In utero exposure to maternal diets containing soy protein isolate, but not genistein alone, protects young adult rat offspring from NMU-induced mammary tumorigenesis

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Abstract

The mechanisms by which dietary factors confer protection against breast cancer are largely unknown, and undoubtedly, complex. However, we (11) and others (12,13) have suggested that induction of the apoptotic pathway that normally opposes uncontrolled cell proliferation is predominantly involved in the reduction of tumor initiation and progression associated with dietary protective effects. The apoptotic pathway is normally dysregulated in cancer cells (14); hence, activation of the ‘death machinery’ and associated ‘death genes’ responsible for the elimination of cells with DNA damage (mutations) accrued through endogenous metabolic (oxidative) stressors or exogenous carcinogenic agents provides a strong rationale to this possibility. Owing to the high number of identified pro- and anti-apoptotic genes that are functionally compromised leading to neoplastic transformation (15), a definitive determination of the likely gene targets for dietary protective effects remains challenging. Nevertheless, our previous studies have indicated that the pro-apoptotic, tumor suppressor phosphatase and tensin homolog deleted in chromosome ten (PTEN), which inhibits Akt activation (16), is induced by pure GEN as well as by serum from rats fed lifetime diets of SPI or supplemented with GEN (11).

The concept of developmental plasticity originally proposed by Prof. Barker (17) considers diet/nutrition as a viable environmental influence contributing to the fine-tuning of genetic susceptibility to adult diseases, including cancer. This hypothesis presupposes that there are critical periods during very early development (e.g. in utero) that are vulnerable to environmental factors that can lead to modified responses to genetically altering agents and that may underlie adult disease states. Using a well-described rat model of mammary carcinogenesis (18,19), we evaluated the hypothesis that exposure only in utero to diets containing the soy isoflavone GEN can confer protection against mammary tumors induced by carcinogen, at a later life stage. We demonstrate here that in utero exposure to soy protein isolate (SPI) used in infant formula was associated with increased tumor latency, decreased tumor multiplicity and decreased tumor pathology relative to the control (CAS) diet. We also show that GEN alone did not mimic the mammary tumor protective effects of SPI when administered under a similar developmental context, and that lifetime exposure to SPI, although tumor protective, elicited higher grade tumors than when exposure was restricted to the gestation period. Further, we provide evidence suggestive of distinct signaling pathways altered by dietary exposure at specific developmental windows.

Introduction

Cancer is a genetic disease elicited by mutations in tumor suppressors, oncogenes and DNA instability genes, allowing for the clonal expansion of damaged cells leading to neoplasia (1). Breast cancer is the second leading cause of death and the most common malignancy among women, with >200 000 new cases predicted in 2006 in the USA alone. The overall survival rate for breast cancer has increased significantly within the last 15 years (2) owing in part to advances in detection procedures and improved and increasingly accessible treatment modalities; however, the goal for the eradication of this disease remains untenable, due largely to the complexity of genetic alterations that are involved in its development and progression (3). There are several well-accepted risk factors for adult onset of breast cancer in a given population, and one such factor is attributable to diet/nutrition. Indeed, the linkage of high intake of soy foods by Asian women, several well-accepted risk factors for adult onset of breast cancer in the USA (4), has promoted vigorous scientific inquiries into the antitumor properties of soy and soy phytochemicals including genistein (GEN) (6,7). Further, the exploration of the market for dietary supplements based on soy protein extracts, as well as other bioactive compounds found in green tea, curry and fruit extracts (8–10), is indicative of the growing acceptance that cancer prevention can be achieved through improved nutrition.

Materials and methods

Animal studies

Animal protocols were approved by and in compliance with guidelines from the University of Arkansas for Medical Sciences Committee on the Use and Care of Animals. Time-mated Sprague–Dawley rats were purchased from Charles Rivers Laboratories (Wilmington, MA) and housed individually in polycarbonate cages under conditions of 24°C, 40% humidity and a 12 h light–dark cycle. Rats at gestation day 4 were randomly assigned to semi-purified isocaloric diets made according to the American Institute of Nutrition-93R formula (20), with corn oil substituting for soybean oil and containing the following as sole protein source: (i) CAS (New Zealand Milk Products, Santa Rosa, CA); (ii) CAS to which GEN was added in the aglycone form (250 mg/kg feed) (Sigma Chemical Co., St Louis, MO) and (iii) SPI (Solae Company, St Louis, MO). The latter contained 394 ± 16 mg total isoflavones/kg diet, including 216 ± 2 mg/kg GEN and 160 ± 6 mg/kg daidzein, both expressed as aglycone equivalents (18,19). Animals were provided food and water ad libitum. Figure 1
summarizes the four dietary regimens evaluated. At delivery [post-natal day (PND) 0], pups from dams fed SPI throughout pregnancy were pooled and randomly assigned to these dams for suckling. The dams were divided into two diet groups as follows: one group was continued on SPI (SPI group), whereas the other group was switched to the CAS diet (SPI–CAS group). Pups assigned to dams fed CAS + GEN throughout pregnancy and switched to CAS during lactation represented a third diet group (GEN–CAS group). A fourth diet group consisted of pups from dams fed CAS during pregnancy and through lactation. Each dam was assigned 10 pups (five per sex) for suckling. Female pups were weaned at PND21 to the same diet as their assigned dams for lactation and were continued on these diets throughout the study. At PND50, female pups (n = 10 each for CAS, SPI and SPI–CAS; n = 5 for GEN–CAS) were killed and the inguinal mammary gland (gland number 4) pair was removed. Portions of the left gland were fixed for paraffin embedding, whereas the right gland was immediately homogenized in TriZol (Invitrogen, Carlsbad, CA) for RNA extraction. Male pups were used in unrelated studies.

**Carcinogen administration and tumor analysis**

At PND51, the remaining female pups were administered the alkylating agent N-methyl-N-nitrosourea (NMU, Lot #AS1-701, Ash Stevens, Detroit, MI) freshly dissolved in 0.9% saline solution (pH 4.6) at a dose of 50 mg/kg body wt, via jugular vein injection. Rats were weighed weekly and beginning at 2 weeks after NMU treatment were palpated on a weekly basis for mammary tumors. For each rat, the initial detection date of palpable tumors, the subsequent detection dates of new tumors and their locations and the total tumor numbers were recorded. Rats from all diet groups were killed when 80% of CAS-fed rats showed palpable tumors (99 days post-NMU). Tumors from each rat were dissected, weighed and preserved in 10% neutral buffered formalin. Tissue sections from the largest tumor for each tumor-bearing rat were stained with hematoxylin–eosin and histologically classified by a board-certified pathologist, as described in previous studies from this group (19).

**Immunohistochemistry**

Mammary glands were fixed overnight in 10% neutral buffered formalin, dehydrated with gradient alcohol and embedded in paraffin. Tissue sections were stained using the following: (i) anti-phospho-p53Ser46 (P-p53Ser46, Cell Signaling Technology, Danvers, MA); (ii) anti-PTEN (A2B1, Santa Cruz Biotechnology, Santa Cruz, CA); and (iii) anti-epithelial cadherin (E-cadherin) (BD Transduction Laboratories, San Jose, CA), following previously described protocols from this group (11,21) or the manufacturer’s instructions. Antigen retrieval in Citra Plus (Biogenex, Milpitas, CA) and incubation with blocking solution (Casblock, Zymed, San Francisco, CA) to minimize non-specific binding were also described previously (21). Immunoperoxidase staining was developed with 3,3′-diaminobenzidine chromagen (Dako Corp, Carpinteria, CA), and slides were counterstained with hematoxylin or methyl green. For PTEN and P-p53Ser46, four randomly chosen fields (×200) per slide per rat (n = 3–4 individual rats per diet) were evaluated for numbers of dark brown color-staining cells, indicating positive expression. For E-cadherin, 10 randomly chosen fields per slide per rat (n = 4–5 individual rats per diet) were independently scored for immunostaining intensities by three laboratory members who were presented with the slides in a blinded fashion. A scoring range of 1 (moderate staining) to 3 (highest staining) was used.

**RNA extraction and quantitative real-time reverse transcription—polymerase chain reaction**

Total RNA was isolated from mammary glands using TriZol reagent according to the manufacturer’s instructions. Integrity of isolated RNAs was confirmed using the RNA 6000 Nano LabChip kit with the Agilent Bioanalyzer System (Agilent Biotechnologies, Palo Alto, CA). The CDNA was generated using 1 μg total RNA with random hexamers and MultiScribe Reverse Transcriptase kit (Bio-Rad Laboratories, Hercules, CA). The primer sequences for rat PTEN, Bax, p21 and 18S have been described in previous studies (11,22). Polymerase chain reaction mix (25 μl) contained 10 nM of primers, 10 ng of cDNA and 2× SYBR Green PCR Master Mix (Bio-Rad Laboratories). Polymerase chain reaction conditions were as follows: 50°C, 2 min and 95°C, 1 min and 40 cycles of 95°C, 15 s and 60°C, 1 min. Relative levels of messenger RNA were normalized to 18S ribosomal RNA and expressed as arbitrary units.

**Results**

**Diet effects on mammary tumor parameters**

Female offspring exposed to lifetime CAS or SPI served as negative and positive controls, respectively, for the mammary tumor protective effects of lifetime-fed diets (19). The appearance of palpable tumors occurred earlier with the CAS group, with 50% of rats showing at least one tumor by 63 days post-NMU (Figure 2A). In contrast, for the other groups fed SPI or GEN, the appearance of at least one tumor in 50% of the rats was not observed until 71 (GEN–CAS), 77 (SPI–CAS) and 78 (SPI) days post-NMU, respectively (Figure 2A). Therefore, whereas rats in the SPI–CAS and GEN–CAS groups rapidly accumulated tumors, reaching tumor incidence comparable with those of the CAS group by 99 days post-NMU, the number of tumor-bearing rats in the SPI group remained relatively constant (Table I and Figure 2A). At the conclusion of the study (PND149), the SPI group had ~20% lower tumor incidence (P = 0.062) compared with the other diet groups.

Analysis of tumor multiplicity among the diet groups indicated lower tumor numbers per tumor-bearing rat in the SPI–CAS group, relative to the other diet groups (Table I). Indeed, whereas only 2% of the tumor-bearing rats in the SPI–CAS group showed more than seven tumors, reaching tumor incidence comparable with those of the CAS group by 99 days post-NMU, the number of tumor-bearing rats in the SPI group remained relatively constant (Table I and Figure 2A). The largest mammary tumor from each rat of all diet groups was histologically analyzed and scored as benign (normal mammary tissues with fibrosis), precancerous (intra-ductal proliferation) or cancerous [ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC)] lesions. The incidence of benign and precancerous lesions did not differ among the diet groups (Table I). Further, the diet groups showed comparable incidence (percentage) of tumors designated as cancerous lesions (DCIS + IDC) (Table I). However, the SPI group showed numerically higher percentage of the more invasive tumor (IDC), whereas the SPI–CAS group had the lowest percentage of this tumor grade (Table I) among the other three groups. In particular, SPI–CAS-fed rats exhibited a greater percentage of IDC (60%) than IDC (30%) when compared with those fed lifetime SPI (P < 0.004).

**Diet effects on mammary apoptosis**

Expression of Tp53 phosphorylated at Serine 46 (immunoreactive P-Tp53Ser46) was used as a readout for apoptosis since phosphorylation of Tp53 at this serine residue is a hallmark of apoptotic pathway activation (23). Mammary terminal end buds (TEBs) of PND50 rats fed lifetime SPI had higher numbers of cells showing Tp53Ser46 immunoreactivity relative to those of the other diet groups (P < 0.001).
which exhibited comparable levels (Figure 3). Ductal epithelium (DE) structures showed minimal immunostaining for Tp53Ser46, albeit the trend (higher for SPI than for all the other diet groups; P = 0.067) was similar to that observed for TEBs.

Diet effects on mammary gene expression
The transcript levels of the pro-apoptotic genes Bax, p21 and PTEN did not differ in mammary tissues of rats from the different diet groups (data not shown). Consistent with our previously published studies (11), lifetime dietary exposure to SPI (SPI group) had no effect on mammary expression of p21 and Bax, relative to those of rats fed the control diet CAS. Dietary exposure to SPI only in utero (SPI-CAS group) mimicked the patterns of p21 and Bax expression of the SPI group. In contrast, whereas lifetime exposure to GEN increased the mammary transcript levels of p21 and Bax, relative to those of CAS (11), in utero exposure only to GEN (GEN-CAS) did not affect the expression of these genes when compared with the CAS group.

Diet effects on mammary PTEN protein levels
The number of PTEN immunopositive cells was evaluated in mammary TEB and DE of PND50 rats exposed to lifetime SPI or only in utero (SPI-CAS and GEN-CAS). The number of cells with nuclear and cytoplasmic PTEN immunostaining was recorded separately for each mammary structure, since cellular localization of PTEN has been linked to distinct effects on cell cycle arrest and apoptosis (24). Expression of PTEN in TEB (quantified as numbers of PTEN immunopositive cells per structure) was significantly higher (P < 0.05) for the SPI group relative to the two other diet groups, irrespective of whether nuclear or cytoplasmic PTEN immunoreactivity was evaluated (Figure 4A). A similar trend for DE was observed between SPI and the other two diet groups (Figure 4B), although the differences were not significant.

Diet effects on E-cadherin expression
PTEN is reported to form a complex with membrane-bound E-cadherin, a tumor suppressor, and β-catenin, leading to lower β-catenin activation (25) and decreased tumorigenesis (26). Because increased PTEN immunoreactivity in mammary structures is associated with lifetime dietary exposure to SPI, but not with in utero-only exposure to SPI or GEN (Figure 4), we evaluated E-cadherin distribution in mammary tissues of rats of the different diet groups by immunohistochemistry. E-cadherin localized at sites of cell–cell contacts in mammary TEB and DE and was not detected in the cytoplasmic compartment (Figure 5). Mammary TEB and DE of rats of the four diet groups showed greater membrane E-cadherin immunoreactivity in SPI-CAS and GEN-CAS groups relative to SPI and CAS groups, respectively. (Figure 5A–C).

Discussion
The present study evaluated the consequence of limited early developmental exposure (in utero) to diets containing the soy isoflavone GEN on mammary tumor development in a rat model of mammary carcinogenesis. We report here that for young adult female rats exposed to the carcinogen NMU, in utero-only exposure to SPI (SPI–CAS group) was associated with increased tumor latency, decreased tumor multiplicity and lower incidence of more invasive tumors (e.g. IDC), relative to the control diet CAS. Moreover, in utero exposure to supplemental GEN alone (GEN–CAS group) largely mimicked the tumor parameters observed for the CAS group, with the exception of higher tumor latency. Further, lifetime dietary SPI exposure (SPI group) decreased tumor incidence that was not mimicked by in utero

Table I. Mammary tumor parameters and developmental stage of dietary exposure

<table>
<thead>
<tr>
<th>Rats/diet</th>
<th>Tumor incidence</th>
<th>Tumor multiplicity</th>
<th>Benign</th>
<th>IDP</th>
<th>DCIS</th>
<th>IDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS</td>
<td>47</td>
<td>80.85%</td>
<td>4.4 ± 0.533</td>
<td>1 (2.6%)</td>
<td>2 (5.3%)</td>
<td>15 (39.5%)</td>
</tr>
<tr>
<td>SPI</td>
<td>44</td>
<td>61.36%</td>
<td>3.3 ± 0.492</td>
<td>2 (7.4%)</td>
<td>0 (0%)</td>
<td>7 (25.9%)</td>
</tr>
<tr>
<td>SPI-CAS</td>
<td>47</td>
<td>85.11%</td>
<td>2.2 ± 0.231</td>
<td>2 (5%)</td>
<td>2 (5%)</td>
<td>24 (60.0%)</td>
</tr>
<tr>
<td>GEN-CAS</td>
<td>39</td>
<td>82.05%</td>
<td>3.6 ± 0.502</td>
<td>4 (12.5%)</td>
<td>0 (0%)</td>
<td>12 (37.5%)</td>
</tr>
</tbody>
</table>

*Tumor incidence = percentage of tumor-positive rats at 99 days post-NMU.
*Tumor multiplicity = average number of tumors/tumor-bearing rat.
*IDP, intra-ductal proliferation.
*Number of rats with designated tumor grade (percentage of the total tumor-bearing rats).
*P < 0.05, relative to CAS (by one-way analysis of variance); and **P = 0.004, relative to SPI (by Fisher's exact test).
exposure alone, and which was distinctly associated with increased mammary TEB apoptotic status and PTEN expression prior to NMU administration. Finally, increased membrane E-cadherin localization in mammary structures prior to NMU insult could partly underlie the tumor protective effects of in utero exposure to soy diets. Taken together, these results suggest that restricted dietary exposure to SPI at a very early developmental window can attenuate mammary tumor progression later in life, and which may involve the induction of mechanisms for the maintenance of the E-cadherin adhesion complex (27). Moreover, these findings provide evidence for the distinct effects of diet on signaling pathways associated with the carcinogenic process, namely PTEN and E-cadherin, and set the groundwork for further identification of bioactive compounds in soy foods other than GEN, with mammary tumor protective effects (28). As regards the latter, the soy isoflavone daidzein is a likely candidate, given its ability to delay 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor development in the mouse mammary tumor virus-neu mouse model (29) and to inhibit DNA adduct formation in mammary glands of mice administered DMBA (30).

Our studies were conducted to address the developmental context of the cancer preventative effects of dietary SPI, as gleaned from epidemiological studies of Asian women with relatively low incidence of breast cancer (4,5,28). Since soy food consumption in this population occurs mostly for lifetime, the consequence of limited exposure was higher than that of lifetime exposure, when the period of dietary intake of SPI, relative to that of lifetime SPI exposure, does not pick up mammary tumorigenesis, when the period of exposure is restricted to pregnancy. An important practical implication of these studies is that the period of dietary intake can be manipulated for maximal health benefits.

The lower tumor multiplicity and tumor grade with in utero-only exposure to dietary SPI, relative to that of lifetime SPI exposure, suggest the ability of SPI to influence fetal mammary gland development with positive outcome against adult risk of mammary cancer. In rodents, late gestation (6 to 7 days before birth), when primary ducts begin to form at the epithelial bud site in response to signals from the surrounding fat pad, is considered a critical period of mammary gland development (33,34). The comparable developmental period for human fetal breast occurs earlier at around 12–14 weeks of embryonic life (34,35). Our data indicate that in utero events leading to the induction of E-cadherin-mediated cell–cell adhesion by SPI in young adult (PND50) mammary gland may contribute to tumor protection (27,36), and raise the possibility of epigenetic changes involving a subset of mammary epithelial cells that are preserved (imprinted) during development (37). Although in utero-only GEN exposure similarly elicited induction of membrane E-cadherin expression at a level comparable with that of SPI–CAS in PND50 mammary glands, the lack of a significant mammary tumor protection with this dietary

In utero SPI exposure and mammary tumor protection

Fig. 3. Expression levels of phosphorylated Tp53 protein in mammary glands of PND50 rats of the different diet groups. The numbers of immunopositive cells for Tp53 phosphorylated at serine 46 (p-Tp53Ser46) in mammary TEB and DE structures were counted from four randomly chosen fields (×200 magnification). Values are means ± SEMs from n = 7 (CAS), 3 (SPI), 4 (SPI–CAS) and 4 (GEN–CAS) individual animals. Differences were identified by one-way analysis of variance, followed by Tukey’s test. TEB, means with different superscripts differed at P < 0.05.

Fig. 4. Tumor suppressor PTEN expression in mammary glands of PND50 rats fed lifetime SPI or in utero-only SPI and GEN-based diets. The numbers of PTEN immunopositive cells in nuclear and cytoplasmic compartments of mammary TEB (A) and DE (B) were counted from four randomly chosen fields (×200 magnification) per slide per rat, using two slides per tissue block from each of n = 3–4 rats per diet group. Differences were identified by one-way analysis of variance, followed by Tukey’s test. Means with different superscripts differed at P < 0.05.
regimen indicates the contribution of additional ‘safeguard’ mechanisms induced by SPI, independent of GEN or related metabolites. These may involve perturbations of the developmental programming with positive effects on immune and DNA repair system maturation, ductal differentiation, glandular structure formation and tumor suppressor methylation. Finally, the inability of lifetime SPI exposure to mimic the up-regulation of E-cadherin expression by in utero exposure implies that events occurring post-natally may alter these early effects. In this regard, activation of other signaling pathways during the peripubertal growth of the mammary tissue, another critical developmental window (34), may confer the increased resistance noted for lifetime SPI diet on tumor development. Based on our previous report (11), and which we confirmed here, we posit that one such pathway involves the activation of PTEN-mediated apoptosis, leading to the early elimination of damaged (mutated) cells that can give rise to tumorigenic cells. Consistent with this, we demonstrated higher levels of cytoplasmic PTEN immunoreactivity, which has been linked to regulation of apoptosis (24), in TEB and to a lesser extent in DE, for the SPI group relative to the SPI–CAS and GEN–CAS groups. Although we are unable to provide a molecular mechanism describing post-natal activation of PTEN signaling by SPI at the present time, it is worth noting that lifetime GEN mimics the effects of lifetime SPI on mammary PTEN signaling (11), indicating that GEN induction of multiple estrogen receptor-dependent and -independent pathways (38,39) may be primarily involved in this process.

In keeping with the well-acknowledged premise that the genesis of the neoplastic process is multifactorial (1,40) and that the development of a malignant solid tumor in adults requires at least three distinct mutations (41), we posit that dietary mammary tumor protective effects must involve at least three distinct signaling pathways, which are altered early on during mammary development. Further, given that NMU-induced tumors are more hormone dependent than those induced by DMBA (42), the ability of dietary SPI to similarly inhibit tumor development initiated by both carcinogens suggests distinct mechanisms of tumor protection. The present study identifies two potential pathways, both of which involve the up-regulation of tumor suppressor expression in young adult (PND50) mammary glands prior to NMU insult. The PTEN signaling cascade is primarily involved in apoptosis; hence, its enhanced expression implies a function in the early elimination of damaged cells, thereby preventing the clonal expansion of mutated cells that subsequently acquire additional mutations upon carcinogen administration (43). The tumor suppressor E-cadherin is associated with the adenomatous polyposis coli pathway, and inhibits β-catenin from translocating to the nucleus, where it acts as proliferative stimulus (26,27,36). Interestingly, results of our studies suggest an apparent selectivity in the developmental window at which dietary SPI can initiate alterations in the expression of these tumor suppressors, since in utero-only and lifetime exposure elicited distinct levels of expression of PTEN and E-cadherin, respectively. At the present time, we do not know the distinct chronology of events leading to the up-regulated expression of PTEN and E-cadherin at PND50. Similarly, the significance of the reported association of PTEN with the E-cadherin–catenin complex reported previously for pancreatic carcinoma cells (25) is unclear in the context of the mammary epithelium. Clearly, the functional effects of soy proteins and individual biologically active components in mediating PTEN and E-cadherin expression to endow mammary epithelial cells with resistance to carcinogenic agents must be further evaluated.

Fig. 5. E-cadherin expression in mammary glands of PND50 rats of the different diet groups. Mammary sections representing individual animals (n = 4–5) of each diet group were analyzed. Representative micrographs of TEB (A) and DE (B) of PND50 rat mammary glands immunostained with anti-E-cadherin antibody. Positive staining (dark brown color; indicated by arrowheads) was localized to membranes of mammary epithelial cells. (C) Graphical representation of immunostaining intensities (means ± SEMs), with representative score of 1 for CAS–TEB and of 3 for GEN/CAS-TEB. Means with different superscripts differed at P ≤ 0.05.

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In summary, our results show that limited exposure to SPI in utero can elicit significant mammary tumor protective effects that may have increased advantage over that of lifetime exposure. We further show that exposure to supplemental GEN at a similar developmental window does not result in mammary tumor protection comparable with that of SPI. Additionally, we identified signaling pathways involving the tumor suppressors PTEN and E-cadherin as potential mechanisms underlying the tumor protective effects of dietary soy proteins at two developmental exposure contexts. We suggest that strategic approaches for the prevention of mammary and other types of cancer might be uncovered by careful refinement of the developmental window for exposure to specific dietary factors.

Acknowledgements

The authors thank the following for their contributions to this work: Dr Frank A Simmen for helpful discussions during the course of the study and critique of the manuscript; Matt Ferguson, Trae Pittman and Tammy Dallari for assistance with the animal work; Dr Sohelia Korourian (University of Arkansas for Medical Sciences) for histopathological analyses of mammary tumors; Pamela Treadaway and Mark Robinette for assistance with data collection and members of this laboratory for help in scoring immunopositive slides. Funds for this research were provided by the United States Department of Agriculture-Current Research Information System-6251-S10002-06S to the Arkansas Children’s Nutrition Center.

Conflict of Interest Statement: None declared.

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