Effect of Thyroid Status on Development of Spontaneous Mammary Tumors in Primiparous C3H Mice

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ABSTRACT

Development of mammary tumors in primiparous C3H/HeN mice (mouse mammary tumor virus positive) in various thyroid states was followed for one year after removal of pups. Animals were either euthyroid or made hyperthyroid (by ingestion of thyroxine) or hypothyroid (by ingestion of 2-thiouracil) during involution. These manipulations resulted in significant changes in serum 3,5,3'-triiodothyronine and thyroxine levels without significant alterations in serum prolactin levels. At the end of one year postlactation, 90 to 96% of the euthyroid and hyperthyroid animals had developed mammary tumors, while the hypothyroid groups had only 70 to 72% tumor incidence. In two separate experiments, 50% tumor incidence was reached after 237 and 252 days in the hyperthyroid animals and after 242 and 252 days in the euthyroid groups. However, 50% tumor incidence in the hypothyroid groups was not reached until 290 and 287 days. The involuted mammary glands of all three groups were morphologically indistinguishable 10 weeks after removal of the pups. However, after 30 weeks, differences were seen. While glands from hyperthyroid and euthyroid animals retained a small degree of ductal branching with primitive alveoli, the glands from hypothyroid animals showed less ductal branching and were devoid of alveoli. Thus, the decrease in mammary tumor incidence in hypothyroid primiparous mice may be due to a greater degree of regression of the mammary epithelium in these animals.

INTRODUCTION

Morphological development of the rodent mammary gland has been shown to be affected by the thyroid status of the animal (11, 27, 33, 35, 37, 54, 61). In hypothyroid mice, lobuloalveolar development is severely retarded (11, 37, 61) while hyperthyroidism leads to significantly increased development of the mammary tissue (61). Attempts to correlate risk of breast cancer in humans with alterations in thyroid function have resulted in several epidemiological and clinical studies showing higher tumor incidence associated with hypothyroidism (6, 8, 34, 50, 55). However, other studies do not support these findings (1, 24, 28, 36, 43, 45, 56). Rodent models have been used in an attempt to clarify this situation. However, in these cases too, conflicting results have been reported. (For a complete review, see Ref. 60.) While some studies report increased tumor incidence and growth in hyperthyroid animals (14, 15, 17, 18), others have shown no effect (7, 19) or even a decrease (3, 11, 20, 23, 25, 37, 39). Studies using transplanted cells from established mammary tumor lines in both rats and mice have shown that physiological levels of thyroxine are necessary for establishing and growing the tumors in vivo (52) and that hypothyroidism appears to retard tumor growth (48, 49, 52) and increase animal survival (48, 49).

Severe hypothyroidism is known to result in changes in pituitary (13, 21) and ovarian (11, 13, 21) functions. Both prolactin (31, 40, 62, 63) and estrogens (30, 32) have been implicated in the regulation of development and maintenance of breast cancer in several species. Alterations in these hormones, rather than a direct effect of thyroid hormones, might then explain some of the previous findings. Therefore, we chose to examine the role of thyroid hormones in development and growth of spontaneous mammary tumors in mice made mildly hypothyroid by ingestion of thiouracil or mildly hyperthyroid by ingestion of thyroxine. Under the conditions used, virgin animals had been shown to have normal estrous cycles and no change in circulating levels of prolactin (61). Primiparous animals were used in an attempt to minimize the effects of thyroid hormones on morphological development of the gland. Previous studies have shown that, 7 weeks after the onset of involution, mammary glands from primiparous mice in various thyroid states are morphologically indistinguishable from each other (61). Despite this observation, we have found a significant decrease in the incidence of mammary tumors in the mildly hypothyroid primiparous animals one year after the onset of involution.

MATERIALS AND METHODS

Chemicals. Thyroxine and TU2 were purchased from ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio. 3,5,3'-Triiodothyronine and thyroxine radioimmunoassay diagnostic kits were obtained from Abbott Laboratories Diagnostic Division, North Chicago, Ill. Diet LF/150C was obtained from Dr. John D. Radcliffe (41, 42).

Animals. All mice were of the C3H/HeN strain and carried both MMTV-S (Bittner milk virus) and MMTV-L (nodule-inducing virus) (38). Mice were bred at 10 weeks of age. Only euthyroid animals in their first pregnancy were used. In Experiment A, pups were weaned from the dams at 22 days of age. The day the pups were removed was designated as Day 0. In each experiment, the euthyroid dams were then randomly assigned to one of 3 groups. The groups of animals then received drinking water containing either no additions ( euthyroid), 2 µg thyroxine per ml ( hyperthyroid), or 0.1% TU (hypothyroid) (61) for the duration of the experiment. All mice were fed a diet of Purina rat chow ad libitum (except for food and water consumption study) and were on a regular cycle of 12 hr of light and 12 hr of darkness. Animals were housed at 5/cage (27 x 16.5 x 13 cm) to minimize stress (44). All animals were weighed every 2 weeks.

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2 The abbreviations used are: TU, 2-thiouracil; HAN, hyperplastic alveolar nodules.
for the first 16 weeks after removal of the pups and again after 24 weeks. After 3 and 10 weeks, on the morning of estrus, 10 animals from each group were selected and killed by decapitation. The serum was collected and stored at −20 °C for subsequent hormone analysis. The No. 4 abdominal glands were whole mounted and stained with hematoxylin as described previously (61).

Food and Water Consumption. For food and water consumption studies, 10 animals in each group were placed on “spill-proof” diet LF/150C (41, 42) to assess accurately the amount of food ingested from the third to the tenth week after removal of the pups. The fat and protein content and quality of this diet are similar to that of Purina rat chow (41, 42). For this study, animals were weighed twice weekly, and the amount of food and water consumed was measured every other day. The animals were killed 10 weeks after removal of the pups, and the mammary glands were whole mounted and stained with hematoxylin (61).

Tumor Incidence and Mammary Gland Morphology. Beginning at the 16th week of involution, all animals were palpated twice weekly for the appearance of tumors. The date of appearance of tumors was noted as well as the location. Tumor sizes were measured weekly by vernier caliper measurements of the s.c. masses. When death seemed imminent or after 4 weeks of measurement, animals were killed by decapitation, serum was collected, and nontumorous abdominal glands were whole mounted and stained with hematoxylin (61). Whole-mounted glands were examined under a dissecting microscope and scored for the presence of absence of alveoli as described (26). Portions of randomly selected tumors were prepared for histological examination. All animals without tumors 365 days after removing the pups were killed, serum was collected, and the abdominal glands were whole mounted and stained with hematoxylin (61).

Serum Hormone Levels. Total 3,5,3′-triiodo-L-thyronine and thyroxine concentrations in serum were determined by radioimmunoassays performed according to the instructions of the manufacturer. Prolactin levels were determined by competition for binding of 125I-iodoprolactin to crude membranes prepared from mouse liver using a standard radioreceptor assay as described (47). Due to limited amounts of serum, levels of estrogen were not determined. However, vaginal smears were taken on animals (7 animals/group) between the ninth and 12th weeks after removal of the pups.

RESULTS

Animal Weight Gain and Food Consumption. Either following 10 to 12 days (Experiment A) or 22 days (Experiment B) of lactation after a single pregnancy, pups were removed, and the dams were randomly placed in one of 3 groups. The animals in each group received drinking water with no additions (euthyroid) or containing either 2 μg thyroxine per ml (hyperthyroid) or 0.1% TU (hypothyroid). In both experiments, the euthyroid animals continued to gain weight during the first 13 to 16 weeks after removal of the pups (to 7 months of age) after which their weight remained constant. The average body weight of the hyperthyroid and hypothyroid animals remained relatively constant throughout the experiment. At the 16th week, the average weights of the hyperthyroid and hypothyroid animals [25.8 ± 0.9 (S.D.) and 24.6 ± 1.3 g, respectively] were significantly different (p < 0.01) from that of the euthyroid mice (28.0 ± 0.6 g) but not from each other.

Similar weight differences were also seen in groups of animals placed on diet LF/150C (41, 42) in order to assess the effect of altered thyroid status on food and water consumption. Chart 1 shows that, while hyperthyroid and euthyroid animals ingested similar quantities of food, the hypothyroid group showed a slight decrease in average food consumption. The hyperthyroid animals drank more water per day and the hypothyroid animals less water per day compared to the euthyroid controls.

Morphology of Mammary Glands Prior to Appearance of Tumors. In both experiments, at 3 weeks and 10 weeks after removal of the pups, animals from each group were killed, and whole mounts of the abdominal mammary glands prepared. As seen in Figs. 1 to 3, after 10 weeks, the mammary glands from all 3 groups showed the same degree of regression, and the glands are morphologically indistinguishable from each other.

Serum Hormone Levels. However, at 10 weeks postlactation, the concentration of thyroid hormones in the sera of
animals in the 3 groups is significantly different. Table 1 shows that, 10 weeks after removal of the pups, animals receiving thyroxine in their drinking water had a 2.33-fold and a 1.58-fold increase in serum thyroxine and 3,5,3'-triiodo-L-thyronine, respectively, compared to euthyroid controls. The animals receiving TU in their drinking water showed a 47 and 58% decrease in serum thyroxine and 3,5,3'-triiodo-L-thyronine, respectively. Similar results were obtained at 3 weeks after removal of the pups (data not shown). Prolonged exposure of the animals to the thyroxine or TU in the drinker (water up to 1 year) did not result in greater intensity of the thyroid dysfunction. In addition, the serum prolactin levels in all 3 groups measured at several times throughout the experiment were not significantly different, attesting to the "mildness" of the alterations in thyroid status (61).

Vaginal smears were taken on animals for 3 weeks from the ninth through the 12th weeks after removal of the pups. Six of the 7 euthyroid animals showed normal 4-day estrous cycles with 1 animal having 5- to 6-day cycle. Five of the 7 hyperthyroid animals had normal cycles with one showing a 5- to 6-day cycle with prolonged diestrus and one showing continual estrus. Of the 7 hypothyroid animals examined, one had a normal cycle with prolonged diestrus and one showing continual estrus. Animals with prolonged cycles showed no consistent pattern of irregularity.

**Tumor Incidence.** Table 2 shows that 1 year after removal of the pups, when the animals were 16 months old, there was a significantly lower tumor incidence in the hypothyroid group compared to the euthyroid or hyperthyroid groups. Chart 2 plots the percentage of animals in each group with tumors against the latent days (i.e., days before the tumor first appeared) for Experiment A. In this experiment, tumors seemed to appear in hyperthyroid animals at a faster rate than in either the euthyroid or hypothyroid mice. However, by the end of 1 year postlactation, the euthyroid group as well as the hyperthyroid group had a tumor incidence of over 96%. The hypothyroid mice developed tumors at a generally slower rate and achieved a final tumor incidence of only 72%. The hyperthyroid and euthyroid groups reached a 50% tumor incidence after 237 and 242 days, respectively, while 290 days (p < 0.001) elapsed before 50% of the hypothyroid mice in the study had tumors. Of the tumor-bearing mice, 50% tumor incidence was achieved at 235 and 240 days for the hyperthyroid and euthyroid mice, respectively. With the hypothyroid mice, 50% of the tumor-bearing mice had tumors by 254 days. Similar analysis of data obtained in Experiment B did not demonstrate a more rapid onset of tumors in the hyperthyroid group (not shown). Fifty % tumor incidence was obtained after 252 days for both the hyperthyroid and euthyroid groups while 50% of the hyperthyroid animals had tumors after 287 days. The tumor incidence was 91.0, 89.7, and 69.6% for hyperthyroid, euthyroid, and hypothyroid groups, respectively.

Histological examination of the tumors showed no difference in the types produced in all 3 groups. Generally, the tumors
were type A, well-differentiated adenocarcinomas, with some type B (12). Growth of individual tumors was calculated and plotted. In all 3 groups, tumors were found which grew extremely rapidly, killing the animals within 7 to 10 days, as well as tumors which did not grow significantly during the 4 weeks they were measured. In general, no differences in the pattern of growth of the tumors were found in the 3 groups.

Examination of prolactin receptor levels in tumors from animals in all 3 groups gave widely varying results. No correlation of receptor levels with altered thyroid status was observed, in contrast to results obtained in livers and normal mammary tissue (5). In general, the levels of unoccupied binding sites were low, ranging from 15 to 60 fmol prolactin bound per mg of membrane tissue.

Only one or 2 tumors developed per animal although one euthyroid mouse did develop 4 small tumors. As a result, the tumor burden was not significantly different for the 3 groups.

Morphology of Normal Mammary Glands in Tumor-bearing Mice. Throughout the course of this study, when tumor-bearing animals were killed, the nontumorous abdominal glands were whole mounted and stained with hematoxylin. Upon careful examination of the glands, it was observed that, beyond the 30th week (animals approximately 11 months old), clear differences in the morphology could be scored. While the glands from all 3 groups were morphologically indistinguishable from each other at 10 weeks (Figs. 1 to 3), beyond the 30th week they were distinctly different. The mammary fat pads in all 3 groups, however, were not noticeably different throughout the course of this study. As seen in Figs. 4 to 6, after 44 to 45 weeks (animals approximately 13 months old), extensive regression of the epithelium had occurred in all 3 groups. The glands from the hyperthyroid and euthyroid animals remained morphologically indistinguishable from each other while those from hypothyroid animals appeared to be more extensively involuted. These observations were consistent throughout the glands. While there is evidence in rat breasts that local atrophy occurs with hypothyroidism although other focal areas of hyperplasia are seen concomitantly within the same tissues (2), such focal areas of well-developed normal growth were rarely seen in these studies or in previous studies with mice (11, 61). HAN, however, were regularly noted in the mouse mammary tumor virus-bearing mice of all 3 thyroid groups. On the whole, when all areas of the entire gland were examined by whole-mount techniques, it is clear that the tissues from the hyperthyroid and euthyroid animals contain many branched ducts and retain primitive alveolar structures (Figs. 7 and 8). The glands from hypothyroid animals (Fig. 9), on the other hand, show less ductal branching and are devoid of alveoli. These differences in regression were seen even in the glands of the non-tumor-bearing animals from all 3 groups which were killed 365 days after removal of the pups.

DISCUSSION

Several hormones, especially prolactin (32, 40, 62, 63) and estrogens (30, 32), have been implicated in mammary tumorigenesis. While thyroid hormones have a direct effect on growth and differentiation of normal mammary tissue (51, 58, 59, 61), their role in tumor development has been difficult to assess. This is due to their effects on the circulating levels of other hormones and on general metabolic processes.

Previous studies attempting to assess the role of thyroid hormones in mammary tumor development have been difficult to interpret because the authors have either not reported or not minimized the effects of altered thyroid status on the levels of other hormones, especially prolactin and estrogens. Nor have they always provided information on body weights and food and water consumption throughout the experiment. Most striking is the lack of any definition of the morphology of the mammary gland itself at the onset of the experiment or as a result of the alterations in thyroid status. However, in this study, we have attempted to clarify the issue and to minimize the secondary effects of thyroid hormones by choosing healthy, mature animals with well-developed mammary glands at the onset of the experiment. Similarly, we have chosen conditions which result in mild hyperthyroidism and hypothyroidism characterized by little or no change in circulating levels of prolactin. We have monitored food and water consumption as well as weight gain or loss and have examined the mammary gland morphology throughout the study.

From our results, it would appear that the decreased incidence in spontaneous mammary tumors in the mildly hypothyroid mice is probably not solely due to secondary effects resulting in changes in levels of prolactin or estrogens. Initially, the majority of these animals, like similarly treated virgin mice (61), appear to have nearly normal estrous cycles. Similarly, no significant differences in the serum prolactin levels were detected in the animals in all 3 thyroid states throughout this study. At the onset of the study, shortly after removal of the pups (3 weeks) and before the tumors arose (10 weeks), no differences were observed in the serum prolactin levels for all 3 groups (Table 1). As the study progressed, serum prolactin levels for tumor-bearing animals and animals without tumors after 1 year showed some differences which were not highly significant. The high degree of variability in these latter groups is probably due to killing the animals without regard for the stage of the estrous cycle and the irregularity of the cycle in animals of advanced age.

The lack of correlation of prolactin receptor levels with thyroid status has also been noted in 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats (53). In rats (16, 53), as in mice (5), prolactin receptor levels in livers are subject to regulation by alterations in thyroid status. The low levels of receptors in all tumors examined may reflect the lack of hormone dependence by these tumors once they are established (38).

Severely impaired weight gain or loss of weight due to extreme changes in thyroid status may have contributed significantly to the results obtained in some studies with rats (23, 25). While these considerations cannot be completely ruled out, their effects are considerably reduced by the mild alterations in thyroid status obtained in this study as well as the maturity of the animals at the onset of the experiment. While the euthyroid animals continued to gain weight for the initial 14 weeks of the experiment, the animals receiving TU and thyroxine did not demonstrate significant changes in body weight despite the alterations in circulating thyroid hormone levels. The reasons for the lack of weight gain in these latter 2 groups are probably not the same since the hypothyroid animals consumed less food and water while the hyperthyroid mice

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2 B. K. Vonderhaar, manuscript in preparation.
consumed the same amount of food but drank more water than euthyroid animals. The tumors which developed in these mice were most likely induced by the mammary tumor viruses MMTV-S and MMTV-L. Such "spontaneous" tumor formation has advantages over studies with rats using chemical carcinogens as thyroid status is known to affect metabolism of chemical carcinogens (4). However, thyroid hormones may act in the mouse system by changing the level or mode of viral expression. Indeed, Hosick and Nandi (22) have reported an increase in the number of domes in primary cultures of mouse mammary carcinoma cells cultured in the presence of 3,5,3'-triiodo-L-thyronine. Domes are believed to be the sites of virus production in vitro (29). A recent study by Sellitti et al. (46) showed that 3,5,3'-triiodo-L-thyronine treatment of mouse mammary tumor virus-positive retired breeders markedly increased the rate of mammary tumor incidence but had no effect on tumor growth rates as measured by tumor-doubling time.

It is possible that thyroid hormones control tumor production by affecting the development of HAN, precursors of mouse mammary tumors (9). Inapparent nodules can be recovered in mice as young as 2 to 3 months of age, long before the overt appearance of HAN (10). In this study, we used primiparous mice which already have HAN. While glands from all the animals, even non-tumor-bearing animals, had at least some HAN, the question arises of whether hypothyroidism causes preexisting HAN to regress, or whether it just retards the appearance of further HAN. Similarly, does hyperthyroidism induce the appearance of HAN? If HAN are a result of normal cyclic growth of alveoli and incomplete regression, hyperthyroidism may accelerate this latter process. That this may be so is suggested by the appearance of the glands from hypothyroid animals 30 weeks after removal of the pups. The glands of these animals appear to be more atrophic than those of euthyroid or hyperthyroid animals, resulting in a more ductal nature. Thus, accelerated regression could lead to loss of susceptible cells, thus leaving fewer sites for subsequent tumor formation. The resolution of these issues will greatly enhance our ability to evaluate the role that thyroid hormones play in spontaneous mammary tumorigenesis.

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References

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Figs. 1 to 3. Whole mounts of abdominal mammary glands from primiparous mouse in various thyroid states 10 weeks after removal of pups stained with hematoxylin (× 4). Fig. 1, hyperthyroid; Fig. 2, euthyroid; Fig. 3, hypothyroid.
Figs. 4 to 6. Whole mounts of abdominal mammary glands from primiparous mice in various thyroid states 44 to 45 weeks after removal of pups stained with hematoxylin (X 4). Fig. 4, hyperthyroid; 305 latent days. Fig. 5, euthyroid; 302 latent days. Fig. 6, hypothyroid; 312 latent days.
Fig. 7. Hyperthyroid; 305 latent days.

Fig. 8. Euthyroid; 302 latent days.

Fig. 9. Hypothyroid; 312 latent days.

Figs. 7 to 9. Higher magnification of whole mounts shown in Figs. 4 to 6 (x 35). Fig. 7, hyperthyroid; 305 latent days. Fig. 8, euthyroid; 302 latent days. Fig. 9, hypothyroid; 312 latent days.
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