-2-nonenal) in primary and metastatic tumor foci. In addition to increased tumor-targeted ROS, the number of apoptotic cells was increased approximately 500% (P < 0.01) and terminal dUTP nucleotide DNA end-labeling–positive cells 200% (P < 0.01) in mice fed the antioxidant-depleted diet, whereas the percentage of tumor cells undergoing mitosis was >50% lower than in controls (P < 0.01). The proportional distribution of small (<1.5 cm) and large (≥1.5 cm) primary mammary tumors differed. The mice fed the antioxidant-depleted diet had more small primary tumors (P < 0.05) and fewer large primary tumors (P < 0.05). Importantly, they also had fewer lung metastatic tumor foci compared with mice fed the control diet (4.5 ± 1.3 vs. 15.8 ± 8.5 foci/lung, P < 0.01). These findings may be important in understanding the role of dietary antioxidant vitamins in tumor growth and metastasis.

KEY WORDS: ● antioxidant ● apoptosis ● mammary neoplasm ● metastasis ● reactive oxygen species

Many recent reports indicate that reactive oxygen species (ROS)4 are essential for the triggering and completion of apoptosis (1–3). Importantly, it is becoming clear that scavenging of ROS by antioxidants can delay or inhibit apoptosis (4,5); thus it is reasonable to suggest that dietary antioxidants might favor the survival of premalignant cells and thereby promote tumorigenesis, whereas an increase in ROS accumulation might promote tumor apoptosis and inhibit tumor growth. In our initial studies, we showed that dietary antioxidant depletion, but not supplementation, enhanced the levels of tumor ROS and apoptosis, and inhibited brain tumorigenesis in TgT121 transgenic mice (6). Although the TgT121 brain tumor model is well defined and suitable for investigating the role of targeted genetic alterations in de novo tumorigenesis (7), these tumors do not metastasize. Because morbidity and mortality in human cancer patients are greatly affected by tumor metastasis, it is important to understand how this stage of tumor progression is affected by dietary antioxidant depletion.

Numerous studies showed that tumor growth is inversely related to the rate of tumor apoptosis (8–10). Importantly, in human breast cancers and related tumor models, a low apoptotic index [terminal dUTP nucleotide DNA end-labeling (TUNEL)] or inhibition of apoptosis (e.g., overexpression of Bcl-2 or sFas; p53-defects) is related to increased rates of lymph node and liver metastases (11–13). This was confirmed by animal studies showing that induction of apoptosis inhibits the metastatic ability of xeno-transplanted human breast cancer cells (14). Although the mechanisms are not yet clear, these studies suggest that the availability of regimens to modulate tumor-targeted apoptosis could lead to more successful control of mammary tumor progression and metastasis.

In a variety of hormone-related human cancers, including breast cancer, tumor size and progression increased as serum vitamin E increased and malondialdehyde, a product of lipid peroxidation, decreased (15–17). Moreover, feeding animals a diet supplemented with vitamin E, or a combination of phenolic antioxidants, suppressed apoptosis and enhanced the metastatic ability of carcinogen-induced tumorigenesis (18–20). Because overgeneration of ROS can increase apoptosis, modulation of the intake of antioxidants in the diet could affect the survival of preneoplastic cells and thus the rate of tumor progression.
and metastasis. Here we test the hypothesis that dietary depletion of antioxidant vitamins A and E inhibits de novo mammary tumorigenesis in MMTV-PyV-mice. In this transgenic mouse tumor model, primary mammary tumors are detectable by palpation in 8- to 9-wk-old mice and lung metastases appear by 15 wk (21). Because the rate of tumorigenesis is well characterized in these mice and lung metastases occur in all mice at a predictable age, we used this model to test the effects of antioxidant depletion on mammary tumorigenesis and metastasis.

MATERIALS AND METHODS

Transgenic mice and diets. The MMTV-PyV transgenic mice harbor the polyomavirus middle T antigen gene driven by the mouse mammary tumor virus (MMTV) regulatory elements. Expression of the middle T antigen is targeted to the epithelial cells of the mammary gland and results in de novo mammary alveolar adenocarcinomas due to altered activity of src-related kinases and phosphatidyl inositol-3-kinase (21). Mice were fed a standard (control) AIN-93G diet containing an AIN-93-VX vitamin mix providing the following antioxidants: 0.8 g/kg diet vitamin A (all-trans-retinyl palmitate) and 15 g/kg vitamin E (all-rac-a-tocopherol acetate) (22). The antioxidant-depleted diet was devoid of vitamins A and E as described previously (6). The standard AIN-93G diet contains 2 antioxidant supplements, namely, vitamins A and E, which were excluded from the antioxidant-depleted diet. Vitamin C was not included in the standard diet because mice, like most rodents, synthesize vitamin C and do not require this antioxidant vitamin in their diet.

Tumorigenesis in transgenic mice. Mice at 9 wk of age were fed the standard or depleted diet for 6 wk at which time they were killed and mammary glands and lungs were obtained and prepared for histological examination. All mice remained healthy throughout the course of the experiment. The size of the grossly dissected tumors was measured using the formula: mean diameter = (A + B)/2, where A and B are the maximum orthogonal diameters. Harvested tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and random 5-μm serial sections were prepared in duplicate and stained with hematoxylin and eosin (H&E). The number of metastatic tumors and metastatic tumor foci in lung was determined from duplicate random 5-μm sections stained with H&E based on previously described morphological features of mouse mammary tumors (23).

Analysis of apoptosis and mitosis. The apoptotic index (the percentage of cells undergoing apoptosis) in H&E stained slides was determined from cell counts of 300 cells in randomly selected microscope fields (40X objective) defined by a calibrated ocular grid (J.R. McCrone). The occurrence of apoptosis was confirmed using the TUNEL assay (24) as described previously (25,26). Positive controls were from regressing rat mammary gland (ApopTag, Oncor) in which sections stained with H&E based on previously described morphological features of mouse mammary tumors (23).

Measurement of oxidative stress. Oxidative stress was measured in primary and metastatic tumors and in normal tissues by immunochemistry and an image analysis method. Briefly, tissue sections were probed with a mouse monoclonal antibody (QED Bioscience) that recognizes 8-hydroxy-2′-deoxyguanosine and, to a lesser extent, 8-hydroxynucleoside, end-point biomarkers of oxidatively modified DNA and RNA, respectively (27). Additional sections were probed with a mouse monoclonal antibody (Oxis Health Products) that recognizes 4-hydroxy-2-nonenal (4-HNE), a product of lipid peroxidation. Sites of antibody binding in tissues were visualized using the ABC method (Vector Laboratories). The intensity of generation of oxidatively modified residues was determined by measuring the optical density of brown immunohistochemical reaction using an unbiased image analysis method. Positive controls were from choline-deficient rat liver in which high levels of oxidative DNA impairment and lipid peroxidation occur. Negative controls in which sections received PBS in place of the primary antibody showed no staining. Image capture, measurement, and analysis were performed using a Nikon FXA microscope and the public domain NIH Image program version 1.61 as described previously (6,28).

Statistical analysis. Data are presented as means ± SEM. Differences between the control and depleted mice were assessed at a single time point and were evaluated using an unpaired or paired t test. For all comparisons, the α level was set a priori at 0.05.

RESULTS

There were no differences between the groups in body weight throughout the experiment (Fig. 1). All mice fed the control or depleted diet developed highly vascularized mammary tumors that metastasized to the lungs. To assess the effects of antioxidant level on the growth of primary tumors, we measured tumor size and mitotic activity at the time of killing. The percentage of mitotic tumor cells was lower in mice fed the depleted diet (0.02 ± 0.003 vs. 0.05 ± 0.009%, P < 0.01), compared with mice fed the control diet. To confirm the effects of dietary antioxidant level on tumor growth, we assessed the proportional distribution of small (<1.5 cm) and large (≥1.5 cm) primary mammary tumors at the time of killing (Table 1). There was a significant shift toward the presence of small primary tumors in mice fed the diet depleted of antioxidants, consistent with a reduction in total tumor burden, compared with mice fed the control diet.

Tumor oxidative stress and apoptosis are increased in antioxidant-depleted mice. To understand how dietary antioxidant depletion inhibits tumor growth, we investigated the relation between antioxidant level and tumor apoptosis (Fig. 2). The percentages of morphologically apoptotic cells (Fig. 3A) and TUNEL-positive cells (Fig. 3B) in primary tumors were significantly greater in mice fed the depleted diet compared to mice fed the control diet.

To determine whether increased tumor oxidative stress is...
associated with increased tumor apoptosis and reduced tumor burden, we measured the levels of DNA/RNA-oxidative damage in tissues using a well-characterized antibody that recognizes oxidized guanine residues. Oxidative damage in primary mammary tumors was significantly greater in antioxidant-depleted compared with control mice (Fig. 3C). In contrast, the groups did not differ in the intensity of oxidative stress in normal bronchial epithelium (data not shown).

**Increased tumor oxidative stress and apoptosis are associated with decreased metastasis.** In normal MMTVPyVT mice, primary mammary adenocarcinomas metastasize exclusively to lung tissue. Histological examination of lung tissue from mice fed the standard diet demonstrated the presence of multiple foci of metastatic mammary adenocarcinoma. The intensity of 4-HNE, a stable product of lipid peroxidation, was significantly greater in primary and metastatic tumor foci in mice fed a diet depleted of antioxidants compared with the control diet (Fig. 4). In contrast, a much smaller increase in 4-HNE was found in normal tissue in mice fed the depleted diet compared with controls (data not shown). The diet depleted of antioxidants significantly increased apoptosis detected by TUNEL labeling in metastatic foci (Table 2). To understand how increased tumor apoptosis affects tumor metastasis, we examined the size and frequency of metastatic tumor foci in lung tissue. The size of metastatic foci did not differ between depleted and control mice (data not shown). However, the number of metastatic tumor foci in lung tissue was significantly lower in mice fed the diet depleted of antioxidants (Table 2).

**TABLE 1**

<table>
<thead>
<tr>
<th>Mammary tumor size distribution in MMTVPyVT mice fed a standard or antioxidant-depleted diet¹,²</th>
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<tr>
<td>% of total primary tumors</td>
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<td>Small (&lt;1.5 cm)</td>
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<tr>
<td>(Range)</td>
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<tr>
<td>Large (≥1.5 cm)</td>
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<tr>
<td>(Range)</td>
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¹ Values are means ± SE, n = 9–10 animals of two independent studies, as indicated by the range of data in a given treatment group; * different from standard, P < 0.05.

² The complete experiment was replicated with similar results (~50% reduction in large tumors).

**FIGURE 2** Localization of tumor apoptosis and TUNEL labeling in primary mammary tumors in MMTVPyVT transgenic mice fed a standard or antioxidant-depleted diet beginning at 9 wk of age for 6 wk. Compared with primary mammary tumors of mice fed the standard diet (A, C), more classically apoptotic cells (B) and TUNEL-positive cells (brown label, D) were detected in primary tumors of mice fed the antioxidant-depleted diet. A and B: H&E; C and D: diaminobenzidine chromagen with methyl green nuclear counterstain. Magnification: 630X.

**FIGURE 3** Decreased primary tumor burden associated with increased tumor apoptosis detected by classical apoptotic morphology (A) and TUNEL labeling (B), and increased tumor oxidative stress (C) in MMTVPyVT transgenic mice fed a standard or antioxidant-depleted diet beginning at 9 wk of age for 6 wk. Tumor apoptotic cells were identified using classical apoptotic morphology and the TUNEL assay. Oxidative stress was measured by the rate of oxidation of guanine residues, a well-established marker of oxidative stress, using a monoclonal antibody and an unbiased image analysis method (3). Representative results from 1 data set are shown. The full experiment was replicated with similar results (200–300% increase in tumor apoptosis and oxidative stress in antioxidant-poor mice). Values are means ± SE; number of tumors analyzed: standard, n = 25 tumors/9 mice; depleted, n = 19 tumors/10 mice. **Different from standard, P < 0.01.
Antioxidant-depleted diet. Mice were treated as described in Figure 2 and the localization of 4HNE, a stable product of lipid peroxidation, was determined. Values are means ± SE, n = 6. **Different from controls, P < 0.01.

DISCUSSION

In earlier studies using the TgT121 transgenic brain tumor model, we demonstrated that dietary antioxidant depletion inhibited de novo tumorigenesis due to enhanced tumor-ROS and apoptosis (6). Here, using a de novo transgenic mouse mammary tumor model, we present data that support our original hypothesis and show that dietary antioxidant depletion significantly limits the size of primary mammary tumors. Both primary and metastatic tumor foci exhibited significantly increased, tumor-targeted, oxidative stress and apoptosis in mice fed an antioxidant-depleted diet. These responses were specific to tumor tissues because no increases in ROS or apoptosis were observed in normal tissues. Importantly, feeding a diet depleted of antioxidant vitamins decreased the rate of mammary tumor metastasis in MMTVpYVT transgenic mice.

Studies in a variety of tissues show that an imbalance in proliferation and apoptosis that favors cell survival can occur at very early stages in tumor development. Thus, in rat liver, high proliferation and low apoptosis favored the development of preneoplastic foci (29), whereas in human colonic adenomas, decreased sensitivity to apoptosis was detected before the appearance of actual colon carcinomas (30). Mammary tumor progression, like that in liver and colon, is also regulated by the balance of tumor cell proliferation and apoptotic cell death. The extent of mammary tumor apoptosis may be part a reflection of the function of genes that regulate apoptosis. Studies show that loss of the genes that underlie the completion of apoptosis, or increased expression of genes that inhibit apoptosis, can render tumor cells resistant to common forms of apoptosis. In developing breast cancers, dysregulation of p53 and loss of proapoptotic bax expression favored the acquisition of an invasive phenotype (31). Oxidative stress can decrease cell survival independently of p53 status in human mammary tumor cell lines in cell culture (32,33). Here we showed that switching tumor-prone mice that express wild-type p53 from an antioxidant-replete diet to one that is depleted of antioxidant vitamins A and E resulted in tumor-targeted oxidative stress and apoptosis that had important effects on tumor growth and metastasis.

ROS are principally generated endogenously as a consequence of leakage of electrons from the mitochondrial electron transport chain during the aerobic production of ATP. The major ROS species generated are superoxide anion (O2•−) and hydroxyl radicals (•OH). Production of these highly reactive moieties can result in the formation of hydrogen peroxide (H2O2), a potential source of additional hydroxyl radicals via Fenton-type reactions involving transition metal ions (e.g., Fe2+). Although ROS generated at low intracellular levels can promote the proliferative stimulus provided by mitogens (34), it is well established that increased generation of ROS plays a central role in the initiation and completion of many forms of apoptosis (35–39). Our studies on dietary modulation of transgenic mouse tumorigenesis are consistent with these findings and indicate that mouse mammary tumors are more sensitive than normal tissues to the induction of ROS-mediated apoptosis.

In studies of archival tissues, most types of human cancer had lower levels of antioxidant enzymes and thus increased sensitivity to oxidative stress compared with their normal tissue counterparts (40,41). Importantly, lower catalase activity was found in a series of breast cancer patients compared with healthy controls (42), consistent with reports showing that malignant cells from diverse types of tumors overgenerate ROS and thus are prone to oxidative stress (43–45). Thus, given the important role played by ROS in most forms of aerobic apoptosis, it is reasonable to suggest that the survival of tumor cells could be affected more by a shift in the tissue redox balance than normal cells. Here we report that feeding a diet depleted of antioxidant vitamins A and E had no apparent effects on normal tissues, but increased tumor-ROS and apoptosis, resulting in striking decreases in both primary and metastatic mammary tumor burden.

Tumor metastasis is a major cause of morbidity and mortality in patients with breast cancer (46). Studies in xenograft models show that the rate of metastasis is strongly influenced by the size of the primary mammary tumor (47), whereas in a model of melanoma, increased sensitivity to apoptosis resulted in decreased tumor growth and metastasis (48). Consistent with these reports, our studies in MMTVpYVT mice suggest that reduced mammary tumor metastasis in mice fed an antioxidant-depleted diet may be related to decreased mitosis and increased apoptosis and a resulting decreased size-range of primary mammary tumors. Furthermore, our observation that increased oxidative stress and apoptosis occur in metastatic foci as well as primary mammary tumors suggests that biological factors common to primary and metastatic foci may underlie the effects of diet on tumor growth and metastasis, although the mechanism involved is likely to be very complex.

Human intervention trials using antioxidant supplements have produced mixed results (49,50). This is not surprising given that many so-called antioxidants have both antioxidant

### TABLE 2

**Rate of tumor metastasis and TUNEL labeling (apoptosis) in metastatic lung foci in MMTVpYVT transgenic mice fed a standard or antioxidant-depleted diet**

<table>
<thead>
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<th>Treatment</th>
<th>TUNEL(+) (%)</th>
<th>Foci/Lung, n</th>
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<tbody>
<tr>
<td>Standard</td>
<td>0.6 ± 0.15</td>
<td>15.8 ± 8.5</td>
</tr>
<tr>
<td>Depleted</td>
<td>1.3 ± 0.2**</td>
<td>4.5 ± 1.3**</td>
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1 Values are means ± SE; ** different from standard, P < 0.01. The number of tumors/group: standard diet, n = 95 metastatic foci/6 mice; antioxidant-depleted diet, n = 27 metastatic foci/6 mice.
and prooxidant properties, in addition to other properties unrelated to redox status. In addition, the response to a given type of antioxidant (e.g., vitamin E) is strongly affected by dose level and the presence of multiple and diverse analogs of the antioxidant (51–54). The notion that tumor-targeted increased ROS can be achieved through manipulation of the level of antioxidant vitamins in the diet, resulting in increased tumor apoptosis and delayed tumor progression, does not contradict the protective role for antioxidants against the induction of premutagenic DNA damage in completely normal cells in healthy individuals. Numerous studies showed that oxidatively stressed tumor cells produce higher levels of ROS and related products than normal cells (55). Consistent with these reports, studies showed that feeding mice a diet depleted of vitamin E can prevent de novo mouse liver tumorgenesis (18,20), whereas in humans with colonic adenomas, a high intake of vitamin C was associated with reduced colorectal premalignant DNA damage in completely normal cells (56). Consistent with our findings and suggest that dietary antioxidant depletion could reduce the rate of tumorgenesis in diverse tissues.

LITERATURE CITED


