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A maternal diet high in $n-6$ polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring

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ABSTRACT **We hypothesized that feeding pregnant rats with a high-fat diet would increase both circulating 17β-estradiol (E2) levels in the dams and the risk of developing carcinogen-induced mammary tumors among their female offspring. Pregnant rats were fed isocaloric diets containing 12% or 16% (low fat) or 43% or 46% (high fat) of calories from corn oil, which primarily contains the $n-6$ polyunsaturated fatty acid (PUFA) linoleic acid, throughout pregnancy. The plasma concentrations of E2 were significantly higher in pregnant females fed a high $n-6$ PUFA diet. The female offspring of these rats were fed with a laboratory chow from birth onward, and when exposed to 7,12-dimethylbenz(a)anthracene had a significantly higher mammary tumor incidence (60% vs. 30%) and shorter latency for tumor appearance (11.4 ± 0.5 weeks vs. 14.2 ± 0.6 weeks) than the offspring of the low-fat mothers. The high-fat offspring also had puberty onset at a younger age, and their mammary glands contained significantly higher numbers of the epithelial structures that are the targets for malignant transformation. Comparable changes in puberty onset, mammary gland morphology, and tumor incidence were observed in the offspring of rats treated daily with 20 ng of E2 during pregnancy. These data, if extrapolated to humans, may explain the link among diet, early puberty onset, mammary parenchymal patterns, and breast cancer risk, and indicate that an *in utero* exposure to a diet high in $n-6$ PUFA and/or estrogenic stimuli may be critical for affecting breast cancer risk.**

Exposure to high maternal circulating estrogen levels during pregnancy could play a critical role in affecting breast cancer risk among daughters (1). This hypothesis is supported by data from epidemiologic studies showing increased breast cancer risk in dizygotic twins (2), who have a higher *in utero* estrogenic environment than single fetuses, and in women who were heavy at birth (3, 4). Birth weight correlates with both maternal weight gain (5) and a high intake of polyunsaturated fatty acids (PUFAs) (6) and is suggested to reflect a high fetal estrogenic environment (7). It contrast, the daughters of women who suffered from pregnancy-induced hypertension (pre-eclampsia/eclampsia) have a 5-fold reduction in breast cancer risk (8). Pre-eclampsia/eclampsia is associated with lower circulating estrogen and PUFA levels (9).

The feto-placental unit is a major source of 17β-estradiol (E2), estrone, and estriol, with maternal and fetal concentrations increasing throughout pregnancy to reach their highest levels at birth (10). Estrogen concentrations exhibit a marked inter-individual variability during pregnancy (11), perhaps reflecting variations in the maternal diet. A high-fat diet increases circulating estrogens (12, 13). In contrast, a low-fat diet can reduce serum E2 levels (14, 15). An increase in the serum estrogen levels by a high-fat diet may reflect an increase in the mass of adipose tissue, which is an important site for the aromatization of androstenedione to estrone (16). Arachidonic acid (a metabolite of $n-6$ PUFA) also can increase aromatization via prostaglandin E_2-induced activation of P450 aromatase (17).

We have found that a maternal diet high in $n-6$ PUFA increases estrogen levels during pregnancy (18), which could influence both mammary gland development and subsequent breast cancer risk in the female offspring. In support of this hypothesis, *in utero* exposure to a high $n-6$ PUFA diet increases the incidence of spontaneous, estrogen-dependent tumors in outbred CD-1 mice (19). Dietary fat intake has been linked to an increased risk of breast cancer in both humans (20, 21) and rodents (22). However, the human data concerning high dietary fat and breast cancer risk are controversial (23). Because none of the human studies have investigated the role of dietary fat exposure in early life, an important period when the mammary gland may be sensitive to the effects of a high-fat diet, could have been overlooked.

The initial formation of the mammary bud and primitive mammary epithelial tree occurs during fetal life. The development of these structures appears to be at least sensitive to, if not partly dependent upon, the estrogenic environment of pregnancy (24). In addition, a high fetal estrogenic environment may induce precocious puberty (25), which is a well established risk factor for breast cancer (26). We have explored the hypothesis that an increased *in utero* estrogen exposure, as produced by consumption of a maternal diet high in $n-6$ PUFA during pregnancy, could alter mammary gland development, puberty onset, and breast cancer susceptibility. The fat source was corn oil, which contains 59% PUFA, the majority being $n-6$ linoleic acid. As a nutritionally essential fatty acid, the only linoleic acid source for the fetus (in humans and rodents) is the maternal diet, and a significant positive correlation exists between maternal and fetal linoleic acid concentrations (27). In addition, while the fetus is capable of metabolizing the nonessential fatty acids from the nutritionally essential fatty acids ($n-6$ linoleic and $n-3$ α-linolenic acids) (28), it is estimated that as much as 50% of the nonessential fatty acid requirements of the fetus are maternally derived (29). Maternal estrogens also readily cross the placenta (30). A diet high in α-linolenic acid was not used, because it may protect from breast cancer (31–33), and it does not appear to alter plasma estrogen levels (14).

Our studies were performed using carcinogen-induced mammary tumors in rats as a model of human breast cancer.

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Abbreviations: PUFA, polyunsaturated fatty acid; E2, estradiol; DMBA, 7,12-dimethylbenz(a)anthracene; TEB, terminal end bud; AB, alveolar bud.
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The development and endocrine responsiveness of the normal mammary gland, and the general structure of the critical epithelial components of the gland that are associated with tumorigenesis, are believed to be broadly comparable between rats and humans (34). Rats require exposure to chemical carcinogens to induce a reproducible incidence of mammary tumors. We chose to induce mammary tumors by administration of 7,12-dimethylbenz(a)anthracene (DMBA). While DMBA is an experimental carcinogen, rather than an etiologic factor for human breast cancer, it nevertheless produces mammary tumors that are comparable with human breast tumors in terms of their long relative latency, histotypes, and endocrine responsiveness (34). The current human chemoprevention trials with tamoxifen also are partly based on the findings obtained with the DMBA model in rats (35).

METHODS

***In Utero* Manipulations.** Three-month-old female Sprague–Dawley rats (Charles River Breeding Laboratories) were fed either a high or low $n-6$ PUFA diet, the feeding beginning 5 days before mating and continuing throughout pregnancy. The animals were returned to a Purina laboratory chow 5001, which contains 12% calories from fat (saturated and unsaturated), immediately after delivery. On postnatal day 2, the offspring were crossfostered and kept with nursing dams that were always fed the Purina chow. Crossfostering was done to control for possible differences in the milk content of mothers fed with the high or low PUFA diet during pregnancy. The mean litter-size in each group also was balanced to 8–10 offspring.

The experimental diets, prepared commercially by Food-Tek (Morris Plains, NJ) and Bioserv (Frenchtown, NJ), contained either 43% or 46% of calories from corn oil (high $n-6$ PUFA), or 12% or 16% calories from corn oil (low $n-6$ PUFA) (20). These percentages of calories from fat are comparable to those consumed in the American diet (≈37%) (36), and those achievable in low-fat (15–20%) human dietary intervention studies (37). The diet we address as low $n-6$ PUFA contains optimal levels of this fatty acid as well as n − 3 PUFA for rodent needs (38). However, because the 12–16% calories from fat diet is comparable to a human low-fat diet, the rat diet also was called a low $n-6$ PUFA diet. Formulation of the semipurified animal feeds (AIN-76A) were based on the recommendations of the American Institute of Nutrition (39). The protein, mineral, and vitamin contents of the high and low $n-6$ PUFA diets were similar (19–20% kcal from protein, 50 g of mineral mix, and 35 g of vitamin mix per 1 kg of feed). To achieve the same caloric intake, the high $n-6$ PUFA diets were made isocaloric with the low $n-6$ PUFA diets by adjusting their caloric contents with carbohydrates (corn starch, maltose, and sucrose) and fiber (alphacel). The caloric density of the diets was 3.7 kcal/g feed. Thus, the high $n-6$ PUFA diets contained less carbohydrates (38% versus 64% kcal from carbohydrates) and more fiber than the low $n-6$ PUFA diets. In a separate set of experiments, 3-month-old pregnant rats were injected subcutaneously with 20 ng of E2 (Sigma) and/or oil vehicle on days 14–20 of gestation in the absence of dietary manipulations.

Serum Estradiol. Total plasma E2 levels were measured using a double-antibody kit (Diagnostic Products, Los Angeles, or ICN) according to the manufacturers' specifications. The blood was collected by cardiac puncture from pregnant rats kept on the high or low $n-6$ PUFA diets on gestation day 14, and from their offspring on postnatal days 8 and 60. In the offspring of E2-treated mothers, the blood was collected on postnatal day 64. The estrus cycle was matched among the offspring, most of them being in estrus at the time of blood collection.

Mammary Parenchymal Patterns. We dissected the fourth abdominal mammary glands from 4-, 7-, and 11-week-old rats that were exposed to a high or low $n-6$ PUFA diet *in utero,* and from 4- and 7-week-old rats exposed to E2 *in utero*. Tissues were processed for morphological analysis as whole mounts. By week 4, the occurrence of terminal end buds (TEBs) is high (40). By week 7, the offspring were exposed to DMBA to assess effects on susceptibility to transformation. By week 11, the typical adult pattern of epithelial cell growth can be seen.

Analysis of mammary epithelial structures was made through visual evaluation and computer-assisted image analysis. We have developed and characterized a visual scale to assess the growth patterns of mammary epithelial cells (41). In our scale method, the stained mammary whole mounts were examined under an Olympus dissecting scope. The following characteristics of the coded mammary glands were evaluated double-blind, using a 4-point scale (0 = absent, 1 = few, 2 = moderate, 3 = numerous): the relative density of (*i*) epithelial ducts; (*ii*) TEBs; and (*iii*) alveolar buds (ABs). The inter-individual correlation was 0.7–0.9 for various glandular structures. These epithelial structures are well characterized by Russo and Russo (40). Measurement of the density of epithelial structures also was performed using the Optimas Image Analysis system. Evaluation of mammary gland morphology was based on images of the glands taken from a camera-affixed, Olympus stereo microscope, using Optimas software. At the age of 4 weeks, total epithelial area was used for density measurements with the image analysis (only two glands per group were suitable for the image analysis at this age). At the ages of 7 and 11 weeks, an area of 3×3 mm, obtained from the same location of the gland for each animal, was used.

Mammary Tumorigenesis. Mammary tumors were induced by administering 10 mg of DMBA (Sigma) to the 55-day-old offspring by oral gavage. The animals were checked regularly for mammary tumors by palpation once per week. Tumor growth rates were measured by recording the tumor diameters with a caliper and determining the length of the longest axis and the width perpendicular to the longest axis. Tumor doubling time was determined only for proliferating tumors, tumor volume and tumor doubling time being estimated as described by Rygaard and Spang-Thomsen (42). The animals were sacrificed when detectable tumor burden approximated 10% of total body weight. The surviving animals and animals that did not develop mammary tumors were sacrificed on week 18 after the DMBA administration.

Puberty Onset: Vaginal Opening. Sexual maturation in rodents can be determined by establishing the age when vaginal opening occurs, the first estrus occurring within a few days of this event (43). The animals were examined daily starting on postnatal day 33.

RESULTS

Pregnancy Outcome. Pregnancy was initiated and maintained with a similar success rate in both groups consuming a high- or low-fat diet. Food consumption and body weight gain of the pregnant rats, and the number of pups born and their body weights, did not differ between the two groups (Table 1). Among the pregnant rats exposed to E2 or vehicle, pregnancy- and nursing-related variables also were similar in the two groups (not shown).

Circulating E2 Levels. The plasma levels of E2 were approximately 30% higher in pregnant females consuming the highest-fat feed (46% calories from corn oil) than in pregnant females consuming the lowest-fat feed (12% calories from corn oil) ($t = 3.37$, $df = 9$, $P < 0.01$; Student's t test) (Fig. 1). The plasma E2 levels were not altered in the 8-day-old offspring of the dietary-manipulated mothers, who from the birth onward were exposed to the Purina rodent chow (Fig. 1). This is consistent with the clearance of estrogens from neonates during the first few days postpartum. There were no differences in serum E2 levels between the 2-month-old female offspring of mothers fed with a high and low $n-6$ PUFA diet, or between the offspring of mothers injected with E2 or oil vehicle during pregnancy (data not shown). Thus, an *in utero* exposure to a high-fat diet does not appear to significantly alter adult E2 levels.

Table 1. Pregnancy- and nursing-related variables in female rats exposed to high (46%) or low (12%) dietary $n-6$ PUFA *in utero*

Variables	Dietary fat content	
	Low	High
Body weight before pregnancy, g	295.7 ± 2.9	288.0 ± 3.6
Body weight gain during pregnancy, g	108.1 ± 13.8	100.1 ± 11.0
Food consumption during pregnancy		
g/day	18.6 ± 0.5	19.2 ± 0.3
kcal/day	70.7 ± 1.9	72.7 ± 1.2
Litter		
Successful pregnancies, %	70.0	70.0
Number of pups/litter	14.0 ± 1.2	10.4 ± 1.4
Female/male ratio	0.9 ± 0.2	1.2 ± 0.4
Female pup weight		
At day 4	6.4 ± 0.3	6.3 ± 0.3
At day 20	37.6 ± 1.2	35.3 ± 2.6
At 2 months	246.0 ± 3.3	240.0 ± 2.4

The data presented were obtained from having 10 female rats in both groups that were housed in groups of three containing two females and a male. The values are means ± SEM.

Mammary Parenchymal Patterns. *High-fat diet.* The total mammary fat pad area, as measured by a caliper, was larger in the female rats exposed to a high $n-6$ PUFA diet *in utero* than in the female rats exposed to a low-fat diet ($F(1, 12) = 5.7, P < 0.03$; two-way ANOVA) (not shown). This finding is consistent with the observation that early postnatal exposure to estrogens stimulates growth of the mammary fat pad (44). The visual image analysis indicated that, on week 4, the total epithelial cell density was clearly higher in the high-fat (5, 582 ± 100 mm^2) than in the low-fat group (188 ± 30 mm^2). In addition, in the 7- and 11-week-old offspring of high-fat-fed mothers, the representative epithelial cell area was significantly more dense than that of the offspring of the low-fat fed mothers ($F(1, 8) = 6.6, P < 0.03$) (Figs. 2*A* and 3). The scale-method produced comparable results. The epithelial tree was more dense, as defined by the visual score, in the female rats exposed to a high-fat diet *in utero* than in the rats exposed to a low-fat diet ($F(1, 21) = 13.43, P < 0.001$) (Fig. 2*A*).

The mammary epithelial structures were significantly different between the female rats exposed to a high and low $n-6$ PUFA diets through their pregnant mother (Fig. 2*B*). The relative density of TEBs, the sites of carcinogen-induced malignant transformation (40), was higher on weeks 4, 7, and 11 in the high-fat animals, when compared with the low-fat animals ($F(1, 21) = 15.0, P < 0.0009$). The relative density of ABs was not significantly different between the groups on week 7. However, when examined on week 11, the mammary glands in the offspring of female rats fed with a high-fat diet during pregnancy had significantly more ABs than the low-fat animals ($F(1, 11) = 5.7, P < 0.04$) (Fig. 2*B*).

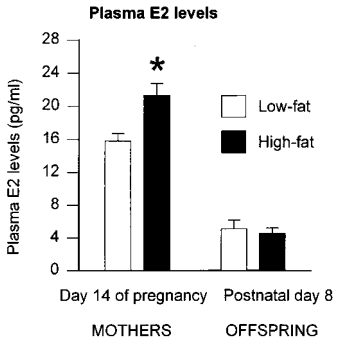

FIG. 1. The plasma levels (mean ± SEM) of total E2 in pregnant females that were fed with isocaloric high (46% calories from fat) ($n = 5$) or low (12% calories from fat) $n-6$ PUFA diet ($n = 6$) throughout gestation, and their 8-day-old offspring (high $n-6$ PUFA: $n = 10$; low $n-6$ PUFA: $n = 15$) that were kept on Purina laboratory chow immediately after birth. * indicates statistical significance at the $P < 0.05$ level.

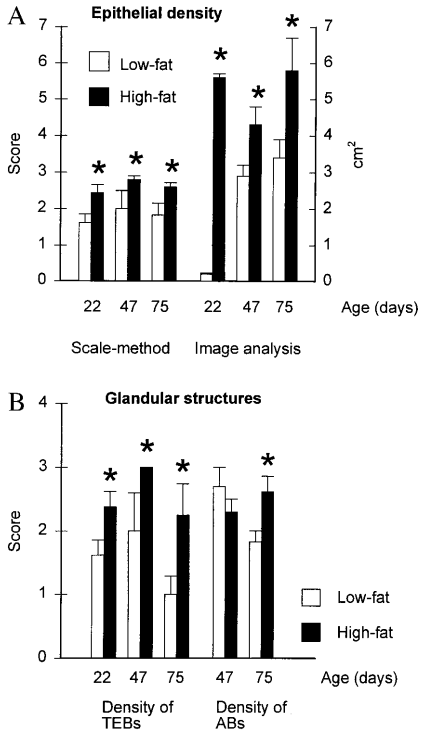

FIG. 2. (*A*) Density of epithelial ducts and (*B*) TEBs and ABs in the fourth abdominal gland of 4-, 7-, and 11-week-old female rats exposed *in utero* to high-fat diet (43% calories from corn oil) or low-fat diet (16% calories from corn oil). Means ± SEM obtained from 3–8 female rats are shown. Significantly different from the controls: *, $P < 0.05$.

E2 exposure. Mammary gland morphology in the offspring of mothers treated with E2 during pregnancy was determined on weeks 4 and 7. As defined by the visual score, the epithelial cell density was significantly higher in the E2-treated offspring at 4 weeks of age, but not at 7 weeks ($F(1, 7) = 12.3, P < 0.01$) (Fig. 4). The number of TEBs was significantly higher in these offspring both on weeks 4 and 7 ($F(1, 7) = 9.5, P < 0.02$). Differentiation of epithelial structures to ABs was lower in the offspring of E2-treated mothers ($t = 4.01, df = 3, P < 0.03$), when the DMBA was administered (by week 7). Because epithelial differentiation protects the mammary gland from malignant transformation (45), *in utero* E2 exposure increased susceptibility to DMBA by increasing the density of TEBs and reducing their differentiation.

Mammary Tumorigenesis. *High-fat diet.* The number of animals in each group with one or more mammary tumors (tumor incidence) was higher among the rats who were exposed *in utero* through a maternal diet high in $n-6$ PUFA, when compared with the offspring of rats fed the low $n-6$ PUFA diet ($z = 4.03$, $P < 0.001$; Gehan–Wilcoxan test) (Fig. 5*A*). On week 18 after DMBA administration, 60% of the female offspring of mothers exposed to a high $n-6$ PUFA diet during pregnancy had developed mammary tumors, whereas 30% of the offspring of low $n-6$ PUFA mothers had mammary tumors. Tumor multiplicity (the mean number of tumors per female) was similar between the two groups. The latency for tumor appearance was significantly shorter in the high-fat diet group ($t = 3.4, df = 51, P < 0.002$) (Table 2). The proportions of histotypes (77% malignant adenocarcinomas, 6% squamous cell carcinomas, and 17% nonmalignant adenomas) were comparable to those previously reported with DMBA (40) and were not affected by the *in utero* dietary manipulations.

Tumor growth rate and size upon first detection did not differ between the offspring of mothers fed a high- or low-fat

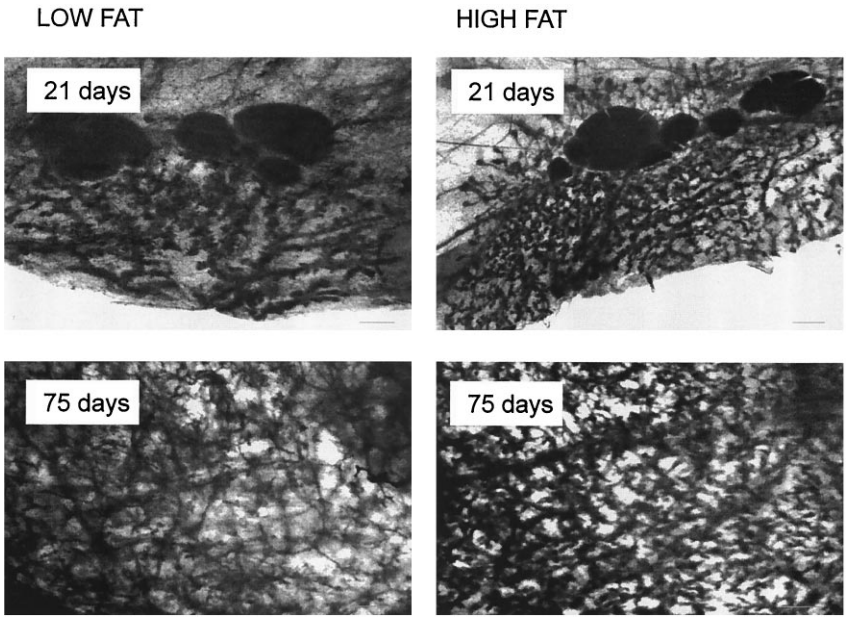

FIG. 3. The epithelial density of the mammary whole-mount preparations (carmine staining) of the fourth abdominal glands obtained from 4- and 11-week-old female rats exposed *in utero* to high-fat diet (43% calories from corn oil) or low-fat diet (16% calories from corn oil) (magnification 2.5 × 25).

diet during pregnancy (Table 2). These endpoints reflect tumor promotion/progression events, i.e., rate of growth of initiated cells. Thus, the effects of a fetal exposure to a high-fat diet through mother on mammary tumorigenesis are more closely associated with factors influencing breast cancer risk.

E2 exposure. The data in Fig. 5*B* demonstrate that the proportion of animals with mammary tumors was significantly higher among the rats who were exposed *in utero* to E2 via maternal injections than in their control group ($z = 2.34$, $P < 0.02$). While tumor latency appeared shorter in the E2-exposed animals, unlike the dietary exposed animals, this did not reach statistical significance (Table 2). One possibility is that the shorter exposure to E2 among the injected animals, where E2 was administered only on gestation days 14 through 20, was not sufficient to alter latency. The high-fat-diet-exposed animals would be expected to have elevated E2 levels throughout pregnancy. Other parameters of tumorigenesis were unaffected by previous E2 exposure, i.e., the multiplicity, size of the DMBA-induced mammary tumors upon first detection, and tumor growth rate (Table 2).

Vaginal Opening. *High-fat diet.* Fig. 6*A* shows that vaginal opening occurred at a younger age in the offspring exposed to a diet high in $n - 6$ PUFA *in utero* (z value $= 3.9$, $P < 0.0004$; Gehan–Wilcoxan test). These data suggest that critical events may occur *in utero* that influence the "priming" of the hypothalamic-gonadal axis for the timing of the onset of puberty. These events may be mediated by estrogens and appear sensitive to dietary influences.

Estrus cycling and uterine wet weights were studied to assess the endocrinologic/estrogenic environment at the time when carcinogen was administered (between 50–60 days of age). The length of the estrus cycle was normal (4–5 days) in 80% of both the rats exposed to a high- and low $n - 6$ PUFA diet *in utero* via the maternal diet. Uterine wet weights were similar in the

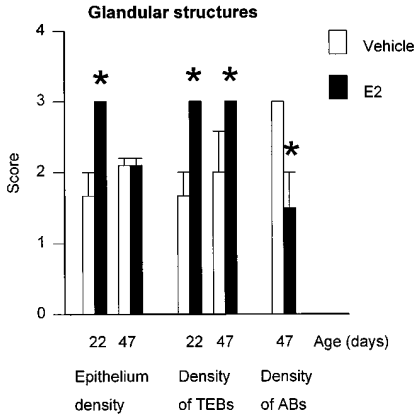

FIG. 4. Density of epithelial ducts, TEBs, and ABs in the fourth abdominal gland of 4- and 7-week-old female rats exposed *in utero* to E2 or oil vehicle injections. Means ± SEM obtained from 2–6 female rats are shown. Significantly different from the controls: *, $P < 0.05$.

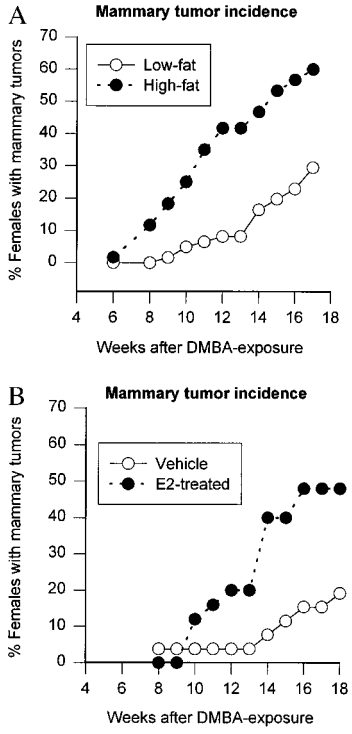

FIG. 5. The proportion of DMBA-induced mammary tumors in offspring of mothers who were (*A*) fed with a high-fat diet containing 46% calories from corn oil ($n = 60$) or a low-fat diet containing 12% calories from corn oil ($n = 60$) during pregnancy, or (*B*) were injected with 20 ng E2 ($n = 25$) or oil vehicle ($n = 26$) daily between days 14 and 20 of gestation. Tumor incidence was significantly higher in the high than in the low $n - 6$ PUFA group ($P < 0.0001$) and in the E2 than in the vehicle control group ($P < 0.02$).

55-day-old offspring of the high- and low-fat groups. Thus, the reproductive hormonal environment at the time of DMBA exposure is unlikely to have influenced mammary tumor incidence between females exposed to a high- or low-fat diet *in utero*.

E2 exposure. Vaginal opening occurred significantly earlier among the E2 group (z value = 3.9, $P < 0.0001$) (Fig. 6*B*). The uterine wet weights, when measured at 8 weeks of age, were not significantly different between the females exposed to E2 *in utero* and the oil controls.

DISCUSSION

The present study provides evidence for the hypothesis that early life plays a critical role in influencing breast cancer risk. We show that in rats maternal exposure to a diet high in $n-6$ PUFA and/or E2 during pregnancy increases the incidence of DMBA-induced mammary tumors in female offspring. The effect of maternal fat intake on an offspring's vaginal opening, mammary gland morphology, and tumor incidence, or lack thereof on tumor growth rate and multiplicity, are essentially equivalent to those observed in the animals exposed *in utero* to E2. These data strongly suggest that the effects of high-fat diet on these parameters are mediated through the fat-associated elevation in estrogen levels in the pregnant mothers. While statistically significant, the increase in the amount of total E2 in the pregnant rats consuming a diet high in $n-6$ PUFA may appear modest. Nevertheless, a 30% increase in the concentration of E2 could have substantial effects upon receptor occupancy, a critical component in mediating the biological response to estrogens. The plasma levels of E2 among Caucasian women are approximately 25% higher than among Oriental women (12), and this may contribute to the significantly higher incidence rate for breast cancer observed in Caucasian women (46). In subsequent studies, we have demonstrated a 75–100% increase in serum E2 levels in other pregnant female rats and male mice by a high $n-6$ PUFA diet (18, 47).

However, we cannot exclude a contribution from other fat-regulated factors. These include alterations in the fluidity and functionality of cellular membranes (48), changes in lipid peroxidation (49), or arachidonic acid (33, 50), prostaglandin E_2 (33, 49), or 12-hydroxyeicosatetraenoic (33) contents. Additionally, metabolic events leading to altered phospholipase C (51) or protein kinase C activity (52, 53) also may occur. Several of these events are regulated by estrogens (54–56).

Our data indicate the ability of an exposure to a high maternal $n-6$ PUFA diet or E2 to alter an offspring's mammary gland morphology. The number of TEBs is significantly increased in both groups, the differentiation to lobular structures is reduced (only E2), or the epithelial cell density is increased (only high fat). Importantly, persistent TEBs also have been reported in transgenic mice that exhibit an increased incidence of hyperplastic or malignant growth in the mammary glands (57, 58). Comparable changes are apparent in women with breast cancer or who are at elevated risk to develop this disease (34, 59). TEBs are considered the primary targets for neoplastic transformation in the rodent mammary gland (40). In humans, most mammary carcinomas originate from terminal ductal-lobular units, which arise directly from the TEBs and are comprised of terminal ducts and ductules (34). Thus, there are striking similarities in the pathogenesis of mammary tumors in rodents and humans (34, 40).

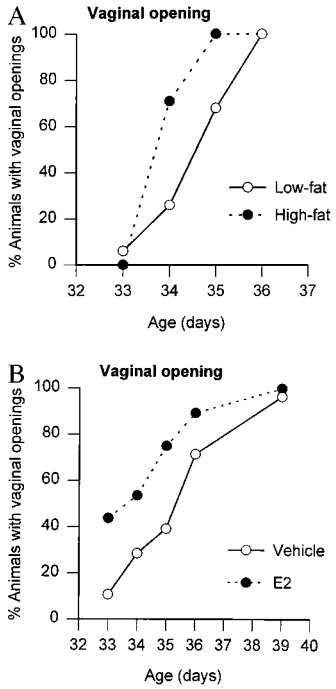

FIG. 6. The proportion of female rats exposed *in utero* via their mother to (*A*) high ($n = 31$) or low $n-6$ PUFA diets ($n = 35$) or (*B*) 20 ng E2 ($n = 28$) or oil vehicle ($n = 28$) between days 14 and 20 of gestation, with vaginal opening between postnatal days 33 and 38 days. Vaginal opening occurred significantly earlier in the high-fat vs. low-fat group ($P < 0.0004$) and in the E2 vs. oil group ($P < 0.0001$).

Rats that have been exposed *in utero* to either a high $n-6$ PUFA diet or E2 reach puberty significantly younger than their respective controls. We also have confirmed this observation in female mice that were treated with E2 or high $n-6$ PUFA diet either *in utero* or immediately after birth (not shown), and that subsequently develop an increased incidence of spontaneous mammary tumors (60). These observations are entirely consistent with the findings in humans that early menarche is linked to increased breast cancer risk (26).

The initiation of puberty is characterized by an altered activity in the hypothalamic gonadotrophin-releasing hormone neurons, and a consequent increase in the release of gonadotrophins from the pituitary, followed by the release of gonadal hormones from the gonads (61). However, the factors determining the timing of the onset of puberty are not known. Familial factors and body fat may contribute (62). Our results suggest that a fetal exposure to a high-fat diet can influence the timing of puberty in rats, and this

Table 2. The latency for the appearance of a tumor after DMBA exposure, number of tumors per animal (tumor multiplicity), mean area of tumors at first detection, and tumor growth rate in DMBA-treated rats that were exposed to low-fat or high-fat diets *in utero* or to oil vehicle or E2 daily during gestation days 14-20

Treatment	Tumor latency, weeks	n	Tumor multiplicity, no. tumors/rat	n	Tumor area, mm^2	n	Tumor doubling time, days	n
Diet *in utero*								
Low fat	14.2 ± 0.6	17	1.2 ± 0.1	17	81.3 ± 11.4	17	8.4 ± 3.7	11
High fat	11.4 ± 0.5*	36	1.4 ± 0.1	36	60.0 ± 12.6	36	13.8 ± 2.2	19
E2, gestation days 14–20								
Vehicle only	14.2 ± 1.7	5	1.0 ± 0.0	5	122.0 ± 80.0	5	30.8 ± 12.9	3
E2	12.7 ± 0.7	13	1.2 ± 0.2	13	67.0 ± 30.0	13	21.0 ± 3.8	10

n = number of tumors. Low-fat diet, $n = 60$, 12% calories from fat. High-fat diet, $n = 60$, 46% calories from fat. Oil vehicle, $n = 26$. E2, 20 ng, $n = 25$, daily during gestation days 14–20.
*$P < 0.002$.

can occur independent of either changes in total body weight or caloric intake before puberty. It is possible that early puberty onset is a risk factor for breast cancer because it increases the cumulative exposure to ovarian estrogens (63). However, based on our findings and those of other investigators, it also is possible that the same biological factor, i.e, a high estrogenic activity during early life, advances puberty onset and increases breast cancer risk.

A diet high in $n - 3$ PUFA may prevent breast cancer (31–33) and, therefore, low dietary $n - 3$ PUFA content may increase the susceptibility to this disease. Whether the increased breast cancer risk seen in the present study is caused by the high *in utero* levels of linoleic acid or low α-linolenic acid content (27) remains unclear. Linoleic acid can suppress the incorporation of α-linolenic acid into phospholipids (64), because these fatty acids compete for the same elongase and desaturase metabolizing enzyme systems (27). However, both the high and low corn oil diets contained adequate levels of $n - 3$ PUFA required during rat pregnancy (38).

The elevated E2 levels associated with the high maternal intake of dietary fat may provide an explanation for the findings in epidemiological studies showing an association between high birth weight and increased breast cancer risk (3, 4). In addition, the $n - 6$ PUFA-induced increase in maternal E2 levels indicates a mechanistic link among diet, mammary parenchymal patterns, puberty onset, and breast cancer risk. These data may have important implications for the human diet and our understanding of the timing of exposure to dietary factors/hormones that can influence breast cancer risk. Currently, even the most educated women consume more fat and less carbohydrates during pregnancy than suggested by the international dietary recommendations (65). The data also implicate a possible role for other exposures that could influence the fetal endocrine environment, including exposure to environmental estrogens and phytochemicals with significant estrogenic activities. The prevention of some breast cancers may have to begin by modifying the dietary intake of some fats in pregnant women.

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