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ORIGINAL ARTICLE

Mammary gland epithelial changes in thyroidectomized female rats

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Summary

Purpose: *Numerous clinical studies have addressed the relationship of hypothyroidism and breast cancer with conflicting results. In the present experimental study we sought to determine whether absolute hypothyroidism established for a long period of time leads to epithelial alterations of the mammary gland.*

Methods: *Thirty five female Wistar rats were allocated to be subjected to either thyroidectomy (N=20) or not (N=15). The rats were kept alive for a period of 3 months in a weather controlled environment. Serum T3, T4, follicular stimulating hormone (FSH) and estradiol levels were measured at baseline and 10 days after thyroidectomy. Mammary glands were obtained at the end of the experiment and reviewed by an expert pathologist.*

Results: *Both serum FSH and estradiol levels were lower 10 days after thyroidectomy; however, only FSH values were significantly lower in the thyroidectomized animals. Pathological analysis revealed significantly increased atrophy and periductal fibrosis of the mammary gland among thyroidectomized animals.*

Conclusion: *This is the first* in vivo *experimental study that reveals an association between the thyroid and mammary glands. Future studies should address the proteomic relationship that connects them.*

Key words: *breast, cancer, hypothyroidism, mammary, wistar rats*

Introduction

Thyroid hormones seem to influence the development and growth of various tissues [1,2]. Thyroid hormone receptors also seem to exert a proliferative effect that includes differentiation of various cell types and homeostasis of malignant lesions [3]. Deiodinase activity has also gained increasing attention in the last years in both normal and malignant tissue development [4,5].

Hypothyroidism has been linked to breast cancer development since in the late 19th century, when Beatson performed thyroidectomies in order to control breast cancer [6]. Since then a significant number of clinical studies have addressed the correlation of thyroid disorders (both hypo and hyperthyroidism) with breast. Results, however, were inconclusive and conficting. In an effort to summarize these studies Angelousi et al. performed a recent meta-analysis and reported that the risk ratio for developing breast cancer among hypothyroid women was slightly higher compared to euthyroid population (RR 1.06, 95% CI 0.82-1.35). However, this result wasn't statistically significant [7]. Hardefeldt et al. in their meta-analysis linked breast cancer with autoimmune thyroiditis (OR 2.92), anti-thyroid antibodies (OR 2.02) and goiter (OR 2.26), without finding a statistically significant prevalence of either hypo or hyperthyroid state [8]. These observations led to significant questions regarding the place of altered thyroid hormone profile in breast cancer de-

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velopment and further progression.

In previous experimental studies in wistar rats researchers showed that hypothyroidism, either surgical or pharmaceutical, resulted in irregular menstrual cycle that was reversed after administration of T4 [9]. Serum FSH and luteinizing hormone (LH) were decreased. Serum estradiol levels were also decreased and this observation was explained by the authors as unresponsiveness of ovarian granulosa cells to FSH stimulation.

However, the impact of hypothyroidism in breast cancer development has been previously questioned by Nogueira et al. who proposed that triiodothyronine mimics the effects of estrogen in a breast cancer cell line [10]. Almost synchronous to this report came another one from Shao et al. who discussed the possibility of cross-talk between T3 and estrogen on breast cancer proliferation [11].

In the present study, we sought to determine whether surgically induced hypothyroidism leads to alterations of mammary gland epithelium.

Methods

Animals

Thirty five female Wistar rats (12 weeks old) (Hellenic Pasteur Institute, Department of Animal Models for Biomedical Research, Greece) were maintained in weather controlled chambers (temperature 20±1 oC, humidity 55± 5%) under controlled light (12 hours light per day) for 5 days in order to adapt to their new environment. ELVIZ 510 food pellets were added *ad libitum*, containing full nutrient supplementation. Animal care, surgical operations and postoperative care were approved by the Athens University Medical School Ethics Committee and by the Veterinary Directorate of the Ministry of Agriculture in agreement with the European Union directive 86/609. The night before the operation food and water were restricted from animals.

Experiment

Twenty female wistar rats were randomly selected for thyroidectomy, using a computer generated system. All the experiments were performed between 8 and 9 a.m. on diestrous day 1 (D-1). Definition of menstrual cycle was assessed using vaginal smears as previously described by Hatsuta et al. [8]. Anesthesia was initially performed by placing the rats in a glass vessel containing cotton dipped in ether. They remained inside the bottle for a median time of 30-50 sec, depending on their response. Intramuscular injection of ketamine solution 0.3 g followed by 0.3 mg of midazolam completed the standard regimen. Before the initiation of thyroidectomy 500-1000 μl of blood sample were obtained from the ocular canthus in order to measure the levels of thyroid hormones, FSH and estradiol. During the procedure of thyroidectomy a mask containing cotton dipped in ether was placed near the animals' nose in order to keep adequate sedation.

Blood samples were collected again approximately 10 days postoperatively and during D-1 day in order to define whether adequate hypothyroidism was achieved in the thyroidectomized rats.

Finally, 3 months after the initiation of the experiment the animals were euthanized with ether and the abdominal wall was removed *en block* in order to obtain adequate samples of mammary gland.

Enzyme-linked immunosorbent assay (ELISA) of serum T3, T4, estradiol and FSH levels

Blood was collected in Vacutainer tubes (BD Diagnostics, NJ, USA). Centrifugation was applied at 3000 rpm for 10 min in order to separate the serum and the specimens were stored at -30 oC until assayed (within 1 month). All measurements were performed in an ELISA photometer (Model 680 Microplate Reader; Bio-RadTM, California, USA). Thyroxine, triiodothyronine, FSH and estradiol levels were assessed using ELISA kits provided by Cusabio Biotech Co. Ltd (Wuhan, Hubei, China).

Pathology

Abdominal wall specimens were immersed and preserved in a 10% solution of formaldehyde for 5 days. After this period they were placed in paraffin blocks and were cut in pieces with the use of microtome. Standard hematoxylin-eosin staining was applied in order to study structural abnormalities of the mammary epithelium.

Statistics

Statistical analysis was performed with SPSS v.20.0 statistical software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Continuous variables were analyzed with either standard two-tailed Student's t-test and were interpreted as mean±SD, or with the non-parametric Mann-Whitney U test and were interpreted as median and range, whereas categorical variables were analyzed with two-tailed chi-square test. The level of statistical significance was set at $p<0.05$.

Results

There was one intraoperative death among thyrectomized rats due to excessive anesthesia that resulted to cardiac arrest.

Thyroidectomy resulted in significant hypothyroidism 10 days after the operation (Table 1). The detectable levels of both T3 and T4 were lower

Table 1. Results of weight and hormone alterations among controls and thyroidectomized (treated animals) prior to the initiation of the experiment (groups designated as thyroidectomized 1 and control 1) and at the end of the experiment in the case of weight (groups designated as thyroidectomized 2 and control 2), or 10 days after the thyroidectomy in the case of biochemical data (groups designated as thyroidectomized 2 and control 2). All values are expressed as mean ± SD or median and range (parentheses). Weight is calculated in grams, T3 (triiodothyronine) in ng/ml, T4 (thryroxin) in μg/ml , FSH (follicle stimulating hormone) in ng/ml and E2 (estradiol) in pg/ml

Indices	*Control 1 (15)*	*Operated 1 (19)*	*p value*	*Control 2 (15)*	*Operated 2 (19)*	*p value*
Weight	190 ± 27	177 ± 21	0.12	234 ± 29	254 ± 19	0.02
T3	98 (87-109)	93 (2 - 218)	0.24	98 (92-118)	15 (2-41)	<0.001
T4	37 (25-49)	26 (16 - 160)	0.02	58 (44-69)	6 (1-12)	<0.001
FSH	248 (145-571)	241 (128 - 33)	0.18	172 (92-222)	109 (53-209)	0.01
E2	98 (66-159)	78 (28 - 317)	0.77	80 (19-140)	78 (20-123)	0.69

Table 2. Results of pathology test revealed statistically higher incidence of periductal fibrosis and of mammary gland atrophy among thyroidectomized animals

		Atrophy				*Fibrosis*			
Group	*Number*	*Normal*	*Moderate*	*Severe*	*p value*	*Normal*	*Moderate*	*Severe*	*p value*
Controls	15	3	10	2	0.040	3	9	3	0.050
Thyroidectomized	19	3	6	10		5	5	11	

than those observed by Hatsuta et al., confirming that adequate hypothyroidism for the purposes of the present experiment could be easily achieved within 10 days postoperatively.

As expected, hypothyroid animals had statistically significant higher weight among treated animals at the end of the experiment (Table 1). This result was intensified when analyzing the differences in weight gain among the two groups because the control group had an insignificant higher weight at the start of the experiment.

In both the thyroidectomized and control rats we observed a drop of both FSH and estradiol levels which was significantly different between groups only in the case of FSH (Table 1).

Pathological analysis showed that operated rats had significantly higher rates of moderate and severe mammary gland atrophy and significantly higher rates of moderate and severe periductal fibrosis (Figure 1, Table 2). In 3 cases of thyroidectomized rats we also observed moderate to severe ductal ectasia. In all 3 cases moderate periductal fibrosis was also present (Figure 2).

We didn't find any abnormalities regarding the nuclear-cytoplasmic ratio nor any other findings indicative of premalignant or malignant disease.

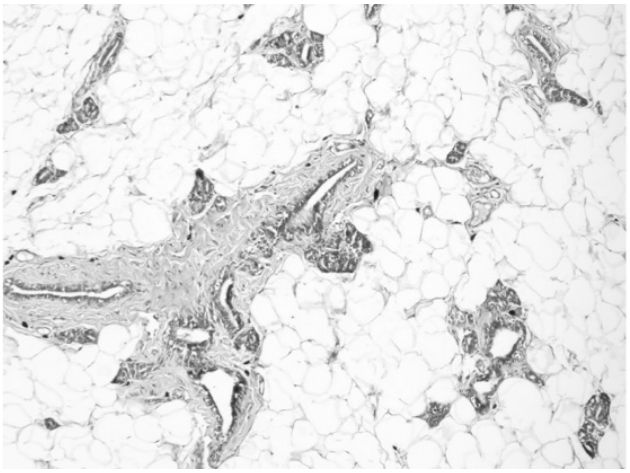

Figure 1. Marked periductal fibrosis in a mammary gland specimen from a thyroidectomized animal (x40).

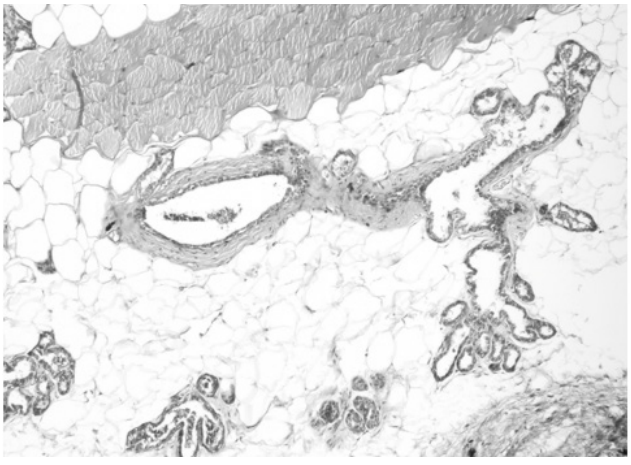

Figure 2. Moderate ductal ectasia in a mammary gland specimen from a thyroidectomized animal (x40).

Discussion

The purpose of the present study was to investigate the effects of hypothyroidism in mammary gland epithelium. We used surgically in-

duced hypothyroidism in order to avoid drugs that could potentially lead to bias regarding our observations.

To our knowledge this is the first *in vivo* experimental study investigating this relationship. Its advantage is the attainment of hypothyroidism with extremely low levels of thyroid hormones that persisted during a long period of time (one tenth of the average wistar rat life).

Previous *in vitro* studies observing the effects triiodothyronine on cell proliferation have proposed that it exerts a direct effect on L-T3-stimulated cell lines [12]. Recently, Cestari et al. proposed that both estradiol and thyroid hormones influence growth of S30 breast cancer cell line by targeting estrogen receptors [13]. They also proposed at their conclusion that, before introducing antiestrogenic therapy to breast cancer patients, T3 levels should be predetermined.

Iglesias et al., on the other hand, observed that although the hypothyroid enviroment decelerated tumor growth in the breast cancer MDA-MB-468 cell line, it seemed to correlate with a more mesenchymal phenotype accompanied by increased invasiveness and metastatic growth [14].

The present study indicates a direct association between the two glands. Fibrosis and duct obliteration had previously been discussed to be present in 90% of both lobular and tubular breast carcinomas [15]. In two consecutive synchronous studies ductal elastosis has been linked to the severity of breast disease [16] and to either invasive lesions or lactation [17]. In a recent systematic review and meta-analysis of the literature McCormack et al. found that increased breast density seems to be an independent risk factor for developing breast cancer and that this effect is independent from other possible confounders, with the exception of age and body mass index [18]. Boyd et al. discussed in their review that mammographic density seems to be influenced by both genetic and environmental factors, with hormones having a predominant role in differences of the breast parenchyma [19]. The same authors also concluded that mammographic breast density seems to be directly connected with the risk of developing malignant breast disease.

Johansson et al. observed that low estradiol levels and high FSH were predictive of mammographic breast density among postmenopausal women [20], a finding that was, however, argued by others [21]. In our study, although FSH levels were significantly lower in thyroidectomized rats, this effect didn't affect estradiol levels. Moreover, we didn't observe elevated levels of FSH after thyroidectomy but rather decreased, when compared with the pretreatment levels, a result that renders the hormonal evaluation more difficult to interprete in conjunction with epithelial changes.

In our study we found that absence of thyroid hormones results both to periductal fibrosis and mammary gland atrophy. While none of these findings is identified as a precancerous lesion, stromal fibrosis has been previously linked to increased risk of developing breast cancer. In previous *in vitro* models hypothyroidism was linked with patterns of increased mesenchymal phenotype that were associated with enhanced potential of growth and invasiveness [14]. As an increasing number of articles [22-26] seems to link malignant development in various tissues with thyroid function, thyroid receptors and deiodinases, and the need for close follow up of thyroid function at least in patients that suffer from breast cancer is becoming imperative.

The results of our study clearly indicate that hypothyroidism exerts a direct effect in mammary tissue. Although hypothyroidism may contribute to the development of breast cancer, the multifactorial etiology of this disease precludes safe conclusions. Future studies should aim towards unveiling the possible molecular pathways that connect hypothyroidism with breast cancer.

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