Calorie Intake during Mammary Development Influences Cancer Risk:
Lasting Inhibition of C3H/HeOu Mammary Tumorigenesis by Peripubertal Calorie Restriction

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ABSTRACT
To test for a relationship between peripubertal calorie intake, mammary development, and tumorigenesis, weaning C3H/HeOu mice were separated into 3 groups: fed diet either ad libitum (AL) and designated group AL (n = 60); fed a similar, calorie-restricted (CR) diet only during mammary development when 4–12 weeks old and then subsequently fed ad libitum when ≥13 weeks old (group CRn-12, n = 24); or continuously calorie restricted (group CR, n = 60). Eight weeks of peripubertal calorie restriction provided CRn-12 mice with lasting protection from mammary tumorigenesis (P = 0.004) and lower cumulative tumor incidence by 33% compared to AL mice. Sustained calorie restriction of group CR mice further reduced mammary tumor incidence compared to both AL (P = 0.000001) and CRn-12 mice (P = 0.009). Calorie intake significantly influenced mammary development and cellular proliferation. Compared to the mammary development of AL mice, calorie restriction reduced the diameter of ductal end buds (189 μm compared to 146 μm; P < 0.01), lowered the end bud [3H]thymidine labeling index from ≥20 to ≤13% (P < 0.001), delayed end bud migration and mammary glandular growth (P < 0.01), reduced alveolar budding (P < 0.001), reduced the proportion of alveoli containing at least one [3H]thymidine labeled cell from ≥50 to ≤22% (P < 0.001), and lowered the alveolar [3H]thymidine labeling index of labeled alveoli from ≥14 to ≤7% (P < 0.001). These findings link peripubertal calorie intake, mammary development, and carcinogenic risk, and show that the abrogation of mammary tumorigenesis by calorie restriction is partially attributable to influences on mammary development.

INTRODUCTION
Calorie restriction in rodents abrogates spontaneous, proviral-associated, carcinogen-induced neoplasia at multiple sites, including the mammary gland (1–9). Mechanisms underlying this antineoplastic effect appear to involve neuroendocrine and immunological and metabolic systems (1). Calorie intake may affect carcinogenic risk by influencing levels and rhythms of hormones, cytokines, and growth factors (5, 10–15), rates of cellular proliferation (16–22), free radical production and the capacity for free radical detoxification (23–25), efficiency of enzyme-mediated DNA repair (26), and levels of immunological responsiveness (1, 27–29).

A degree of breast cancer risk may be established during adolescence by influences of calorie intake on peripubertal breast development (1, 30–38). Calorie restriction inhibits rodent mammary tumorigenesis most effectively when initiated during early peripubertal life (1, 30, 31). Adult body height of women is highly correlated to breast cancer incidence and influenced by adolescent calorie intake (32–38). Definitive evidence that peripubertal calorie intake has a lasting influence on mammary tumorigenesis has not been presented.

Characteristics of the developing mammary gland may contribute to breast cancer risk. Mammary epithelium of the peripubertal rodent is particularly vulnerable to chemical carcinogenic challenge (39, 40) and sensitive to oncogenic mutation (41–43). It is intensely mitotic, undifferentiated, and endowed with limited capacity for enzyme-mediated DNA repair, compared to the mammary epithelium of the adult (39, 40). Although calorie restriction has been shown to lower rates of cellular proliferation within the mature mammary gland and other organs of the adult rodent (16–22), its influence on cellular proliferation during peripubertal mammary development has not been described.

Peripubertal mammary development begins in 4-week-old pubescent mice and involves extension and arborization of the primordial ductal tree formed during embryogenesis (44). Stimulated by estradiol (45) and perhaps progesterone, EGF, and TGF-β (46–51), ducts of the primordial ductal tree develop highly mitotic end buds which invade the surrounding fatty stroma and form secondary and tertiary branches and bud alveoli. Peripubertal mammary development concludes within approximately 8 weeks, when the growing ductal tree extends to the outer margins of the fat pad and end buds involute, perhaps in response to growth inhibiting TGF-β (52, 53). In the present report, with the use of mice of the mammary tumor-prone (C3H/HeOu strain, where mouse mammary tumor virus transcription and protooncogene Wnt-1/Int-2 activation are linked to mammary carcinogenesis (54), influences of peripubertal calorie intake on mammary development and risk of subsequent tumorigenesis are described.

MATERIALS AND METHODS

Animals. One hundred forty-four female 4-week-old C3H/HeOu mice (The Jackson Laboratory, Bar Harbor, ME) were randomly separated into 3 dietary groups. Sixty mice consumed semipurified diet ad libitum and were designated group AL. Sixty mice consumed a similar but calorie restricted diet and were designated group CR to indicate calorie restriction throughout the 60-week study. Twenty-four mice were designated group CRn-12 to indicate that they were calorie restricted for only 8 weeks during mammary development, when 4–12 weeks of age, after which they consumed high calorie diet ad libitum.

Experimental Plan. Six mice each from groups AL and CR in metestrus/diestrus were euthanized when 4, 6, 8, 10, 12, and 16 weeks old. Whole mounts of the mammary gland and histoautoradiographs of the mammary gland, intestine, spleen, and kidney were prepared to determine the extent of mammary development, enumerate ductal structures, and assess rates of cell proliferation in ductal structures and the duodenum, colon, spleen, and kidney. Twenty-four mice in each of the three dietary groups were followed for mammary tumor incidence.

Semipurified Diets. Group AL mice were allowed free access to an excess of high calorie semipurified diet. Calorie restriction of CR and CRn-12 mice was made at increments of 5% fewer dietary calories offered each feeding, relative to the feeding of group AL mice. Group CR and CRn-12 mice were offered 95% of the calories offered group AL mice when 4 weeks old, 90% when 5 weeks old, 88% at 6 weeks, 70% at 7 weeks, and 60% when 8–12 weeks of age. Group CR mice >8 weeks old were offered 40% fewer calories than AL mice. Group CRn-12 mice calorie restricted when 4–12 weeks of age.

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mice were calorie restricted only when 4–12 weeks old, and were subsequently allowed free access to diet when ≥13 weeks old.

Food consumption was determined by weighing the food offered and reweighing the remaining food at the end of each feeding interval. Calorie intake was determined as a product of the weight of diet consumed (grams) times the caloric concentration of the diet offered (calories/gram). Mice were weighed weekly.

The composition of the calorie-restricted diet was formulated with each incremental increase in calorie restriction to ensure an equivalent intake of essential nutrients by all mice, while limiting only total dietary calories. Composition of the diets offered to adult AL and CR mice is presented in Table 1. All dietary constituents were obtained from ICN Biochemicals (Costa Mesa, CA). Diets were low in dietary fat. Calories were restricted principally by limiting calories from protein and carbohydrate.

Vaginal Estrus. Age at first vaginal estrus and the length and regularity of subsequent estrous cycles were determined by evaluating daily vaginal smears of 10 AL and 10 CR mice for cellular composition, degree of squamous epithelial cell cornification, and other standard signs of estrus (55, 56) when mice were 3–6 and 22–25 weeks old.

Rate of Mammary Development. Whole mounts of the left inguinal mammary fat pad were prepared as described (22). The extent of mammary development was determined as a ratio of fat pad area (mm²) containing glandular structures divided by the total fat pad area times 100, using a Zeiss Sterioskop, a SV8 microscope, and a stage micrometer, and by measuring tracings of photomicrographs of whole tumors. Whole mounts were evaluated for the extent of ductal ramification and for characteristics of ductal structures.

[³H]Thymidine Labeling Indexes. Mice were administered 15 μCi/gm body weight [³H]thymidine i.p. 1 h prior to euthanasia. Right inguinal mammary glands and samples of duodenum, colon, spleen, and kidney were fixed in 10% buffered formalin; dehydrated; embedded in paraffin; and sectioned at 3–5 μm thickness. Sections were deparaffinized, coated with NTB-2 nuclear track emulsion (Kodak), and stored for 10 days in a lightproof box at 4°C. Slides were developed with D-19 developer and processed in acid fixed. Sections were stained with hematoxylin and eosin.

Mammary autoradiographs were evaluated for ductal morphology, the number of ductal structures/mm² of fat pad, the percentage of ductal structures with at least one cell which had incorporated [³H]thymidine, and the labeling index of [³H]thymidine-labeled ductal structures. At least three levels from each gland were evaluated. Labeling indexes of [³H]thymidine-labeled structures were determined as a ratio of labeled nuclei/total nuclei counted times 100, with at least 500 nuclei considered labeled.

Labeling indexes of the duodenum and colon were determined by evaluating 10 longitudinally-oriented crypts. Labeling indexes of the crypt cell populations (product of the crypt column and circumferential cell counts) were determined. Crypt column counts were made from the crypt base to the duodenal crypt-villus junction or to the top of the colonic crypt. Labeling indexes of the intestine, spleen, and kidney were recorded as a percentage of labeled nuclei.

Statistical Analysis. Profiles of the tendency to remain mammary tumor free were compared between dietary groups by Kaplan-Meier analysis, using a Peto/Peto nonparametric log-rank test with stringent 95% confidence intervals. Mean calorie intake and body weight, age at onset and intervals between vaginal estrus, extent of mammary development, number and size of ductal terminal end buds and alveoli, percentage of these ductal structures with at least one cell which incorporated [³H]thymidine, and tissue labeling indexes were compared between dietary groups using paired t tests or an analysis of variance.

RESULTS

Calorie Intake. When 4–12 weeks old, group CR and CR₄₋₁₂ mice were offered an average of 29% fewer calories and consumed a mean 19% fewer calories (or 9.9 kcal/day) compared to peripubertal AL mice, which consumed 12.2 kcal/day during this 8-week interval (P < 0.01) (Table 2). Adult group CR mice ≥13 weeks old were offered 40% fewer calories and consumed a mean 32% fewer calories (or 10.3 kcal/day) compared to adult group AL mice, which consumed 15.1 kcal/day (P < 0.01). Adult CR₄₋₁₂ mice ≥13 weeks old were allowed free access to diet and consumed 14.9 kcal/day, similar to that of adult AL mice and greater than the mean calorie intake of adult CR mice (P < 0.01).

Body Weight and Vaginal Estrus. Mean body weights of CR mice ≥10 weeks old were significantly less than those of AL mice (P < 0.01; Table 2). Mean body weights of CR₄₋₁₂ mice were transiently reduced compared to those of AL mice when 8–12 weeks old (P < 0.01), similar to weights of adult AL mice ≥15 weeks of age and greater than those of adult CR mice (P < 0.01). Mice exhibited first vaginal estrus at 29 ± 1.8 (SD) days. Intervals between estrus for peripubertal AL or CR mice 3–6 weeks old were similar (7.8 ± 1.1 days) but were longer and more variable in adult 22–25-week-old CR mice (12.9 ± 2.8 days), compared to adult AL mice (7.8 ± 1.6 days; P < 0.01).

Mammary Development. Numerous, large end buds were present in glands of 4–10-week-old AL mice (Fig. 1). With each subsequent evaluation of mammary whole mounts of AL mice, end buds moved progressively toward the margins of the fat pad and then involuted.
when mice were 12 weeks old. Ducts of these glands were fine and possessed a high order of branching and numerous alveoli.

In contrast, end buds in glands of peripubertal CR mice were smaller, fewer in number, and migrated more gradually in subsequent whole mounts through the fat pad. End buds were still present in glands at 16-week-old CR mice. Mammary ducts of CR mice were dilated and had a low order of branching and rare alveoli.

Mammary development was significantly greater in AL compared to CR mice when mice were 8, 10, and 12 weeks old (P < 0.01; Fig. 2). For example, as evident in Fig. 1, b and c, approximately 80% of the fat pad of an 8-week-old AL mouse contained glandular structures, while only 55% of the stromal fat of an age-matched CR mouse contained developing mammary gland.

Histology and Labeling Indexes of Mammary Ductal Structure.
In successive mammary autoradiographs, end buds were initially more numerous and proliferative in glands of 6-week-old AL mice (P < 0.001), similar in number but still more proliferative in 8-week-old AL mice (P < 0.001), and fewer in number (P < 0.001) and only modestly more proliferative (P < 0.05) in 10 week-old AL mice compared to CR mice (Fig. 3).

All end buds contained at least one cell which had incorporated [3H]thymidine into DNA, but end buds differed significantly in size and labeling index between dietary groups. End buds of AL mice were larger, each with a prominent cap cell layer and a mean labeling index between 20 and 30% (Fig. 4). In contrast, end buds of CR mice were smaller, had an ill-defined or absent cap cell layer, were ensheathed in fibrocytes, and had a lower mean labeling index between 9 and 13% (P < 0.001). Mean end bud diameter was greater in AL mice than in CR mice (189 µm compared to 146 µm; P < 0.01).

These observations depict large, highly proliferative end buds in...
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Fig. 3. Number of ductal terminal end buds (TEB) or alveoli/mm² of fat pad, [³H]thymidine DNA-labeling index (DNA-LI) of end buds, percentage of alveoli with one or more [³H]thymidine-labeled cell [light (AL) and dark (CR) shaded bars], and [³H]thymidine DNA-labeling index of labeled alveoli in AL (O) or CR (L) mice. Points, mean; bars, SD. * P < 0.01; † P < 0.05.

Fig. 4. Mammary autoradiographs showing a large end of an AL mouse (a) with prominent cap-cell layer and numerous labeled cells (black grains), in contrast to the smaller end bud of a CR mouse (b) with indistinct cap-cell layer, few labeled cells, and a surrounding layer of fibrocytes (hematoxylin and eosin; × 630).
glands of AL mice which migrated quickly to the fat pad margin and then involuted, in contrast to the smaller, less proliferative end buds of CR mice which moved more gradually through the stroma.

Mammary ducts of CR mice had few alveoli, and of the few alveoli that developed, ≤22% had even 1 cell which had incorporated \(^{3}\)H-thymidine into DNA (Figs. 3 and 5). Mean labeling indexes of labeled alveoli in glands of CR mice were less than 7% at all ages of assessment. In glands of AL mice ≥8 weeks old, significantly more alveoli developed (P < 0.001), ≥50% of alveoli contained \(^{3}\)H-thymidine-labeled cells (P < 0.001), and alveolar labeling indexes exceeded 14% (P < 0.001).

**Labeling Indexes of Duodenum, Colon, Spleen, and Kidney.**
AL and CR mice had similar duodenal and colonic crypt dimensions and crypt cell populations, and labeling indexes were comparable when mice were 6–10 weeks old (data not shown). The mean duodenal labeling index of 12–16 week old AL mice (24.7 ± 0.8) was greater than that of CR mice (19.4 ± 0.9; P < 0.01), as was the mean colonic labeling index (7.9 ± 0.8 compared to 5.1 ± 0.6; P < 0.01). Labeling indexes of spleen and kidney were similar for AL and CR mice (data not shown).

**Analysis of Tumor Incidence.**
Multiple comparisons of Kaplan-Meier generated curves depicting the tendency of each group to remain mammary tumor free were made (Fig. 6). Overall, the three profiles were significantly different (P = 0.000005). Peripubertal calorie restriction of CR\(_{4-12}\) mice reduced mammary tumor risk (P = 0.004) and lowered cumulative tumor incidence by 33% compared to AL mice. Sustained calorie restriction throughout the 60-week study reduced mammary tumor risk of CR mice compared to both AL mice (P = 0.000001) and CR\(_{4-12}\) mice (P = 0.009). Cumulative numbers and percentages of 24 mice in each group which developed mammary tumors were: CR, 3/13%; CR\(_{4-12}\), 12/50%, and AL, 20/83%.

**DISCUSSION**
Controlling breast cancer requires an understanding of breast development and the cellular and molecular consequences of risk factors which influence normal breast development and contribute to carcinogenesis. That calorie intake during adolescence may influence breast carcinogenesis by affecting breast development has been suggested by both epidemiological (32–38) and experimental data (1, 4, 30, 31, 57), but has not been demonstrated.

In the present report, details of the relationship between calorie intake, mammary morphogenesis, and tumorigenesis are described. Peripubertal calorie intake influenced ductal end bud formation, rates of epithelial proliferation, ductal arborization, and alveolar budding. Delayed mammary development with calorie restriction does not impair subsequent functional differentiation of the mammary gland during prelactational mammogenesis, provided the energy needs of the gravid and lactating female are met (16, 58). Normal litters are produced and weaned, and normal lactating amounts of mammary...
α-casein mRNA are transcribed by calorie-restricted mice which are permitted free access to diet at mating through parturition (16, 58).

Sustained calorie restriction of the adult mouse further lowered cumulative mammary tumor incidence; increased the irregularity of vaginal estrus; and has been shown to lower and delay peak levels of estradiol and progesterone associated with ovulatory cycles (10); to lower serum levels of prolactin (5), growth hormone (11), insulin (12), and insulin-like growth factors I and II (11, 12); and to reduce cellular proliferation in the mature mammary gland and other organs (16, 19–22). Abrogation of mammary tumorigenesis by lifelong calorie restriction may be attributed in part to lowered developmental and adult mammary mitogenesis (16, 41, 42), with consequent reduction in oncogenic mutagenesis (41–43), including, in the C3H/HeOu mouse, reduced mouse mammary tumor virus transcription (58) and impaired proviral insertional activation of oncogenes (16).

Delayed mammary development and lasting protection from tumorigenesis with peripubertal calorie restriction may also be mediated by alterations in levels of hormones or expression of growth factors or inhibitors, such as lowered estradiol, progesterone, EGF, or TGF-α, or elevated TGF-β. Regression of mammary end buds, which occurred in glands of calorie-restricted mice, also occurs in mice following ovariectomy (34, 35) or submandibular sialoadenectomy (59), which removes a principle EGF source, or with the intramammary administration of TGF-β (36, 37). Mammary tumor incidence in mice is reduced by submandibular sialoadenectomy and reelevated by administering EGF to the sialoadenectomized mice (59). Intestinal hypoplasia, which results from i.v. feeding, is reversed by the administration of EGF (60). Administering EGF to calorie-restricted mice may reelevate lowered mammary and intestinal epithelial proliferation, expedite mammary development, and reelevate mammary tumor risk.

In this report, we demonstrate that calorie intake levels, which either abrogate or encourage tumorigenesis, influence mammary morphogenesis and thereby provide evidence for a link between calorie intake, mammary development, and carcinogenesis. These observations provide a basis for investigations of the relationship between calories, hormones, growth factors, and the events of mammary morphogenesis, and for determining which mammary developmental events and mediators are responsible for the lasting influence of peripubertal calorie intake on carcinogenic risk.
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