Dietary Walnut Suppressed Mammary Gland Tumorigenesis in the C(3)1 TAg Mouse

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Walnuts contain multiple ingredients that, individually, have been shown to slow cancer growth, including omega-3 fatty acids, antioxidants, and phytosterols. In previous research, consumption of walnuts has slowed the growth of implanted breast cancers. We wanted to determine whether regular walnut consumption might reduce the risk for developing cancer. Homozygous male C(3)1 TAg mice were bred with female SV129 mice consuming either the control AIN-76 diet or the walnut-containing diet. At weaning, the female hemizygous pups were randomized to control or walnut-containing diets and followed for tumor development. Compared to a diet without walnuts, consumption of walnuts significantly reduced tumor incidence (fraction of mice with at least one tumor), multiplicity (number of glands with tumor/mouse), and size. Gene expression analyses indicated that consumption of the walnut diet altered expression of multiple genes associated with proliferation and differentiation of mammary epithelial cells. A comparison with another dietary intervention indicated that the omega 3 content alone did not account for the extent of tumor suppression due to the walnut. The results of this study indicate that walnut consumption could contribute to a healthy diet to reduce risk for breast cancer.

INTRODUCTION

Many scientists now think that diet can alter carcinogenesis (1). Epidemiology studies have tried to identify individual components of whole foods that might reduce risk for cancer; however, these studies often indicate little or no benefit to consuming a specific dietary component. Clinical trials that have used specific supplements [i.e., calcium (2), calcium and wheat bran (3), selenium, and vitamin E (4)] often demonstrate little or no effect of the supplement. However, people consume whole foods; the multiple ingredients in individual foods, as well as accumulated amounts of these components within a whole diet, could act additively or synergistically to contribute to reduction of risk for disease, including cancer. In support of this idea, one preclinical study reported that very low doses (more similar to dietary doses) of selenium and docosahexaenoic acid in combination were more effective against cancer than either of these components individually at high doses (5).

Walnuts contain multiple ingredients (6) that individually have been reported to reduce cancer risk or growth rate. These ingredients include omega-3 fatty acids (7,8); phytosterols, especially β-sitosterol (9,10); and antioxidants (11,12). We have previously reported that walnut in the diet of mice (human equivalent of 2 servings a day) would reduce the growth rate of implanted human breast cancers (13). We have also reported that canola oil—which contains α-linolenic acid, the 18C omega-3 fatty acid incorporated in the maternal diet during gestation and lactation of offspring—significantly reduced mammary gland cancer risk in the offspring (14). Walnuts contain the highest fraction of α-linolenic acid of any tree nut (15). The current study was designed to determine whether the risk for developing breast cancer might be reduced by regular walnut consumption, perhaps due to the high α-linolenic acid content.

In designing mouse diets that were applicable to human consumption, we used a 10% corn oil diet as the control diet. This diet has an omega-6 fatty acid (linoleic acid) content that is approximately equivalent to the linoleic acid content in the American diet. It turned out that the α-linolenic acid content of the walnut diet was equivalent to the α-linolenic acid content of the canola oil diet, giving us the added value of comparing 2 interventions containing the same amount of α-linolenic acid to determine if the α-linolenic acid content of the walnut diet might be the primary cancer suppressive component of walnut.

The C(3)1 TAg transgenic mouse is a well-characterized breast cancer model and the females of these mice develop mammary gland cancer at a predictable rate (16,17). As in human tumors, the early stages are estrogen receptor α (ERα) positive but become ERα negative in later stages (17). The cancer development is slow enough that a dietary modification can make a difference in tumor development. Use of this model allowed us to control the maternal diet as well as the diet of the experimental
offspring. However, as with any dietary modification study, we cannot determine if subtraction of a detrimental component or addition of a beneficial component determined the difference in tumorigenesis.

When a dietary component is routinely consumed within a population, the offspring are exposed to the component during gestation and lactation as well as by consumption after weaning. This notion is duplicated by the experiment reported here. Exposure to the walnut included in the maternal diet during gestation and lactation, as well as by individual consumption after weaning, provided significant protection from mammary gland cancer in the offspring. The reduction in cancer risk cannot be explained solely by the omega-3 content of the diet. Increased consumption of walnut could be part of a healthy diet and reduce risk for cancer in future generations.

**METHODS AND MATERIALS**

**Animals**

Breeding pairs of mice bearing a transgene for the SV40 large T antigen with a C3(1) rat prostate steroid binding protein promoter were obtained from Dr. Jeffrey Green. The female transgenic mice are expected to develop mammary gland cancer due to expression of the large T antigen in the mammary gland (17). The transgenic line is maintained in the laboratory and all mice were genotyped to ensure presence of the transgene. Twenty female SV129 mice, 6 wk old, were obtained from Charles River Laboratories (Wilmington, MA), quarantined for 2 wk, and then moved to a study room. All animal work was approved by the Marshall University School of Medicine Institutional Animal Care and Use Committee.

**Study Design**

SV129 females (breeder females) were split into 2 groups and numbered for identification. Ten female mice were placed on a diet containing 10% w/w corn oil (control diet, see below) and 10 female mice were placed on a diet containing walnuts (test diet). The compositions of the diet and of the dietary fat are listed in Table 1. After 2 wk, these females were bred with homozygous C3(1)/TAg male mice. The hemizygous female pups from these breedings were the experimental mice—not the wild-type mother mice. Pups were weaned at 21 days old and were randomized to the 2 diets, generating 4 experimental groups: corn oil/corn oil (CO/CO), corn oil/walnut (CO/walnut), walnut/walnut, or walnut/corn oil (walnut/CO) (the first diet was the maternal diet; the second diet was the pup’s diet). The offspring were housed 3 to 4 in a cage, individually numbered for identification, and weighed weekly.

**TABLE 1**

Compositions of the diets

| Diet Compositions | Corn Oil (Control) Diet | Both Diets | Walnut Diet (18% of calories from walnut) |
|-------------------|------------------------|------------|----------------------------------------|
|                   | g/kg diet              | Calories/g | % of total energy | g/kg diet | protein: 17.2 g | Fat: 73.7 g |
| Cassein           | 200                    | 0.80       | 19.2                  | 183 g     | 183 g           | Fat: 73.7 g (4.6 g water in walnut) |
| Sucrose           | 450                    | 1.80       | 43.4                  | 450 g     | 450 g           | Fiber: 2.0 g |
| Corn starch       | 150                    | 0.60       | 14.6                  | 135 g     | 135 g           | Carbohydrate: 15.5 g |
| Alphacel          | 2.0                    | 0.00       | 0                     | 2 g       | 2 g             | Carbohydrate: 15.5 g |
| Choline bitartrate| 3.0                    | 0.00       | 0                     | 3 g       | 3 g             | Carbohydrate: 15.5 g |
| DL-methionine     | 0.10                   | 0.00       | 0                     | 10 g      | 111 g           | Carbohydrate: 15.5 g |
| AIN-76 Mineral mix| 0.35                   | 0.02       | 0.4                   | 35 g      | 35 g            | Carbohydrate: 15.5 g |
| AIN-76A Vitamin mix| 0.10                  | 0.030      | 0.7                   | 10 g      | 10 g            | Carbohydrate: 15.5 g |
| Ground walnut     | 0                      | 0.00       | 0                     | 111 g     | 111 g           | Carbohydrate: 15.5 g |
| Fat (10% total fat)|                       | 0.90       | 21.7                  | 26.3 g    | 26.3 g          | Carbohydrate: 15.5 g |
| Totals            | 1,000 g                | 4.15 cal/g | 99.9                  | 1,003 g   | 1,003 g         | Carbohydrate: 15.5 g |

Fat in diet

| Fat composition (g/100 g) of corn oil and walnut from Ref. 15. Small amounts of various lipids make up the remainder of the fat and are not listed. Shown are the compositions of the corn oil and walnut-containing diets of this article. The canola oil diet, referred to in Fig. 4, had 10% canola oil instead of 10% corn oil and is detailed in Ref. 21. |
Diet

Diet compositions were prepared in the Marshall University School of Medicine animal diet prep room. Diet composition is shown in Table 1 and was formulated to be isocaloric, isonutrient, and more relevant to human consumption than the very high-fat diets used in many studies. If a Western diet contains about 14.7 g of linoleic acid/day (18), the calories from linoleic acid are 6.6% of a 2,000 calorie diet. The 10% corn oil mouse diet contained 10.9% of calories from linoleic acid. The walnut-containing diet was formulated to contain 18% of calories from walnut. This approximates a human diet that includes 2 servings (2 ounces) of walnuts per day. (Two ounces of walnuts, 28 halves, equals 370 calories; 370 calories = 18.5% of a 2,000 calorie diet.) The AIN-76A diet is adequate for the nutritional support of the mice (19). The dry ingredients of the diet, except sugar, were purchased locally (100% corn oil, no additives or preservatives). Walnuts were provided by the California Walnut Commission and kept frozen at −20°C until used. Walnuts (with brown pellicle but without shells) were ground fine in a blender for mixing into the diet. Batches of diet were prepared as needed, about each 2 wk. The diet mixture was pressed into trays and cut into small squares. Individual cage-sized portions (25–30 g) were stored in sealed containers at −20°C to prevent oxidation of the fat and bacterial growth in the food. Mice had free access to food and water and were fed fresh food 5 days per wk. Food removed from cages was discarded.

Transgene Copy Number

Real-time PCR was used to verify the presence of the transgene in all experimental pups, as previously described (14). Primers for the transgene (SV40 forward: ATA TGC CTT CAT CAG AGG AAT ATT C; SV40 reverse: TAA AGT TTT AAA CAG AGA GGA ATC TTT GC) and the VIC labeled SV40PROBE (VICCCC AGG CAC TCC TTT CAA GAC CTA GAA GGMMBFQ), β-actin primers and PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). The rTPCR assay was performed according to the Applied Biosystems instructions on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) instrument.

Body Weights

Body weights were measured each week and terminally. Statistical differences in mean body weight change between groups were determined using analysis of variance (ANOVA) and Prism (Graphpad, Inc.) software.

Tumor Incidence, Multiplicity, and Weight

Mice were palpated for tumors 3 times weekly from 90 days of age. Total tumor incidence, multiplicity, and weights were determined at necropsy. The differences between groups and across time were statistically analyzed by Kruskal-Wallis, 2-way ANOVA, χ², Fisher exact test, or Mann-Whitney test as appropriate using Prism software (Graphpad, Inc., La Jolla, CA).

Necropsy

Mice were euthanized at 110, 130, and 145 days of age. The earliest time for tumors was expected to be 110 days of age; mice were euthanized thereafter to follow the increase in tumor incidence and multiplicity. The left fourth mammary gland was quickly removed and frozen in liquid nitrogen. All 10 mammary glands were examined for the presence of a tumor 1 mm or larger. All tumors detected were measured, removed, and weighed; thus total tumor weight and numbers include tumors that were too small to be detected by palpation. If a tumor was large enough for further assay, it was flash-frozen in liquid nitrogen. The number of tumors in each gland and the number of glands with tumor were recorded for every mouse. Some mice in the CO/CO group had to be euthanized (due to large tumor size) before a scheduled 145 days, thus slightly skewing (to a smaller size) the results for tumor mass in this group.

Gas Chromatography

The fatty acid compositions of mammary glands at 130 days of age were analyzed by gas chromatography. Frozen tissues were thawed and homogenized in distilled water containing 0.1% BHT to prevent oxidation of the fatty acids. Lipids were extracted with chloroform/methanol, and the fatty acids were methylated followed by separation and identification using gas chromatography, as previously described (20). ANOVA followed by a Bonferroni posttest was used to determine statistical differences of individual fatty acids between dietary groups.

Gene Expression Assay

The Mouse Signal Transduction Pathway Finder RT² Profiler PCR Array, PAMM-014 (SuperArray Bioscience Corporation, Frederick, MD) was used to analyze the expression of 84 genes in 3–4 mammary glands per group at 130 days of age mice. (The complete list of genes on the plate can be found at www.sabiosciences.com/rt_pcr_product/HTML/PAMM-014A.html.) The Signal Transduction pathway finder array was used because, a priori, we did not know what genes or pathways might be influenced by consumption of the walnut diet. Mammary gland without macroscopic tissue was analyzed to obtain changes due to the diet not to the presence of a tumor. This array profiles genes in 18 different pathways that could be applicable to cancer development. Frozen tissue was homogenized in Tri Reagent (Sigma-Aldrich, St. Louis, MO) following the protocol of the manufacturer to isolate the RNA. RNA quality control was performed for all samples to ensure the purity and integrity of the RNA on an Agilent 2100 Bioanalyzer (Santa Clara, CA). The RT2 First Strand Kit was used to make cDNA; the cDNA was then quantitatively amplified by real-time PCR using an ABI Prism 7000 (Applied Biosystems, Foster City, CA) and RT2 qPCR Master Mix (Superarray) according to the manufacturer’s protocol. The protocol and software provided by SuperArray were followed to determine relative fold difference in gene expression using
some of the extra weight gain in the CO/walnut groups. However, there was no significant difference in tumor incidence and size were the CO/CO and walnut/walnut groups. However, there was no significant difference in body weight (mean ± SD; CO/CO = 24.85 ± 1.6 g vs. walnut/walnut = 24.25 ± 1.4 g) at 135 days (before any tumor cachexia). The mean tumor mass of the CO/CO group was 1/3 of the variance in body weight; thus it does not seem that the tumor mass was a significant portion of the mouse weight gain though certainly some of the extra weight gain in the CO/CO group could be due to tumor growth. An important point here is that free access to walnut, a high-calorie food, in the diet did not increase weight gain as would be a concern of many readers. Clinical trials have also indicated that addition of walnut to the diet does not increase body weight or cause weight gain in people (21–23).

Fatty Acid Composition of the Diet and of the Mammary Gland

Table 1 shows that the corn oil diet contained more oleic acid (monounsaturated) and saturated fat but that the walnut diet contained more linoleic acid and α-linolenic acid. The fat composition of the mammary gland (Fig. 1) reflects the fat composition of the diet; that is, the mammary glands of mice that consumed the walnut diet after weaning contained significantly less oleic acid and significantly more linoleic acid, α-linolenic acid, and total omega-3 fats than did the mammary glands of the mice that consumed the corn oil diet after weaning [2-way ANOVA (fatty acid and diet group) followed by Bonferroni posttest, P < 0.05]. There were no other fatty acids that were significantly different due to diet.

Tumor Incidence and Multiplicity

At 110 days of age, the first euthanasia point, no mice had tumors. Fig. 2 shows the tumor size, incidence, and multiplicity at 130 and 145 days of age. At 130 days it appears that consumption of walnut is increasing the latency of tumors; however, largely due to the early stage of tumorigenesis, neither the tumor mass (Fig. 2A, Kruskal Wallis, P value for walnut/walnut vs. CO/CO = 0.06), multiplicity (Fig. 2B, number of glands with
FIG. 2. Mammary gland tumors were quantified at euthanasia at 130 and 145 days of age, \( n = 10-13 \) mice per time point and diet group. By 145 days, the total tumor mass, number of glands with tumor (multiplicity), and tumor incidence of the walnut/walnut group was significantly less than of the corn oil/corn oil group. Consumption of walnut after weaning (corn oil/walnut group) also decreased the multiplicity, mass, and incidence or tumors (groups which share a letter are not significantly different \( P < 0.05 \), statistical test stated in Results).
DIETARY WALNUT SUPPRESSED BREAST CANCER

By 145 days, suppression of tumorigenesis by the walnut-containing diet was clearly evident. The median tumor size of the walnut/walnut group was significantly less than of the CO/CO group (Fig. 2D, \( P < 0.05 \), Kruskal-Wallis followed by Dunn’s multiple comparison test). (Some mice in the CO/CO group had to be euthanized due to large tumor size before the scheduled 145 days, thus slightly skewing the results for tumor mass in this group to a smaller mass than if the mice had lived to 145 days old.) The multiplicity of tumor was decreased in both groups that consumed walnuts after weaning, with the multiplicity of tumor in the walnut/walnut group being significantly less (Fig. 2E, \( P < 0.05 \), Kruskal-Wallis followed by Dunn’s multiple comparison test) than in the group not exposed to walnut (CO/CO). The tumor incidence was less in both groups that consumed walnut after weaning than in the group not exposed to walnut (Fig. 2F, CO/CO) with the tumor incidence in the walnut/walnut group being significantly less (Fig. 2E, \( P < 0.05 \), Kruskal-Wallis followed by Dunn’s multiple comparison test) than in the group not exposed to walnut (CO/CO). Tumor incidence was decreased 40% compared to the CO/CO group when walnut was consumed after weaning; however, with 10 mice in a group this did not reach significance.

PCR Array Analyses for mRNA Expression Followed by Western Blot

The mRNA from a total of 84 genes was quantitatively assayed by the Mouse Signal Transduction Pathway Finder RT\(^2\) Profiler PCR Array. Table 2 lists the 41 genes of these genes in which expression in the mammary gland was changed either more than twofold or with a \( P \) value < 0.05 by \( t \)-test (using the manufacturer’s statistical analyses software). Genes in the arrays are listed in the applicable pathways at the bottom of Table 2 with genes that were significantly altered by walnut consumption shown in italics. The expression of many genes in pathways associated with mitogenesis, survival, or NfkB and other signaling has been altered by exposure to dietary walnut.

We noted that a number of the genes with altered expression are in the NfkB signaling pathway, so 3 of these genes [NfkB inhibitor alpha (IkB, Nfkbia), IkB kinase (IKK, Ikbkb) and nitric oxide synthase 2 (iNOS or Nos2)] were chosen for Western blot analyses. Western blot results (Fig. 3) were in general agreement with the PCR array analyses for these genes and with
| Symbol                                                      | Fold Difference CO/Walnut vs. CO/CO | Fold Difference Walnut/Walnut vs. CO/CO | Fold Difference Walnut/CO vs. CO/CO |
|-------------------------------------------------------------|-------------------------------------|----------------------------------------|-------------------------------------|
| Activating transcription factor 2 (ATF2)                    | 2.597                               | 1.737                                  | 1.838                               |
| Bcl2-associated X protein (BAX)                              | 107.535                             | 119.263                                | 105.420                             |
| B-cell leukemia/lymphoma 2 (Bcl2)                            | 27.338                              | 11.867                                 | 24.098                              |
| Baculoviral IAP repeat-containing 2 (Birc2)                  | 4.328                               | 1.896                                  | 3.375                               |
| Bone morphogenetic protein 2 (BMP2)                         | 17.182                              | 19.865                                 | 12.914                              |
| Bone morphogenetic protein 4 (BMP4)                         | 3.254                               | 3.592                                  | 4.228                               |
| Cyclin-dependent kinase inhibitor 2A (Cdkn2a or p16)         | 950.691                             | 216.092                                | 308.865                             |
| CCAAT/enhancer binding protein (C/EBP), beta                 | 2876.968                            | 790.342                                | 2152.305                            |
| Chemokine (C-X-C motif) ligand 1 (Cxc11)                     | 9.309                               | 4.728                                  | 3.732                               |
| Early growth response 1 (EGR1)                              | 472.608                             | 363.002                                | 168.410                             |
| Engrailed 1 (En1)                                            | 21.449                              | 6.895                                  | 43.236                              |
| Fas (TNF receptor superfamily member)                       | 5.794                               | 1.782                                  | 5.084                               |
| Fatty acid synthase (Fasn)                                   | 7.350                               | 5.280                                  | 8.649                               |
| FBJ osteosarcoma oncogene (FOS)                              | 67.275                              | 59.356                                 | 29.446                              |
| Growth arrest and DNA-damage-inducible 45 alpha (Gadd45a)   | 3.519                               | 3.156                                  | 2.362                               |
| Gene regulated by estrogen in breast cancer protein (Greb1)  | -2.328                              | -4.923                                 | -4.131                              |
| Glycogen synthase 1, muscle (Gys1)                           | -2.717                              | -2.213                                 | -2.085                              |
| Hedgehog-interacting protein (Hhip)                         | 2.111                               | -3.308                                 | 4.285                               |
| Hexokinase 2 (Hk2)                                           | 35.886                              | 24.499                                 | 29.719                              |
| Heat shock factor 1 (Hsf1)                                  | 15.378                              | -1.034                                 | 13.462                              |
| Insulin-like growth factor binding protein 4 (Igfbp4)        | 18.214                              | 24.584                                 | 19.904                              |
| Inhibitor of kappaB kinase beta (Ikbkb)                     | -2.727                              | -6.874                                 | -3.456                              |
| Interleukin 2 (Il2)                                          | 2.749                               | 4.986                                  | 1.260                               |
| Interleukin 2 receptor, alpha chain (Il2ra)                  | -4.650                              | -12.622                                | -5.448                              |
| Interleukin 4 receptor, alpha (Il4ra)                        | -6.221                              | -8.601                                 | -6.317                              |
| Jun oncogene (Jun)                                           | 506.529                             | 2.230                                  | 465.456                             |
| Transformed mouse 3T3 cell double minute 2 (Mdm2)            | 71.937                              | 29.135                                 | 53.199                              |
| Matrix metalloproteinase 10 (Mmp10)                         | 2.350                              | 3.487                                  | 1.275                               |
| Matrix metalloproteinase 7 (Mmp7)                           | 2.285                              | 10.664                                 | 3.647                               |
| Myelocytomatosis oncogene (Myc)                              | 1.739                              | -4.569                                 | 1.360                               |
| Ngn-A binding protein 2 (Nab2)                              | -7.938                              | -2.961                                 | -8.042                              |
| Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (Nfkbia) | 20.971                              | 12.874                                 | 18.358                              |
| Nitric oxide synthase 2, inducible, macrophage (Nos2)       | -3.080                              | -2.922                                 | -1.813                              |

(Continued on next page)
the actual tumor incidence results. That is, compared to protein abundance in the mammary glands of the CO/CO group, protein abundance of Nfkbia was increased and of Ikbkb was decreased in the walnut/walnut group. Since Nfkbia binds and prevents activation of NFkB and Ikbkb cleaves NFkB to allow activation of NFkB, these coordinated changes would be expected to decrease NFkB activation and to be associated with suppression of cancer risk (24). Nfkbia was also increased in the CO/walnut and in walnut/CO groups but Ikbkb was not changed. These results would be expected to be associated with less tumor suppression in the CO/walnut and walnut/CO groups than in the walnut/walnut group. Inducible nitric oxide synthase (Nos2) mRNA was slightly decreased but protein was barely detectable in any group and was not changed by a walnut-containing diet.

**Comparison of Tumor Multiplicity With a Diet Containing the Same Omega-3 Amount as the Walnut Diet**

We have previously reported partial results of a study in which incorporation of canola oil instead of corn oil was the dietary intervention (14). The experimental design was the same
Epidemiology studies indicate that some populations have lower incidences of cancer (1,25), the challenge is to identify the foods that contribute to reducing cancer risk given the complex compositions of whole foods. Another challenge in all dietary studies is to determine whether it is the addition of a beneficial component or the subtraction of a detrimental component that provided the benefit against cancer.

The primary aim of this study was to assess the effects of walnut consumption on mammary gland cancer risk. The results presented herein indicate that walnut consumption could significantly alter expression of multiple genes and mammary gland cancer development. Walnut consumption by both mother and offspring, as would naturally occur when walnuts are consumed as part of the usual diet of a population, did significantly reduce mammary gland cancer development and reduced multiplicity in this transgenic mouse. Consumption of walnut after weaning, as might occur when children migrate to a different environment or choose to add walnut to their diet, also slowed mammary gland cancer development and showed a 40% reduction in tumor incidence and a 44% reduction in multiplicity compared to mice not exposed to walnut. The next question is: What component of walnut was effective at slowing carcinogenesis?

Our first hypothesis was that the increased omega 3 content and decreased omega-6 content of the diet-reduced carcinogenesis. Long-chain omega-3 fatty acids have been shown to slow breast cancer growth in multiple animal studies (7,26–30) and have been proposed for cancer prevention (30). Conversely, omega 6 fatty acids, especially linoleic acid as found in corn oil, have been shown to increase carcinogenesis (31–33). In order to maintain balanced fat in the diet, if one species is increased (α-linolenic) then another (linoleic) has to decrease. We had data from another recently completed study (14) that provided information that indicated that increasing the α-linolenic content contributed to reduced mammary gland cancer risk. After doing the calculations we realized that the canola oil and walnut diets contained the same amount of omega-3 in the form of α-linolenic acid. The canola oil containing diet did significantly suppress tumorigenesis compared to the corn oil containing diet; however, the walnut diet resulted in an additional significant suppression of tumorigenesis. Thus, some component of the diet, in addition to the increased α-linolenic content (or reduced linoleic acid), was functioning to suppress carcinogenesis.

There are reports that β-sitosterol can suppress cancer cell growth (34–36) and walnuts contain a significant amount of β-sitosterol (6,37). However, in assessing the β-sitosterol composition of the diets (using composition of the diet and Ref. 15), we found that the β-sitosterol content for the corn oil diet was 968 mg/kg; for the canola oil diet was 413 mg/kg; and for the walnut diet was 71 mg/kg. The total β-sitosterol content of the walnut diet was less than that of the corn oil diet. Most of the references for benefit of β-sitosterol against cancer do not use breast cancer cells, which might be stimulated by binding of β-sitosterol to the estrogen receptor, but use other cell types that are not usually considered estrogen dependent. Interestingly, we did find 1 paper (9) indicating that β-sitosterol may increase breast cancer cell growth in estrogen responsive cells; this would agree with our data. The tumors formed by this C3(1)/SV40 TAg model have been well characterized (16,17). Green et al. reported that

Atypia of the mammary ductal epithelium develops at about 8 wk of age, progressing to mammary intraepithelial neoplasia (resembling human ductal carcinoma in situ) at about 12 wk of age with invasive carcinomas at about 16 wk in 100% of female mice. The tumors appear hormone responsive at early stages, invasive carcinomas are hormone independent, which corresponds to loss of ERα during progression. (16) (p. 1020)
Tumors were evident sooner in the mice that consumed the corn oil diet, perhaps in response to the \( \beta \)-sitosterol stimulation of ER\(\alpha\). At later times, when tumors became invasive and should be ER\(\alpha\)-, the growth of tumors of mice that consumed the corn oil diet was not as influenced by corn oil diet. More research will be needed to decide this question.

Assessment of vitamin E in the diets did provide a lead to an additional active component that was common to the diets. We found the gamma tocopherol is associated with slowing cancer cell growth (38–40) whereas alpha tocopherol did not have benefit against cancer and may block some of the activity of gamma tocopherol. Assessing the tocopherol content of the diets revealed that for the corn oil diet, alpha tocopherol = 14.3 mg/kg and gamma tocopherol = 0; for the canola oil diet, alpha tocopherol = 17.46 mg/kg, gamma tocopherol = 27.34 mg/kg; for the walnut diet, alpha tocopherol = 1.77 mg/kg and gamma tocopherol = 22.9 mg/kg. The changes of alpha and gamma tocopherol are clearly in the direction that according to the work of others would indicate benefit against cancer. Studies could be devised to test this question.

**CONCLUSION**

These data indicate that exposure to a small amount of walnut in the diet of this transgenic mouse slowed the development and reduced the multiplicity of mammary gland cancers but does not define the mechanism of action for the walnut nor an active ingredient of the walnut. Walnut in the diet was associated with alterations in cell signaling pathways involved in proliferation, cell differentiation, and apoptosis. The signaling pathways altered in mammary glands of these mice have been identified as important in the development of human breast cancer, thus this study should be relevant to humans. The fatty acid composition of the mammary glands was altered but comparison to another study with the same amount of omega 3 fatty acids in the diet indicates that increased omega 3 fatty acids in the mammary gland does not explain the altered tumor incidence. However, alterations in dietary gamma tocopherol were inversely associated with tumorigenesis.

More work will need to be done to determine the components of walnut and the mechanisms associated with tumor suppression. However, humans eat the whole nut, not specific components. It seems likely that incorporation of walnuts as part of a healthy diet could reduce the risk for breast cancer in humans.

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