The immunohistochemical demonstration of parafollicular cells and evaluation of calcium-phosphate balance in patients with thyroid hemiagenesis

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Abstract: Thyroid hemiagenesis (TH) is characterized by the congenital absence of one thyroid lobe. The aim of this study was to evaluate the calcium-phosphate balance in TH. Twenty patients with TH and 20 controls with a bilobed thyroid were studied. Serum concentrations of total calcium, parathormon and calcitonin were measured. Additionally, the immunohistochemical expression of calcitonin, chromogranin A (chA), neuron-specific enolase (NSE) and calcitonin gene-related peptide (CGRP) was evaluated in surgical specimens from patients with TH and controls. There were no significant differences in biochemical parameters between TH and controls. Positive staining for calcitonin was demonstrated in 3/8 thyroid sections from three patients with TH, but only in 2/33 sections from four controls (p < 0.005). All sections from patients with TH positive for calcitonin also expressed chA, NSE and CGRP. Two sections from controls positive for calcitonin presented an additionally positive reaction for chA, and one of them also for NSE. None presented positive staining for CGRP. Of three TH sections, in one, hyperplasia of C cells of medium grade, and in another hyperplasia of C cells of high grade, could be detected. In the controls, hyperplasia of C cells of low and medium grade was observed. TH was associated with slightly enhanced C cells hyperplasia compared to controls, which might indicate compensatory proliferation. However, the calcium-phosphate balance does not seem to be significantly affected. (Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 2, pp. 299–305)

Key words: immunohistochemistry, parafollicular cells, calcium-phosphate balance, thyroid hemiagenesis

Introduction

Histologically, the thyroid gland is formed by follicular and parafollicular cells. Although sharing localization, these cells differ considerably in terms of their origin and physiological role. The former constitute thyroid follicles, and are engaged in the process of production and release of thyroxine and triiodothyronine. Parafollicular cells are scattered in small clusters at the periphery of the follicles, predominantly in the central part of thyroid lobes. Their main function is the production of calcitonin, the hormone involved in regulating the calcium-phosphate balance [1].
Precursors of follicular cells originate from the endodermal epithelium, lining the floor of the mouth between the 1st and 2nd pharyngeal arch [2]. The thyroid primordium is formed on 20th–22nd day of embryogenesis, and then begins its descent along the anterior pharyngeal wall, gradually gaining its bilobed structure [3, 4]. On the 48th–50th day of development, the gland reaches its final prelaryngeal localization. Meanwhile, parafollicular cells precursors migrate from the neural crest bilaterally towards the 4th and 5th pharyngeal pouches and subsequently locate within the ultimobranchial bodies. These are twin structures, which develop independently of thyroid anlage, migrate towards the midline and on about the 44th day fuse with the descending thyroid primordium. Eventually, they constitute about 1–30% of thyroid mass [5]. Following the fusion, parafollicular cells carried by ultimobranchial bodies disperse between the thyroid follicles and, therefore, the final histological structure of a thyroid gland is formed, while ultimobranchial bodies disappear.

Thyroid hemiagenesis (TH) is a rare congenital anomaly in which one of the thyroid lobes fails to develop [6]. The etiopathogenesis of this entity is still unknown. It is thought that impaired development of ultimobranchial bodies may underlie the disturbed formation of a bilobed structure of the thyroid in cases of TH. It is not yet known whether TH is an isolated abnormality or whether concomitant developmental anomalies of other structures derived from pharyngeal apparatus may coexist. One recently described experimental model of TH are TBX-1 gene knock-out mice, characterized by developmental failure in II–VI pharyngeal arches and II–IV pharyngeal pouches [7]. Therefore, the animals present with a unilateral thyroid, devoid of parafollicular cells and parathyroid glands, associated with a lack of ultimobranchial bodies formation [7, 8]. The bioavailability of parafollicular cells in subjects with TH is unknown, although it has been hypothesized that their survival or activity might be deteriorated [9]. To the best of our knowledge, neither the function of parafollicular cells nor that of parathyroid glands in humans with TH has yet to be systematically evaluated.

The aim of this study was to assess the possible association between TH and concomitant alterations of other structures derived from pharyngeal apparatus, which might potentially affect the calcium-phosphate balance.

**Material and methods**

The studied group consisted of 20 patients with TH (16 women, four men), aged 35.4 ± 17.4 (mean ± SD), with median value of 32 years, while the control group comprised 20 subjects with a normally developed thyroid gland, matched for age and gender. Patients with a history of treatment with monoclonal antibodies, chronic renal failure, pernicious anemia or pregnant women were excluded from the study.

A fasting morning specimen of blood was obtained from each patient and kept on ice. Specimens affected by visible hemolysis or lipemia were avoided. Red cells were separated within 30 minutes of collection and serum was frozen immediately.

Serum concentration of total calcium, parathormon and calcitonin were measured in basal conditions. Estimation of total calcium concentration was performed with complexometric assay and Roche kits. Parathormon concentration was determined with electrochemiluminescent assay (ECLIAs), via Roche Elecsys 1010/2010 analyzer, while calcitonin concentration was assessed using radioimmunoassay (RIA) and Brahms kits.

The obtained results were subjected to statistical analysis. Serum concentrations of total calcium, parathormon and calcitonin in patients with TH were compared to those in the control group of subjects with a fully developed thyroid gland. The significance of differences of the parameters measured in an interval scale was performed with Student’s t-test for unpaired data. All calculations were done with STATISTICA version 8.0. Differences were assumed to be significant at the level of p < 0.05.

Additionally, an immunohistochemical study of calcitonin expression in eight surgical specimens, obtained from three patients with TH subjected to thyreoidectomy was performed. The same was performed in 33 control sections from surgical specimens obtained from four patients with a normally developed, bilobed, thyroid gland. All subjects were operated upon owing to medical indications (non-toxic nodular goiter). Patients with the diagnosis of colloid nodular goiter, and with malignancy excluded upon routine histopathological examination, were included in the study.

The thyroid sections were fixed in a 10% formalin solution and subsequently embedded in paraffin, and cut into 5 μm thick sections. The immunohistochemical staining was performed according to ABC (Avidin–Biotynylated peroxidase Complex) method. On the first day, sections were deparaffinized in xylene and hydrated in a row of alcohol solutions of decreasing concentrations (100%, 90%, 85%, 80%, 70%), and subsequently washed in running water for ten minutes. The next step involved blocking endogenous peroxidase activity by incubation with 1% solution of hydrogen peroxide for 30 minutes. After subsequent washing in running and, then in distilled, water, each for ten minutes, sections were incubated in a normal goat serum diluted 1:20 for 30 minutes. The key step was incubation with adequately diluted (1:5) polyclonal rabbit anti-human anti-
bodies to calcitonin (Dako) for about 18 hours at a temperature of 4°C. On the next day, the incubation was continued for one hour at room temperature and the sections were washed in a phosphate buffered saline (PBS) three times for three minutes in a shaker. Then, the sections were washed in a Dako REALTM solution (Envision) for 30 minutes and, subsequently, in PBS three times for three minutes and, then, incubated with 3,3’-diaminobenzidine for 5–7 minutes, washed for ten minutes in running water, and then for ten minutes in distilled, water. Consecutively, the sections were counterstained with hematoxylin (for two minutes) and then washed in running water for ten minutes. Next, the preparations were subjected to dehydration in a number of alcohol solutions of increasing concentrations (70%, 85%, 90%, 95%, 100%) and treated with xylene. The final step was closing each preparation with the use of Canadian balm and cover glass. Each time a negative control was performed, where specific antibodies were replaced with PBS.

In the preparations presenting positive staining for calcitonin, additional assessment of chromogranin A (chA), neuron-specific enolase (NSE) and calcitonin gene-related peptide (CGRP) expression was performed, with the use of polyclonal rabbit anti-human antibodies to calcitonin and chA (DAKO), as well as monoclonal mouse anti-human antibodies to NSE (DAKO) and CGRP (Santa Cruz).

The study was approved by the local Ethics Committee, and all patients gave informed consent for participation in the project.

**Results**

The serum calcitonin concentration in patients with TH was slightly lower compared to control subjects, while the concentrations of calcium and parathormon were comparable. However, there were no statistically significant differences between serum concentrations of calcitonin, total calcium and parathormon between the two groups (Table 1).

All three patients with TH referred for surgery presented agenesis of a left thyroid lobe. Positive staining for calcitonin was demonstrated in three out of eight (37.5%) thyroid sections from three patients with TH, but only in two out of 33 (6.06%) sections from four control patients (p < 0.005). All preparations from patients with TH, which were positive for calcitonin, presented also positive reactions for chA, NSE and CGRP. Two preparations from control subjects, positive for calcitonin, presented additionally a positive reaction for chA, and one of them also for NSE. None, however, presented positive staining for CGRP. Of three preparations from patients with TH, in one preparation hyperplasia of parafollicular cells of medium grade, and in another preparation hyperplasia of parafollicular cells of high grade, could be detected (Figures 1). In comparison, in control preparations, hyperplasia of C cells of low and medium grade could be observed (Figure 2).

**Discussion**

An extensive search of the literature has revealed about 300 cases of TH described to date. Most of the patients presented with concomitant thyroid pathologies [6], including simple goiter [10], non-toxic [11] and toxic nodular goiter [12], autonomous nodule [13], Graves’ disease [14], Hashimoto’s thyroiditis [15], subacute thyroiditis [16], congenital hypothyroidism [17], accessory lingual thyroid [18], thyroglossal duct cyst [19], papillary cancer [11] as well as follicular cancer [20]. Interestingly, among numerous cases of HT associated with different thyroid pathologies, there were no reports of medullary cancer [20, 21]. In a number of patients, HT was accompanied by extrathyroidal abnormalities, including parathyroid adenoma [22], autoimmune polyglandular syndrome type III [23], Williams syndrome [24], pituitary adenoma [25], Down syndrome [26], Marfan syndrome [27], right aortic arch [28] or dysmorphic face with short stature [29]. However, it has never been investigated whether TH is associated with disturbances of the calcium-phosphate balance.

Although parathyroid glands develop in close proximity to the thyroid, it is unknown whether development of these structures is directly connected or occurs independently. Parathyroids are structures derived from 3rd and 4th pharyngeal pouches, while thyroid follicular cells develop from endoderm between the 1st and 2nd pharyngeal arch [2, 4]. It has been hypothesized that secondarily to disturbed development of a thyroid lobe in TH, the formation of

### Table 1. Comparison of serum concentrations of calcitonin, total calcium and parathormon in patients with thyroid hemiagenesis and control group of subjects with normally developed bilobed thyroid gland

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<th>Thyroid hemiagenesis</th>
<th>Control group</th>
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<tbody>
<tr>
<td>Calcitonin [pg/ml]</td>
<td>6.84 ± 3.8</td>
<td>8.54 ± 6.3</td>
<td>0.408</td>
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<tr>
<td>Total calcium [mg/dl]</td>
<td>9.62 ± 0.8</td>
<td>9.89 ± 0.6</td>
<td>0.331</td>
</tr>
<tr>
<td>Parathormon [pg/ml]</td>
<td>40.99 ± 16.8</td>
<td>42.96 ± 14.4</td>
<td>0.978</td>
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surrounding structures derived from the pharyngeal apparatus is aberrant, which might influence calcium, parathormon or calcitonin levels in these subjects. In some cases of HT, on the side of the agenetic lobe, no parathyroids or thyroid artery have been demonstrated [22, 28, 30].

On the other hand, the number and localization of parathyroid glands can vary greatly even among subjects with a normal thyroid. Thus even the lack of two parathyroid glands on the side ipsilateral to the agenetic lobe might not be reflected in detectable disturbances in calcium or PTH concentrations, due to compensatory functioning of the remaining two. In a patient described by Sakurai et al., HT was diagnosed during diagnosis of symptoms suggesting hyperparathyroidism [22]. During the surgery, pre-operative diagnosis of one thyroid lobe agenesis, as well as a lack of parathyroid glands at the unilateral side, was confirmed. No thyroid arteries were seen, but a recurrent laryngeal nerve of typical localization was localized. Similarly, a lack of thyroid arteries was detected on the side of a lobe agenesis in the studies by Mariani et al. as well as by Konno and Kanaya [28, 30]. This might be a confirmation of a theory of developmental failure of one thyroid lobe due to disturbed vascularization. For the same reason, parathyroid glands on the same side may not develop. On the other hand, Woods et al. demonstrated the presence of two parathyroid glands also on the side of thyroid lobe agenesis during the surgery of a patient with TH [31].

In our three patients with TH who were operated upon, on the side of a single thyroid lobe two normal parathyroid glands were seen, with no evidence of hyperplasia and presenting typical location on the posterior wall in the poles of a thyroid lobe. Due to pre-operatively confirmed agenesis of one thyroid lobe and lack of medical indications for exploration of a contralateral side, the surgeons did not investigate the other side of the neck, hence we could not confirm the presence or absence of parathyroid glands on the contralateral side. However, none of the patients presented (either pre- or postoperatively) symptoms of disturbed parathyroid function.

Data on the presence of C cells nests in the thyroid or parathyroid glands, and a described case of a medullary cancer developing in the lingual thyroid, indicate that parafollicular cells migration is occasionally subject to aberration [5]. The localization of C cells in subjects with thyroid agenesis is not known. In these patients, cystic structures in the thyroid bed are often visualized on ultrasound examination, although these residues have not been evaluated immunohistochemically for the presence of parafollicular cells [32]. Thus, the proximity of parathyroid...
glands and the thyroid, as well as reports on experimental animals in which HT was associated with disturbed C cell development, prompted us to evaluate the calcium-phosphate balance in subjects with TH [7].

DiGeorge syndrome and Williams syndrome are genetic disorders that have been found to be often accompanied by TH. The former is associated with the deletion of 7q1l1.23, containing genes implicated in the SHH signalling [33, 34], while in the latter haploinsufficiency of TBX1 gene is responsible for the major developmental defects [35]. In mice with the knock-out of TBX1 or SHH gene, thyroid develops as a unilobate gland resembling TH in humans [7, 36]. In TBX1 –/– mice with TH, the proper histogenic pattern and functional differentiation of follicular cells were confirmed by the expression of thyroglobulin, TTF1 and TTF2, while no positive reaction for calcitonin in the immunohistochemical examination was demonstrated. This was because that thyroid is devoid of the presence of parafollicular cells, presumably due to disturbance of ultimobranchial bodies formation [7]. Moreover, in TBX1 –/– mutant embryos, number of C cells precursors in the surroundings of thyroid anlage was significantly reduced. Therefore, it was suggested that the proper bilobation of the thyroid gland requires the presence of cells of neuroectodermal origin. In addition, it has been demonstrated that follicular cells begin to express thyroid-specific antigens only at the moment of fusion with ultimobranchial bodies (Zabel M et al., unpublished data). This hypothesis was confirmed in a study of chick embryos, in which neural crest ablation led to thyroid agenesis or hemiagenesis [37]. On the other hand, Demeester-Mirkine et al. hypothesized that proper development of parafollicular cells requires the presence of factors secreted by follicular cells, while cells which did not migrate adequately would not present a proper function [38]. In a study of SHH knock-out mice, the expression of calcitonin in the unilateral thyroid was not evaluated [36]. However, embryos deficient in SHH or TBX1 present similar defects in the head and neck region. Moreover, it has been demonstrated that TBX1 is positively regulated by SHH and its expression is abrogated in SHH deficient mice [39].

Apart from the work by McHenry et al., where a group of seven patients with HT was studied according to the calcium concentration, and no abnormalities were found [20], in the studies performed to date, larger groups of subjects with TH have not been evaluated according to the state of the calcium-phosphate balance and expression of parafollicular cells in a hemiagenetic thyroid preparation.

Therefore, our study aimed to demonstrate the presence or absence of C cells in surgical preparations from patients with TH, and to compare it to the result of biochemical assessment of serum calcitonin concentration. By immunohistochemical examination, the presence of C cells in thyroid preparations from subjects with TH was demonstrated in amounts suggesting even compensatory hyperplasia in comparison to thyroid sections obtained from patients with a bilobed gland. We hypothesize that observed hyperplasia represents a compensatory mechanism in response to the congenital absence of one of the thyroid lobes. Further studies on larger groups of patients are required to confirm these results.

The clinical assessment of the calcium-phosphate balance in subjects with thyroid dysgenesis has only occasionally been studied. Daripa et al. demonstrated that subjects with congenital hypothyroidism due to thyroid dysgenesis present comparable basal concentrations of calcium, parathormon and calcitonin, but the calcium-stimulated calcitonin response is blunted [9]. This corresponds to the results of our study, performed on the group of patients with TH, where no significant differences in these parameters were observed compared to normal subjects. This is probably because of compensatory hyperplasia of parafollicular cells in the single thyroid lobe. It is also possible that in subjects with TH, extrathyroid production might play the role of a compensatory mechanism in response to the decreased number of C cells in a single thyroid lobe as described in some patients following total thyreoidectomy [40]. However, one limitation of the study is that provocative tests, with assessment of calcitonin level followed by administration of calcium or pentagastrin, were not performed. On the other hand, Demeester-Mirkine et al. evaluated the basal and calcium-stimulated calcitonin concentration in subjects with congenital hypothyroidism and found that the basal concentration of calcitonin, as well as the secretory reserve of parafollicular cells, were decreased [38]. Similar findings were obtained by Carey et al. who demonstrated calcitonin deficiency, as well as altered calcitonin response, to calcium or pentagastrin infusion in children with non-goitrous cretinism [41].

To summarize, our study provides the first immunohistochemical demonstration of the presence of parafollicular cells in subjects with TH. The analysis of surgical specimens from patients with TH reveals slightly enhanced parafollicular cells hyperplasia compared to control preparations, which might be a result of compensatory stimulation in the presence of a single thyroid lobe. However, the calcium-phosphate balance does not seem significantly affected in patients with TH compared to subjects with a normally developed thyroid gland.
Funding

This study was supported by the State Committee for Scientific Research (grant no. N N402 016736).

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Submitted: 18 March, 2011
Accepted after reviews: 1 May, 2011