Adolescent Vitamin A Intake Alters Susceptibility to Mammary Carcinogenesis in the Sprague-Dawley Rat

Richard P. Metz, Mark Kaeck, Maria Stacewicz-Sapuntzakis, Terry Mitrenga, Heidi McCarty, and Pepper Schedin

Abstract: We tested the hypothesis that adolescent dietary vitamin A intake impacts mammary gland development and subsequent sensitivity to carcinogenesis. Sprague-Dawley rats were fed a purified diet that was vitamin A deficient, adequate (2.2 mg retinyl palmitate/kg diet), or supranutritional (16 mg retinyl palmitate/kg diet) from 21 to 63 days of age, the period of adolescent mammary gland development. At 73 days of age, rats were given 1-methyl-1-nitrosourea (25 mg/kg body wt ip) and monitored for mammary tumors. Tumors appeared earlier and more frequently in rats fed vitamin A-deficient or -supplemented diets. Vitamin A deficiency during adolescence was associated with alveolar mammary gland development and precocious milk protein expression, while supplementation was associated with ductal gland development and suppression of milk protein expression. Differences in circulating estradiol and mammary gland estrogen receptor-α, and estrogen-responsive progesterone receptor mRNA were not observed, suggesting that the effects of vitamin A on mammary gland development and carcinogenesis are estrogen independent. Mammary expression of another hormone receptor that regulates milk protein expression, the glucocorticoid receptor, was also unaffected. These results demonstrate that vitamin A intake during adolescence alters mammary gland differentiation and indicate that a narrow range of vitamin A intake during adolescence protects against carcinogenesis.

Introduction

The American Cancer Society estimates that >190,000 new cases of invasive breast cancer will be diagnosed in 2001. It is estimated that 39,600 women will die of breast cancer in the United States in 2001, making it the third leading cause of death for American women. Recent efforts to reduce breast cancer-associated mortality and morbidity have focused on prevention. The years between onset of puberty and first childbirth have been identified as the most crucial in establishing future risk of developing breast cancer (1). On the basis of these observations, strong arguments have been made for shifting the focus of breast cancer prevention strategies to youth (1–3).

The etiology of breast cancer is intimately associated with breast organogenesis (4–11), which occurs primarily during the adolescent growth spurt. In the United States, mammary development occurs on average between 8 and 16 yr of age (12). Exposure to radiation (4,6–8) or elevated levels of putative carcinogens (9) during adolescence increases breast cancer risk more than similar exposures later in life. Research has confirmed that the actively maturing mammary gland is highly sensitive to cancer initiation events (13,14). Cumulatively, these studies strongly suggest that events occurring during the period of mammary gland development are critical determinants of an individual’s subsequent breast cancer risk. A better understanding of adolescent mammary development and how it relates to breast cancer risk, is therefore, anticipated to provide valuable insights into possible chemopreventive strategies.

Substantial data suggest that retinoids, natural or synthetic derivatives of vitamin A, have chemopreventive activity in the breast. Evidence that retinoids are effective anti-breast-cancer agents in rodents was first published in 1976 (15). Since then, extensive experimental evidence has demonstrated that retinoids can prevent and reverse mammary tumorigenesis in rodent models (see Refs. 16 and 17 for review). In addition, human epidemiological studies have suggested an inverse relationship between dietary retinoid intake and breast cancer risk. The Harvard Nurses’ Study followed the eating habits and medical histories of >84,000 female nurses over an 18-yr period. The researchers concluded that women with a family history of breast cancer who consumed higher amounts of α-carotene, β-carotene, and vitamin A had a slightly decreased risk of breast cancer compared with women with similar histories who consumed low levels of these retinoids (18).
On the basis of laboratory and epidemiological evidence of retinoid chemopreventive activity, human clinical trials designed to investigate the efficacy of retinoids against breast cancer were initiated. The Italian fenretinide study enlisted 2,972 women with surgically removed Stage I breast cancer or ductal carcinoma in situ and randomly assigned them to receive 4-hydroxyphenylretinamide (4-HPR) or placebo for 5 yr (19). The final end point was the occurrence of contralateral or ipsilateral breast cancer. Overall, there was not a significant reduction in breast cancer due to treatment, but there was a slight protective effect in premenopausal women who received 4-HPR (20).

The chemopreventive mechanism(s) of retinoids is not entirely understood. It is known that retinoids cause cancer cells to differentiate, thus reducing their proliferative capacity (16). In addition, inhibitory effects of retinoids on mammary tumor cell proliferation in vitro are well documented (16,17). Much of our understanding of retinoid function comes from studies evaluating the role of vitamin A during embryonic morphogenesis. In normal-developing epithelial tissues, vitamin A deficiency results in lack of differentiation and excessive cellular proliferation or epithelial hyperplasia (21,22). This hyperplasia can be reversed by vitamin A supplementation, indicating that retinoids play a key role in epithelial morphogenesis (see Ref. 22 for review). Although the role of vitamin A during embryonic epithelial development has been characterized, very little is known about retinoid effects on postembryonic epithelial development, such as that in the adolescent mammary gland. The purpose of this study was to determine whether adolescent dietary intake of retinyl palmitate would impact mammary gland development and susceptibility to carcinogenesis. We have found that mammary gland development and susceptibility to carcinogen-induced tumorigenesis are significantly affected by vitamin A intake during adolescence. Our data suggest that a narrow range of vitamin A intake during adolescence provides protection from carcinogenesis, with levels above or below resulting in increased mammary cancer risk.

Materials and Methods

Animals and Diets

The basal diet consisted of modified AIN-93G, a diet optimized for rapidly growing rats, which was formulated without vitamin A and with alcohol-extracted casein to be free of residual vitamin A (TD 98368, Harlan Teklad) (23). Vitamin A, in the form of retinyl palmitate (Sigma, St. Louis, MO), was added back to the base diet to produce the vitamin A-adequate and -supplemented diets. Female Sprague-Dawley rats (Taconic Farms, Germantown, NY) received the experimental diets from 21 to 63 days of age. The first group received the vitamin A-deficient (TD 98368) diet. The second group was fed the vitamin A-deficient (TD 98638) diet containing 4,000 IU retinyl palmitate/kg (2.2 mg/kg or 4 µM) to match vitamin A levels found in the standard AIN-93G diet. The third group was fed the vitamin A-deficient (TD 98368) diet containing 29,000 IU retinyl palmitate/kg (16.0 mg/kg or 30 µM), which provides, on average, an additional 500 IU of vitamin A per rat per day. This dose was designed to be roughly equivalent to the increased vitamin A levels attained by humans who consume over-the-counter vitamin A supplements (24). From 64 days of age until study termination, all rats received a vitamin A-adequate diet (AIN-93M, Harlan Teklad), thus limiting dietary intervention to the period of adolescent mammary gland development. The care and use of animals were in accordance with National Institutes of Health guidelines and AMC Cancer Research Center Animal Care and Use Committee regulations.

Carcinogenesis Study

On the basis of power calculations, in which a difference in tumor incidence of 20% can be detected with 95% confidence and 90% power, 45 rats were assigned to each diet group (25). Animals received the experimental test diets described above from 21 to 63 days of age and were fed a vitamin A-adequate diet for the remainder of the study. At 73 days of age, rats received a single injection of 1-methyl-1-nitrosourea (MNU, 25 mg/kg body wt ip), as previously described (26). This dose was chosen because it allows detection of decreased as well as increased tumor incidence. Rats were palpated twice per week for 6 mo for detection of mammary tumors. All tumors were excised, weighed, and processed for routine histological examination. Mammary tumors were classified histologically by the criteria of Young and Hallowes (27), and only tumors classified as adenocarcinomas were included in subsequent analyses. MNU was provided by the National Cancer Institute’s Chemical Carcinogen Reference Standards Repository operated under contract by the Midwest Research Institute (N02-CB-07008).

Analysis of Tumor Data

Analysis of variance (ANOVA) was performed to determine which group(s) differed. Tumor data generally follow a Poisson distribution, requiring square root transformation before ANOVA is done. A nonparametric k-sample test described by Lee (28) was used to test the significance of differences. Differences among groups in final cancer incidence were evaluated as described by Peto (29). Values are means ± SEM.

Developmental Study

One hundred eight 21-day-old female Sprague-Dawley rats (Taconic Farms, Germantown, NY) were randomly placed into one of the three dietary groups: vitamin A deficient, adequate, or supplemented, as described above. Twelve rats per group were sacrificed at 35, 49, and 63 days.
of age for histological and biochemical analyses. Left mammary gland chains 4–6 were harvested for whole-mount histological analyses. Right mammary gland chains 4–6 were removed, stripped of lymph node tissue, snap frozen in liquid nitrogen, and stored at −80°C for biochemical analyses. Stage of estrus was determined by vaginal smear cytology and cervical histology analyses of tissue taken at the time of sacrifice, as previously described (30).

Serum Retinol, Retinyl Palmitate, and Estradiol Determinations

Serum samples were obtained from CO₂-stunned animals by orbital eye bleed at the time of sacrifice. Blood was collected under low-light conditions into lithium heparin-containing tubes. Samples were centrifuged at 1,200 g for 10 min at 4°C, and supernatant was removed and stored at −80°C until high-performance liquid chromatography (HPLC) analysis, as previously described (31,32). Sera from animals in the proestrus (2 animals/group) and estrous (3 animals/group) stages of the estrous cycle from each group at each time point were analyzed to control for potential variation across groups due to estrous cycle. For retinol and retinyl palmitate analyses, serum (0.2 ml) was deproteinized with an equal volume of ethanol and extracted twice with 2 ml of hexane containing butylated hydroxytoluene (0.01% wt/vol). Samples were evaporated under vacuum and reconstituted with 50 μl of stabilized ether and 150 μl of mobile phase. Ten microliters of sample were injected into the HPLC system and eluted isocratically at a flow rate of 1 ml/min using methanol-acetonitrile-tetrahydrofuran (50:45:5) as the mobile phase and a Waters 15-cm NovaPak column. A Waters 490 programmable multiwavelength detector was used to detect retinol and retinyl palmitate at 325 nm. Data were processed using the Millennium computer program and custom-made software. For estradiol determinations, whole blood was collected under CO₂ anesthesia via orbital eye bleed. Blood was allowed to clot for 2 h and then centrifuged at 2,500 rpm for 20 min, aliquoted to cryovials, and stored at −80°C until analysis. Serum was analyzed from five animals per group at each time point (2 proestrus- and 3 estrous-staged animals) to control for potential variation across groups due to estrous cycle. Values are means ± SEM. Estradiol was measured in duplicate using Diagnostic System Laboratories (Webster, TX) Ultra Sensitive 4800 RIA with a minimum detection level of 2.2 pg/ml. Intra- and interassay coefficients of variation were 3% and 9.6%, respectively.

Morphological Characterization of Mammary Gland Development

For developmental characterization and terminal end bud (TEB) analyses, whole-mount preparations of mammary gland chain 4–6 were prepared and photographed as previously described (30). Total numbers of TEBs in mammary gland chain 4 were counted directly from coded photographs of whole mounts by two separate investigators. Values are means ± SEM. For histological analyses, mammary tissue adjacent to mammary gland lymph chain 4 was evaluated to minimize differences in mammary gland morphology due to regional variation. Mammary tissue was fixed in 10% neutral buffered formalin for 6 h, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin. Mammary glands were classified as ductal or lobuloalveolar, according to the methods originally described by Russo et al. (13) and Welsch and O’Connor (33). Mammary glands with small lobules composed of ≤10 acini per lobule were scored as ductal, and those with ≥11 acini per lobule were scored as alveolar.

Isolation of RNA and RNase Protection Analyses

Total RNA was isolated from pooled mammary tissue samples (mammary gland chains 4–6 with lymph nodes removed, 5 animals/group) using RNAwiz (Ambion, Austin, TX). For each test group, only mammary tissues from animals in the proestrus (2 animals) and estrous phases (3 animals) of the estrous cycle were utilized to control for potential estrous cycle variation across groups. In addition, mammary gland RNA was pooled from 21-day-old rats to represent baseline expression levels before adolescence and differential vitamin A intake. RNase protection assays were performed using RPA III kits (Ambion) according to the protocol supplied by the manufacturer. Probes were generated by reverse transcriptase-polymerase chain reaction cloning of DNA fragments amplified from adult rat mammary gland RNA into pGEM-T Easy (Promega, Madison, WI). Primers used to generate the whey acidic protein (WAP) probe amplified a DNA fragment corresponding to bases 160–385 of the reported sequence (34). Primers used to generate the progesterone receptor (PR) probe amplified a DNA fragment corresponding to bases 1580–1762 of the reported sequence (35). The sequences of the primers used to amplify estrogen receptor (ER)-α and glucocorticoid receptor (GR) DNA fragments are reported elsewhere (36,37). Probe-containing plasmids were sequence verified (University of Colorado Health Sciences Center DNA Sequencing Core Facility, Denver, CO) and linearized by restriction enzyme digestion before T7 RNA polymerase-mediated probe generation in the presence of [α-32P]UTP using Maxiscript kits (Ambion). The rat β-actin probe pTRI-β-actin-125-rat was purchased from Ambion.

Results

Effects of Dietary Retinyl Palmitate Intake on Serum Retinol and Retinyl Palmitate Levels

Rats were fed vitamin A-deficient, -adequate, or -supplemented diets from 21 to 63 days of age, the period of adolescent mammary gland growth in the rat. The effects of the 42-day dietary intervention on circulating levels of retinol and retinyl palmitate are shown in Table 1. In 63-day-old rats fed the vitamin A-adaptable diet, circulating serum reti-
levels were ~350 ng/ml. This level is slightly lower than 360–440 ng/ml reported for healthy adolescent-age girls (38).

In rats receiving the vitamin A-deficient diet, average serum retinol levels were ~93 ng/ml, significantly lower than the vitamin A-adequate group. This level is two- to threefold lower than the 270–320 ng/ml reported for healthy adolescent girls whose serum vitamin A levels fall within the bottom 10th percentile (38). Despite the level of deficiency achieved in the vitamin A-deficient rats, there was no evidence of growth suppression as measured by body weight gain or total body weight (Fig. 1, top). Retinol levels were slightly higher in the supplemented group than in controls; however, this difference was not statistically significant. In rats receiving the vitamin A-deficient diet, retinyl palmitate was undetectable, while in the rats receiving the vitamin A-supplemented diet, retinyl palmitate levels were four- to fivefold higher than in the vitamin A-adequate group. Thus, after 42 days of dietary intervention, circulating levels of retinol and retinyl palmitate were significantly altered.

**Effects of Dietary Retinyl Palmitate on Body Weight Gain**

It has been reported that chemopreventive doses of retinoids can reduce body weight (39,40). Reduced body weight

<table>
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<tr>
<th>Animal No.</th>
<th>Retinol, ng/ml</th>
<th>Avg Retinol, ng/ml</th>
<th>Retinyl Palmitate, ng/ml</th>
<th>Avg Retinyl Palmitate, ng/ml</th>
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<td>3</td>
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<td>4</td>
<td>73</td>
<td>0</td>
<td>0</td>
<td>ND</td>
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**Vitamin A adequate**

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**Vitamin A supplemented**

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<th>Retinyl Palmitate, ng/ml</th>
<th>Avg Retinyl Palmitate, ng/ml</th>
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<td>487</td>
<td>45</td>
<td>111</td>
<td>58 ± 14</td>
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<td>12</td>
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<td>58 ± 14</td>
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<td>346</td>
<td>51</td>
<td>60</td>
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<tr>
<td>15</td>
<td>342</td>
<td>60</td>
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*a:* Rats were fed vitamin A-deficient, -adequate, or -supplemented diets from 21 to 63 days of age. Serum samples were obtained from 5 rats/group determined to be in estrous (3 rats/group) or proestrous (2 rats/group) phase of estrous cycle on Day 63 and analyzed for retinol and retinyl palmitate levels by high-performance liquid chromatography. ND, not detectable.

**Figure 1.** Effects of adolescent dietary retinyl palmitate intake on 1-methyl-1-nitrosourea (MNU)-induced mammary carcinogenesis. Female Sprague-Dawley rats (45/group) received experimental diets from 21 to 63 days of age and a vitamin A-adequate diet from Day 64 to end of study. MNU (25 mg/kg body wt ip) was administered at 73 days of age. Top: body weight. Rats were weighed weekly from 21 to 250 days of age. Values are means ± SEM. Significant differences in body weight gain or total body weight were not observed at any time. Middle: mammary tumor incidence. After MNU injection, rats were monitored twice weekly for mammary tumors. Only confirmed adenocarcinomas were included in analyses. Values are means ± SEM. Significant differences in body weight gain or total body weight were not observed at any time. Bottom: mammary tumor multiplicity. Average number of tumors per animal per group was determined. Values are means ± SEM. Differences in tumor multiplicity are statistically different between vitamin A-deficient and -adequate group (P = 0.042) and between vitamin A-adequate and -supplemented group (P = 0.036, by analysis of variance and nonparametric k-sample test).
can decrease carcinogen susceptibility, producing an artificial protective effect associated with treatment that is independent of retinoid activity (41,42). The levels of retinoids utilized in chemoprevention studies are usually 300–1,000 mg/kg diet (see Ref. 43 for review). The levels of vitamin A supplementation in our studies were ~20- to 60-fold less than chemopreventive doses (2.2 and 16.0 mg/kg diet for vitamin A-adequate and -supplemented diets, respectively) and well within a physiological range. As shown in Fig. 1, top, body weight gain and total body weight were unaffected by our dietary supplementation strategy. This observation indicates that physiological doses of retinyl palmitate do not alter body weight and eliminates the possibility that our carcinogenesis data are confounded by body weight differences.

**Effects of Vitamin A Intake During Adolescence on Subsequent Mammary Tumorigenesis**

After the 6-wk period of dietary intervention, all the rats were fed a vitamin A-adequate diet for the remainder of the study. Thus the effects of ingesting different levels of retinyl palmitate were limited to adolescence. Ten days after termination of experimental diets, at 73 days of age, rats were given a single injection of MNU (25 mg/kg body wt ip). Rats were monitored for mammary tumors for 6 mo. Mammary tumors were detected in the vitamin A-deficient and -supplemented groups as early as 39 days after MNU administration (Fig. 1, middle). In contrast, mammary tumors were not detected in the vitamin A-adequate group until 81 days after MNU administration. At 6 mo after carcinogen administration, the incidence of mammary tumors was highest in the vitamin A-supplemented and -deficient groups and lowest in the adequate group (Fig. 1, middle; Table 2). Although over-and underingestion of retinyl palmitate was associated with a higher tumor incidence than the vitamin A-adequate group, the χ² test for overall difference in proportions was not statistically different. However, by 170 days after MNU administration, statistically significant differences in tumor multiplicity were noted between the vitamin A-adequate animals and the vitamin A-deficient and -supplemented animals (Fig. 1, bottom; Table 2). Vitamin A deficiency and supplementation resulted in approximately twice the average number of tumors per animal than in the vitamin A-adequate group. Mammary tumor burden was also affected by adolescent retinyl palmitate intake (Table 2). Vitamin A deficiency and supplementation resulted in increased tumor size and overall tumor burden compared with animals receiving adequate levels of vitamin A during adolescence. Cumulatively, our tumor data indicate that MNU-induced mammary tumors formed sooner and more frequently and were larger in vitamin A-deficient and -supplemented rats than in rats fed a diet containing an adequate level of vitamin A.

**Time Course of Dietary Retinyl Palmitate Intake and Serum Retinol Levels**

In the carcinogenesis study, after the 42-day adolescent feeding intervention, we observed that serum retinol levels were significantly depleted in the vitamin A-deficient group while retinyl palmitate levels were significantly elevated in the supplemented group (Table 1). To better characterize the effects of dietary retinyl palmitate intake on circulating levels of retinol and retinyl palmitate, a time-course study was initiated. Twenty-one-day-old rats were randomized into one of the three dietary groups described above (vitamin A deficient, vitamin A adequate, and vitamin A supplemented). Serum samples were obtained from rats at 35, 49, and 63 days of age for retinol and retinyl palmitate analyses. Circulating retinol levels did not differ significantly between the groups until 63 days of age, when the average serum retinol level of the vitamin A-deficient group was approximately fourfold lower than that of the vitamin A-adequate group (Fig. 2, top). Significant differences in circulating retinol between the vitamin A-adequate and -supplemented groups were not noted at any of the time points analyzed. On the basis of these data, depletion of endogenous vitamin A stores began to affect circulating retinol levels after 4 wk of dietary deficiency, while vitamin A supplementation did not affect circulating retinol levels. In contrast to the effects of dietary intervention on circulating retinol, significant differences in retinyl palmitate levels were obvious by 2 wk of dietary intervention (Fig. 2, bottom). Although low levels of retinyl palmitate were detected in the vitamin A-deficient animals at 35 days of age, retinyl palmitate was undetectable at 49 and 63 days of age. In the vitamin A-supplemented group, serum retinyl palmitate was three- to fourfold higher

**Table 2. Influence of Adolescent Vitamin A Intake on MNU-Induced Mammary Cancer**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body Wt, g</th>
<th>Cancer Incidence, %</th>
<th>Time to 1st Tumor, wk</th>
<th>Avg No. of Cancers/Rat</th>
<th>Avg Tumor Wt per Animal, g</th>
<th>Avg Tumor Wt per TBA, g</th>
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<tbody>
<tr>
<td>Vitamin A deficient</td>
<td>214.49</td>
<td>41</td>
<td>5.6</td>
<td>0.89 ± 0.20†</td>
<td>2.91 ± 0.81</td>
<td>6.89 ± 1.50</td>
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<tr>
<td>Vitamin A adequate</td>
<td>216.44</td>
<td>32</td>
<td>11.6</td>
<td>0.49 ± 0.15</td>
<td>0.87 ± 0.39</td>
<td>2.62 ± 0.89</td>
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<tr>
<td>Vitamin A supplemented</td>
<td>213.07</td>
<td>49</td>
<td>5.6</td>
<td>1.00 ± 0.26†</td>
<td>4.50 ± 1.31†</td>
<td>9.00 ± 2.25†</td>
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</tbody>
</table>

*a: Values are means ± SEM; n = 45/group. MNU, 1-methyl-1-nitrosourea; TBA, tumor-bearing animal.

*b: Statistical significance is as follows: *, P = 0.042; †, P = 0.036 vs. vitamin A-adequate group (based on Poisson distribution); ‡, P < 0.01; §, P < 0.02 vs. vitamin A-adequate group (by analysis of variance).

c: Body wt was measured at 70 days of age.

d: Only confirmed adenocarcinomas are reported.
Effects of Dietary Retinyl Palmitate on Mammary Gland Morphology

The effects of vitamin A intake on adolescent mammary gland development were assessed from whole-mounted preparations and 4-µm sections of mammary glands from rats sacrificed at 35, 49, and 63 days of age. Glands were scored as primarily ductal or alveolar on the basis of established criteria (13,33). Although mammary gland morphology ranged from ductal to alveolar in all groups, trends in development within each group were evident. Fifty-five percent of rats receiving the vitamin A-deficient diet were classified as lobuloalveolar (Fig. 3a). Mammary gland morphology of vitamin A-adequate rats was mixed, with 48% of rats having glands with primarily ductal architecture and 52% having glands with primarily alveolar architecture. When present, the alveoli seen in the vitamin A-adequate mammary glands were generally smaller and less complex than the structures present in vitamin A-deficient mammary glands (Fig. 3b). In contrast, only 33% of rats receiving the vitamin A-supplemented diet had mammary glands that were characterized as lobuloalveolar in nature (Fig. 3c). Histological analysis of thin sections demonstrated that, in vitamin A-deficient animals, lobuloalveoli were, in general, larger and had more secretory material within the lumen than mammary glands from vitamin A-adequate or -supplemented animals (Fig. 3, d–f).

It has been hypothesized that susceptibility to mammary carcinogenesis correlates with the degree of mammary gland development (13,14,44). Before ovarian hormone stimulation, which occurs between 4 and 5 wk of age in the Sprague-Dawley rat, the mammary gland consists of a simple ductal system that elongates into the fat pad at a rate equal to the growth of the animal (isometric growth). Beginning with onset of estrous cycling, the process of mammary organogenesis is rapidly accelerated. The mammary gland fills the fat pad through a process of elongation and branching of the specialized TEBs (see Ref. 45 for review). TEB number decreases rapidly from 21 to 63 days of age as the gland extends and TEBs differentiate (13). In rats, cells of the TEB have been identified as primary targets of carcinogenesis, with susceptibility to carcinogen directly relating to TEB number (13). Therefore, we determined whether the observed changes in susceptibility to mammary carcinogenesis with vitamin A intake might be due to changes in TEB number. Specifically, we would predict that TEB number might be lower in vitamin A-adequate animals than in vitamin A-deficient or -supplemented animals. Total number of TEBs was counted from photographs of whole-mounted mammary gland preparations of animals sacrificed at 35, 49, and 63 days of age (Fig. 4). The number of TEBs decreased with age as expected (13); however, significant differences in TEB number due to dietary vitamin A intake were not observed. The lack of an effect of vitamin A intake on TEB number suggests that the protective effects of the diet adequate in vitamin A were not due to a reduction in the number of TEBs, i.e., a reduction in the number of potential targets for carcinogenic transformation.

Effects of Adolescent Vitamin Intake on Milk Protein mRNA Levels

The resulting differences in mammary gland morphology due to vitamin A intake suggest that vitamin A deficiency stimulates, while supranutritional intake of vitamin A inhibits, mammary gland differentiation (Fig. 3). To further evaluate the effects of vitamin A intake on adolescent mammary gland differentiation, expression of WAP was determined. WAP is the principal whey protein in milk and provides a...
molecular marker for functional differentiation of alveolar cells. In rodents, the WAP gene is tightly regulated, with high levels of expression being detectable during mid to late pregnancy through lactation (46). WAP is also expressed at low levels during the metestrous stage of the rat estrous cycle (30). RNase protection assays were performed on mammary gland total RNA to determine whether vitamin A intake altered WAP mRNA levels. The highest level of WAP expression was observed in the vitamin A-deficient animals and the lowest in the vitamin A-supplemented animals at all time points analyzed (Fig. 5). These observations are consistent with the morphological data (Fig. 3) and suggest that low levels of vitamin A support, while high levels of vitamin A suppress, morphological and biochemical differentiation of alveoli.

Figure 3. Effects of vitamin A intake on rat mammary gland morphology after 6 wk of experimental diet. Alum carmine-stained whole-mount preparations (a–c) and hematoxylin-and-eosin-stained 4-µm sections (d–f) of 49-day-old rat mammary glands illustrate morphological changes after ingestion of diets containing deficient, adequate, and supplemented levels of retinyl palmitate from 21 to 49 days of age. Representational data are shown. a: Mammary gland from vitamin A-deficient rat demonstrating predominant alveolar development. b: Mammary gland from vitamin A-adequate rat showing moderate alveolar development. c: Mammary gland from vitamin A-supplemented rat showing predominant ductal development. d: Mammary gland from a vitamin A-deficient rat. e: Mammary gland from a vitamin A-adequate rat. f: Mammary gland from a vitamin A-supplemented rat. Scale bar, 25 µm.

Effects of Vitamin A Intake on Circulating Estradiol

Lobuloalveolar mammary gland development is primarily dependent on complex interactions between ovarian and pituitary hormones. Therefore, a plausible mechanism through which vitamin A intake alters mammary gland alveolar differentiation may involve modulation of the pituitary-ovarian hormone axis. In this study, we initially investigated whether circulating levels of estrogen were affected by vitamin A intake. Estrogen has been demonstrated to be an upstream mediator of progesterone in mammary epithelial cells (47), and progesterone plays a key role in alveolar differentiation (48,49). In addition, in vitro evidence has demonstrated that growth inhibition by retinoids occurs preferentially in estrogen-responsive cells (50–52). Further-
more, hypovitaminosis A has been reported to cause menorrhagia in women, presumably by reducing circulating levels of estradiol (53). These investigators reported that vitamin A supplementation therapy resulted in increased circulating estradiol in these patients. On the basis of these observations, we investigated whether vitamin A intake altered circulating estradiol levels in the adolescent rat. Blood samples from rats in each dietary group were obtained at 35, 42, and 63 days of age. Sera from five estrous stage-matched rats per group (2 rats in proestrus and 3 rats in estrus) were analyzed for estradiol levels by radioimmunoassay. Preliminary experiments did not detect significant differences in estradiol levels between proestrus and estrus (data not shown). Significant differences in serum estradiol levels with vitamin A dietary intake were also not detected (Fig. 6).

**Effects of Vitamin A Intake on Hormone Receptor Steady-State mRNA Levels**

An alternative mechanism by which vitamin A could modulate estrogen signaling in the mammary gland is through altered expression of ERs. To address this possibility, steady-state mammary gland mRNA levels of ER-α were determined. As shown in Fig. 7, significant differences in ER-α mRNA levels due to vitamin A intake were not detected at any time point. To further investigate whether vitamin A may be modulating estrogen function in the mammary gland, we analyzed mammary RNA for differences in expression of a known estrogen-inducible gene, the PR. The PR gene contains multiple functional estrogen-responsive elements throughout the 5′-untranslated region (54). Discernable differences in mammary PR mRNA were undetectable, indicating that ER signaling, as measured by PR mRNA levels, is unaffected by dietary vitamin A (Fig. 7). Finally, the observed effects of vitamin A on WAP mRNA levels (Fig. 4) suggest that vitamin A may modulate transcription factors required for WAP expression. The WAP promoter contains several glucocorticoid response element half-sites, which are critical for transcriptional activation (see Ref. 46 for review). Therefore, we analyzed mammary tissues for GR mRNA levels. Similar to ER-α and PR, we found no evidence of vitamin A modulation of GR during adolescent mammary gland development (Fig. 7).

**Discussion**

The purpose of these studies was to directly address the effects of dietary intake of retinyl palmitate on adolescent mammary gland development and subsequent mammary cancer risk in the Sprague-Dawley rat. The ability of retinoids to reduce mammary carcinogenesis in rodent models...
occurred in the absence of quantifiable changes in TEB

Interestingly, the observed differences in tumor multiplicity in the vitamin A-adequate rats (Fig. 1, bottom; Table 2). In the vitamin A-deficient and -supplemented rats than in the vitamin A-adequate rats (Fig. 1, middle; Table 2). Tumor multiplicity and tumor burden were also significantly higher in the vitamin A-deficient and -supplemented rats than in the vitamin A-adequate rats (Fig. 1, middle; Table 2). These observations suggest that the target population of cells susceptible to MNU may be altered by low, adequate, and high vitamin A intake during adolescence.

Our observation that vitamin A deficiency during adolescent mammary gland development increases sensitivity of the gland to chemical carcinogenesis is novel. However, this observation is consistent with results from embryonic studies, which have shown that vitamin A deficiency causes epithelial hyperplasia (21,22), a known risk factor for tumor development. In addition, our observation is consistent with epidemiological data demonstrating a correlation between low vitamin A intake and breast cancer risk in women (18). Whether vitamin A deficiency results in mammary epithelial hyperplasia in the adolescent rat mammary gland and whether such hyperplasia contributes to carcinogenesis remain to be determined.

Our observation that retinoid supplementation can increase mammary carcinogenesis in rodents is contradictory to the vast majority of chemopreventive studies, which demonstrate chemopreventive efficacy of retinoids (16,17,22,43). An important difference between our study and others, which report chemopreventive efficacy of retinoids against mammary carcinogenesis, relates to timing of retinoid treatment with respect to carcinogen administration. Our study is novel, in that retinoid treatment was restricted to adolescence, with dietary intervention ceasing 10 days before carcinogen administration. Therefore, our study evaluates the effect of vitamin A on mammary gland development and the subsequent susceptibility of the gland to carcinogen exposure. Most studies demonstrating retinoid chemopreventive efficacy have evaluated the ability of retinoids to slow or inhibit progression of existing disease by administering retinoids after carcinogen exposure (see Ref. 43 for review). Retinoids may also block the ability of a known carcinogen to initiate tumor development. Numerous studies have shown that vitamin A treatment before carcinogen administration has preventive efficacy against chemical- and hormone-induced mammary cancers in rats (56–59). However, one study has reported increased mammary carcinogenesis in response to retinoid treatment. In that study, retinyl acetate (328 mg/kg diet) or 4-HPR (728 mg/kg diet) was administered to female rats for 2 mo before carcinogen exposure (40). Retinoids were given from 40 to 100 days of age, thus overlapping the period of adolescent mammary gland development (21–63 days). Similar to our observations, these researchers found an increase in mammary adenocarcinomas in rats pretreated with retinyl acetate or 4-HPR. However, they also found that tumor number decreased if retinoid treatment was continued after carcinogen administration, demonstrating the complexity of the relationships between

![Figure 7. Retinyl palmitate intake did not alter mammary gland mRNA levels of steroid hormone receptor superfamily members estrogen receptor (ER)-α, progesterone receptor (PR), and glucocorticoid receptor (GR). ER-α, PR, and GR mRNA levels, normalized to β-actin, were determined by RNase protection analyses of total mammary gland RNA isolated at 35, 49, and 63 days of age. RNA samples were pooled from 3 rats in estrous and 2 rats in proestrous stage of estrous cycle. Several repeats of experiment failed to demonstrate differences in mRNA levels due to vitamin A intake or age. B, baseline (21-day-old virgin rats); see Fig. 5 legend for other abbreviations.](image)
time of dietary intervention, duration of retinoid ingestion, and susceptibility to chemically induced mammary carcinogenesis. These authors concluded that newly synthesized retinoids should be evaluated for chemopreventive activity against mammary cancer initiation as well as for their anti-promotional activity (40).

In addition, discrepancies between studies demonstrating chemopreventive effects of vitamin A against mammary carcinogenesis and our data may be explained, in part, by changes in body weight gain. A recent study has investigated the effects of retinyl acetate and 4-HPR on food intake, weight gain, and tumor incidence in comparison to similar reductions in weight gain induced by food restriction (42). These investigators, as well as others (39,40), have found that reduced body weight can decrease carcinogen susceptibility and may produce an artificial “protective” effect. 4-HPR (782 mg/kg) and retinyl acetate (328 mg/kg) were found to reduce mammary tumor incidence by 42% and 84%, respectively, in comparison to weight-matched control animals, which had a tumor incidence of 92% (42). Although the inhibitory effect of food restriction on tumorigenesis does not approach the level of protection provided by chemopreventive doses of retinoids, it is important to separate body weight from retinoid effects. Low doses of vitamin A were administered in our study (20- to 60-fold lower than those routinely used for chemoprevention studies). Under these dietary conditions, circulating levels of retinyl palmitate were elevated in the supplemented group (Fig. 1, middle). However, total body weight and body weight gain were unaffected (Fig. 1, top). Therefore, the observed responses to carcinogen reported here are not confounded by weight gain variables.

We have also observed that low vitamin A intake was associated with mammary alveolar development and precocious milk protein mRNA levels, while high vitamin A intake was associated with ductal development and suppression of milk protein expression (Figs. 3 and 5). Precocious mammary gland development with mild vitamin A deficiency has not been previously reported and requires further investigation. On the other hand, our observation that retinyl palmitate supplementation reduces alveolar development in the mammary gland is consistent with numerous reports describing alveolar inhibition after treatment with chemopreventive doses of retinoids (43,60). Retinoids have been shown to suppress alveolar mammary gland development in adult mice (61,62) and rats (60,63–65). Organ culture data are more variable, with inhibition (66) and stimulation (67) of alveolar differentiation reported. Our data extend these observations and suggest that physiological levels of retinoid are sufficient to inhibit mammary gland lobuloalveolar development during adolescence. The differences observed in mammary gland development between vitamin A-deficient and -supplemented animals are very similar to differences seen with high or low circulating ovarian hormone levels, respectively (30,68,69). These developmental similarities indicate that retinoids may modulate mammotrophic hormone signaling, with the actions of mammotrophic hormones being potentiated by vitamin A deficiency and suppressed by supplementation. Numerous in vivo and in vitro studies have suggested that retinoids modulate estrogen and progesterone signaling. We previously demonstrated that retinyl acetate at 1 mmol/kg diet inhibited estrogen- and progesterone-stimulated lobuloalveolar development and β-casein expression in adult rat mammary tissue without inhibiting cell proliferation (60,63). In women, the ability of 4-HPR to protect against contralateral breast cancer was only observed in menstrual cycling, premenopausal women, implicating an interaction between retinoid chemopreventive efficacy and hormone action (20). In cell culture, the growth of breast tumor cells that are ER positive is more likely to be inhibited by retinoids than ER-negative cells (50,51). In addition, ER-negative, retinoid-resistant breast tumor cells have been demonstrated to regain retinoid sensitivity when transfected with a functional ER (52). Cumulatively, these studies implicate a cross-talk mechanism between mammotrophic hormones and vitamin A signaling in mediating the effects of vitamin A on mammary gland development and carcinogenesis.

To test estradiol modulation as a potential mechanism of retinoid action, we analyzed blood for differences in circulating estradiol levels due to adolescent vitamin A intake. We found that plasma estradiol levels did not differ significantly between groups, regardless of amount of vitamin A ingested or age of the animals (Fig. 6). An alternative mechanism by which vitamin A could alter estrogen signaling without affecting circulating estrogen levels involves alterations in ER abundance. In this study, we found no evidence that ER-α levels are altered by dietary vitamin A intake during adolescence (Fig. 7). To further evaluate the potential effect of circulating retinoid on estradiol signaling within the mammary gland, we examined mammary tissue for expression of a known estrogen-responsive gene, the PR (Fig. 7). The rat PR gene has multiple functional estrogen-responsive elements throughout the 5′-untranslated region (54). As with ER levels, expression of PR was unaffected by vitamin A. Together, these observations suggest that the effects of vitamin A on mammary gland development are independent of circulating estrogen levels and indicate that the level of active estradiol within the mammary tissue itself, as measured by PR levels, is also unaffected by vitamin A intake. However, our results do not rule out the possibility that vitamin A alters estrogen signaling within specific target cells in the mammary gland. An alternative mechanism by which retinyl palmitate promotes mammary ductal development is through effects on mammary growth factors that contribute to ductal development. Retinoic acid has been reported to increase transforming growth factor-α (TGF-α) expression (70) and enhance TGF-α-mediated increases in expression of the epidermal growth factor receptor in human mammary carcinoma cells (71), both of which enhance ductal mammary gland development and are proposed to play a role in carcinogenesis (72). Whether adolescent vitamin A intake affects mammary TFG-α expression in vivo is unknown.

The prominent ductal development and inhibition of milk protein expression by supranutritional vitamin A intake
could also be the result of retinoid-mediated inhibition of alveolar mammary gland development, rather than promotion of ductal development. Alveolar mammary gland development is mainly controlled by the actions of progesterone (48,49) and prolactin (73). In PR knock-out and prolactin receptor knock-out mice, lobuloalveolar development and milk protein expression were inhibited when animals were treated with lactogenic hormones or stimulated by pregnancy (48,49,73). It has been reported that retinoids can decrease expression of the PR in mammary tumor cells in culture (74,75). Although we did not detect changes in PR mRNA expression with vitamin A intake (Fig. 7), alterations in PR protein levels or function cannot be ruled out. Recently, it has also been shown that retinoids can inhibit expression of prolactin receptors in vitro (76). Whether prolactin or prolactin signaling was affected by dietary vitamin A intake in this system remains to be determined.

The effects of vitamin A intake on WAP expression suggest that retinoids affect one or more pathways regulating milk protein expression. Regulation of milk protein genes (primarily the casein and whey proteins) is governed by complex interactions between multiple transcription factors at composite response elements in their promoters (see Ref. 46 for review). Glucocorticoids, such as hydrocortisone, play an important role in expression of milk proteins through activation of the GR transcription factor (46). The promoters of WAP and β-casein contain glucocorticoid response element half-sites that are adjacent to nuclear factor I and CAATT enhancer binding protein (C/EBP) binding sites, respectively. As a result, increased expression of WAP and β-casein occurs when GR is activated by glucocorticoids. The WAP gene promoter also has a signal transducer and activator of transcription-5 (Stat-5) binding site, which is the primary prolactin-responsive regulatory element (46). GR has been shown to interact directly with prolactin-stimulated Stat-5 to synergistically increase expression of the β-casein gene (77). It has been predicted that GR may interact similarly with nuclear factor I in the WAP promoter to increase its expression (46). Because glucocorticoids are so essential for milk protein expression, we began to address whether vitamin A intake modulates this pathway by measuring the effects of vitamin A on GR mRNA levels in the mammary gland. Our data indicate that vitamin A intake does not affect GR at the mRNA level (Fig. 7). As with our observations demonstrating lack of a change in expression of two other members of the steroid hormone receptor transcription factor superfamily, ER-α and PR, a lack of detectable changes in GR message does not rule out the possibility that vitamin A affects GR protein function, thereby modulating milk protein expression. Although we have not identified specific hormone, growth factor, or steroid superfamily transcription factor targets of retinoids that could account for the observed effects of retinoids on mammary gland development and carcinogen susceptibility, the apparent relationship between vitamin A intake and hormone-dependent mammary gland development remains a key area of future investigation.

In summary, we have demonstrated that adolescent mammary gland development and subsequent mammary gland carcinogenesis are influenced by dietary intake of retinyl palmitate. Our data are consistent with the hypothesis that adolescence represents a critical window for setting susceptibility to mammary carcinogenesis in the adult by modulating mammary gland development. Precocious and delayed mammary gland development were associated with elevated sensitivity to mammary carcinogenesis. Importantly, a modest deficiency or supplementation of retinyl palmitate (7.25-fold higher than the recommended daily allowance) during adolescence increased sensitivity of the rat mammary gland to MNU-induced carcinogenesis. These data highlight the necessity for further research into mechanisms of action of retinoids in normal and neoplastic mammary gland development.

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