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Alteration of parafollicular (C) cells activity in the experimental model of hypothyroidism in rats.

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Abstract: Our previous study has shown the alteration of C cells activity in rats with experimental model of hyperthyroidism. The aim of the present study was the evaluation of parafollicular cells activity in rats with hypothyroidism evoked by propylthiouracil (PTU) given in drinking water over 21 days. Histological, ultrastructural and immunocytochemical studies using specific antibodies against calcitonin and CGRP were performed on thyroid glands taken from experimental and control groups of rats. Moreover, in all animals the calcitonin plasma levels were evaluated by radioimmunoassay. After chronic administration of PTU, thyroid image showed predominant microfollicular hyperplasia and attenuated density of parafollicular cells. The intensity of immunocytochemical reactions for CT and CGRP were weaker in the majority of C cells in comparison to the control rats, in which strong immunocytochemical reaction was observed. Examination in the electron microscope reveals the features of hypoactivity both in follicular and parafollicular cells, in which the quantity and electron density of secretory granules were smaller in comparison to the control group. These microscopic changes were accompanied by a significant decrease of calcitonin plasma concentration. Alteration of C cells activity in the experimental model of hypothyroidism, accompanied by microfollicular hypertrophy, may point to the mutual cooperation between parafollicular and follicular cells.

Key words: C cells - calcitonin - CGRP - hypothyroidism - rats

Introduction

In the thyroid gland of mammals except the basic follicular cells, irregularly distributed cells were described. The most common name of them is parafollicular cells, or C cells (calcitonin cells) [24]. According to Pearse [17] they belong to disperse neuroendocrine cells of APUD system (amine precursor uptake and decarboxylation). The role of parafollicular cells in the function of the thyroid gland has not been clarified till now. Despite controversial data, one could presume that co-localisation of follicular and parafollicular cells in the thyroid gland is not accidental. It seems to be possible that there is an interaction between them mediated by the releasing of peptidergic hormones [3,23].

There is support for the notion that parafollicular cells synthesise and release many of regulatory peptides like: catacalcins, gastrin releasing peptide, somatostatin, thyreoliberin (TRH), ghrelin and helodermin [3,13,23,24]. Moreover, parafollicular cells produce neuromediators like: calcitonin-gene-related peptide (CGRP), N-terminal fragment of CT/CGRP gene expression, neuromedin, cholecystokinin, secretory petide I, vasoactive intestinal peptide, neuropeptide Y and histydylo-methionin peptide [3,23,24]. The kind and amount of released hormones depend on many agents i.e. age, sex and state of health [3].

As the essential indicator of parafollicular cells activity, 32 amino-acids hormone - calcitonin (CT), product of CT/CGRP gene expression is proposed [25,29]. The basic action of calcitonin is diminution of concentration of Ca ions, by inhibition of reabsorption activity of osteoclasts and by facilitation of calcium excretion in kidneys [3,8,20]. The finding that in homozygotic mice devoid of gene coding calcitonin osteopenia is developed may indicate that this hormone plays an important role in bone tissue homeostasis [11,12]. Thus, calcitonin is applied in hypercalcemia, Paget's disease and osteoporosis

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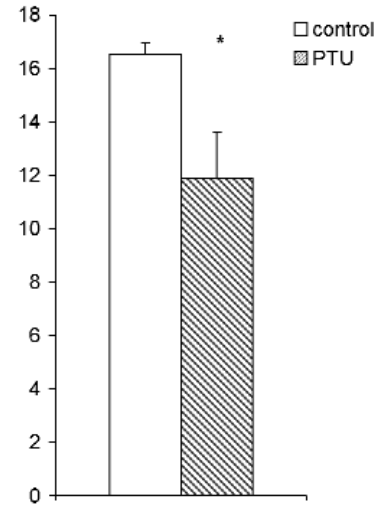

Fig. 1. The influence of PTU on calcitonin plasma concentration (pg/ml). Values are means ±SD, *p<0.001 vs control animals (Student's test).

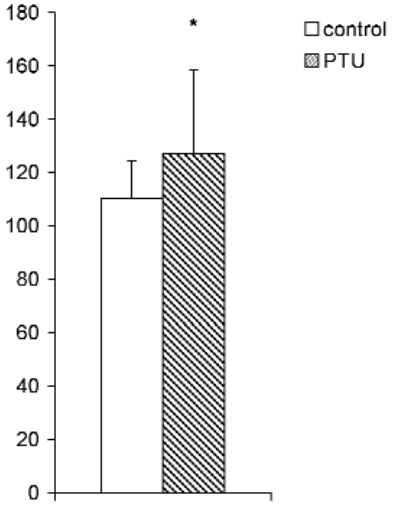

Fig. 2. Average optical density of immunocytochemical staining for calcitonin in parafollicular cells after PTU administration. Values are means ±SD, *p<0.001 (Mann-Whitney test).

[20,25]. There are a few publications concerning the evaluation of the structure and function of parafollicular cells in the thyroid gland diseases [2,5]. There is also only several data dealing with the problem of mutual relation between parafollicular and follicular cells in physiological and also in pathological conditions [4,6] and only a single observation concerning ectopic production of CT in other tissues [3].

The aim of this study was the evaluation of the activity of thyroid parafollicular cells in experimental model of hypothyroidism.

Materials and methods

Animals. The study was performed on thirty, male Wistar rats weighing approximately 90-100 g at the beginning of the experiment. All animals had free access to standard granulated diet and drinking water. The animals were housed in plastic cages at 22 ± 1°C and constant humidity, with a 12/12 light/dark cycle, beginning at 7 am. The rats were randomly divided into 2 groups with 15 animals in each group. Experimental model of hypothyroidism was evoked in 15 rats by 0.1% propylthiouracil (PTU) given in drinking water over 21 days; remained 15 rats served as controls.

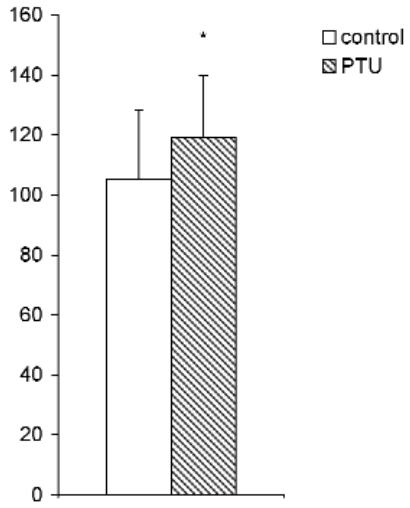

Fig. 3 Average optical density of immunocytochemical staining for CGRP in parafollicular cells after PTU administration. Values are means ±SD, *p<0.001 (Mann-Whitney test).

At the end of experiment, the animals were anaesthetized with pentobarbital sodium (50 mg/kg b. wt.), their abdomen was opened by midline incision and the blood was taken from the abdominal aorta of each rat for measurement of calcitonin serum concentration. Subsequently, all rats were thyroidectomized. The tissues taken from 10 animals of each group were fixed in Bouin's fluid and were prepared to histological and immunocytochemical investigations. The thyroid glands from remained animals were prepared to ultrastructural evaluation.

All procedures were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Local Ethics Committee in Bialystok.

Determination of calcitonin plasma concentration. The blood taken from abdominal aorta of each rat was collected into polypropylene tubes without anticoagulant and was incubated in room temperature until the clot was formed and then centrifuged (2500 × g for 15 min). The supernatant (serum) was removed and stored at -20°C until a consequtive analysis. Calcitonin levels were determined by the double-antibody radioimmunoassay technique. The protocol for radioimmunoassay kit is accessible on the web site: www.phoenixpeptide.com. The sensitivity of this kit was IC50 ~20 pg/tube with a low limit ~4.9 pg/tube.

The immunocytochemical study. The thyroid glands were fixed in Bouin's fluid for 1 day at 4°C. After thorough washing in 0.1 M phosphate buffer (pH=7.4) at 4°C, the tissues were routinely embedded in paraffin, and 5-μm-thick sections were cut. For blocking of the endogenous peroxidase activity Peroxidase Blocking Reagent (Dako Poland) was used over 10 minutes. In these studies a specific antibody against CT (Dako Poland) and CGRP (SIGMA-Aldrich) were used. After washing with distilled water and 0.05 M TRIS-HCl pH=7.4, three times for 5 minutes, the sections were incubated with the antibody for 15 minutes (CT) and 1

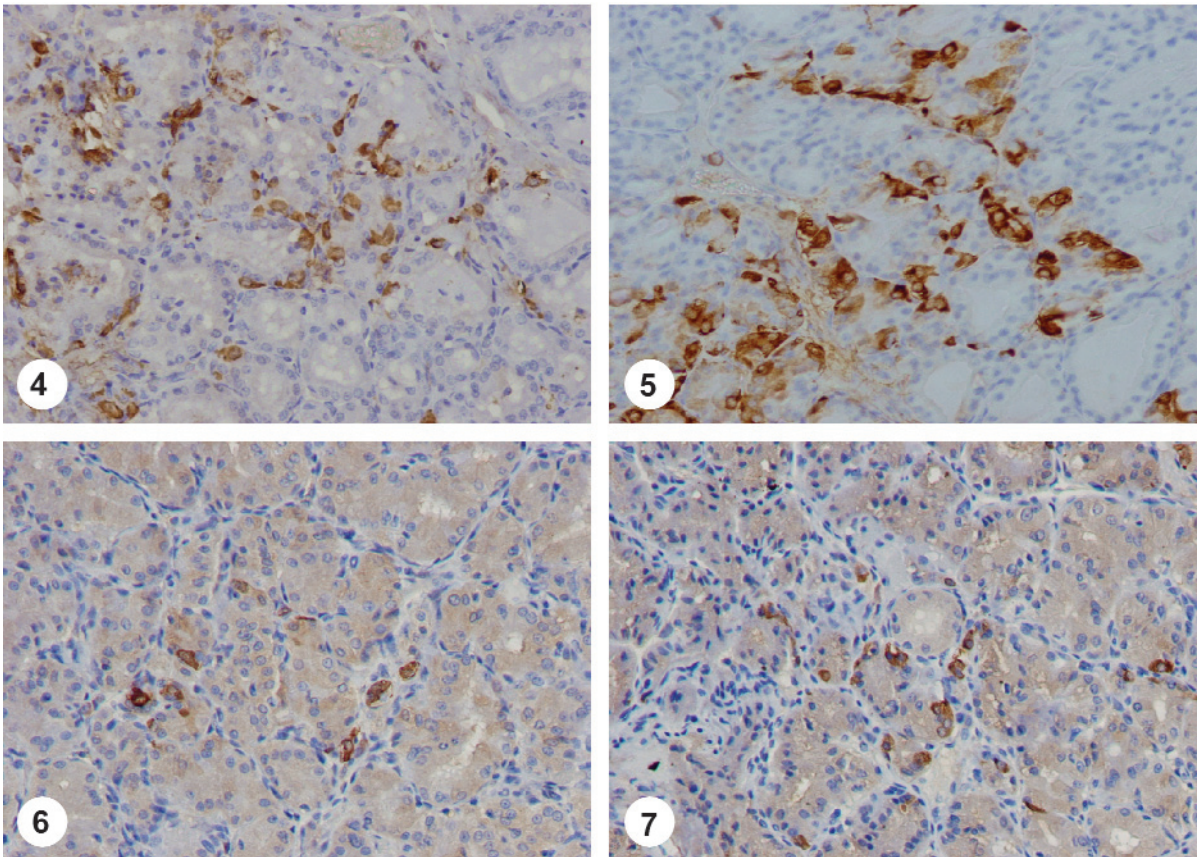

Fig. 4. Light micrograph of thyroid gland of control rat. Positive immunocytochemical reaction for calcitonin was observed in majority of C cells (magnification ×200). **Fig. 5.** Light micrograph of thyroid gland of control rat. Positive immunocytochemical reaction for CGRP was observed in majority of C cells (magnification ×200). **Fig. 6.** Light micrograph of thyroid gland of rat treated with PTU. The attenuation of immunocytochemical reaction for calcitonin was observed in majority of C cells (magnification ×200). **Fig. 7.** Light micrograph of thyroid gland of rat treated with PTU. The attenuation of immunocytochemical reaction for CGRP was observed in majority of C cells (magnification ×200).

hour (CGRP) at room temperature. Then, sections were washed three times in TRIS buffer. The LSAB2 (Labelled Streptavidin-Biotin 2 System) method (CT) and EnVision method (CGRP) was applied, according to the protocol for identification of the immunocytochemical reaction [7,9]. The sections were counterstained with Mayer's haematoxylin. In the negative control, the specific antibody was omitted in the staining procedure. Positive control was done for specific tissue recommended by the producer. The histological preparations were subjected to analysis, using Olympus B ×50 microscope.

Ultrastructural study. The thyroid glands from 5 animals of each group were cut into approximately 1 mm^3 pieces and then fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (CB, pH=7.4) for 2 h at 4°C. The specimens were thoroughly washed in CB at 4°C and then post-fixed in 1% osmium tetroxide in CB for 2 h at 4°C. After dehydration through a graded ethanol series, the samples were embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Ultrathin sections were mounted on nickel grids and evaluated in a transmission electron microscope OPTON 900 PC.

Image analysis. To quantify immunoreactivity of the examined markers, computerized image analysis was performed. Images were captured via video link to an Olympus BX50 microscope at 20 objective magnification, so the tissue fully occupied each field, and was scanned by the computer. Pictures were adjusted for optimal contrast, fixed at the same brightness levels, and saved in a buffering system. Staining was analyzed using Olympus 3.2 version image analysis computer system as described in details by Postek *et al.* [21]. Eight fields per tissue section were scanned and the mean values were used subsequently. Than, the average optical density was analyzed for parafollicular cells expressing CT or CGRP in both, experimental and control rats. The average optical density is the method measured values range from 0 to 255 where 0 means black and 255 white colors.

Statistics. All values were given as mean SD. The Mann-Whitney test was used for testing the differences between both groups in the intensity of immunocytochemical reactions. The Student-t test was used for the evaluation of the differences between groups in calcitonin plasma concentrations. The value $p<0.05$ was considered to be significant.

Results

Radioimmunoassay

After 21 days of PTU treatment plasma calcitonin concentration (11.9 pg/ml ± 1.72) was significantly reduced as compared to the respective value in control rats (16.5 ± 0.46) (Fig. 1).

Results of histological study of thyroid gland

The thyroid glands had a follicular, encapsulated structure in the control and the experimental rats. However, the differences between the central and the peripheral follicles in thyroids obtained from control rats were observed. The central follicles had a smaller diameter, the colloid was less dense, and the follicular epithelium was higher, whereas the peripheral follicles were larger, delimited with a flat cuboid epithelium and fulfilled with homogenous, intensively stained colloid (Fig. 4 and 5). Examination of thyroid sections of animals treated with PTU pointed to the blocking of thyroid function, expressed by the predominance of microfollicular hyperplasia. The hyperplastic follicles had irregular shape and were poor in colloid (Fig. 6 and 7).

In the thyroid glands of both, control and experimental rats except the basic follicular cells, irregularly distributed C cells were observed. The small groups or a single C cells were placed at the periphery of follicles in the central regions of thyroid lobes (Fig. 4 and 5). In all rats C cells presented different shapes with predominance of oval cells. Moreover, a single elongated parafollicular cells placed along epithelial lining of follicles were also observed in both groups of animals. However, in the hyperplastic thyroid glands obtained from PTU treated rats, the density of C cells was attenuated in comparison to the control rats (Fig. 6 and 7).

In the thyroid glands of control animals the immunocytochemical reactions for CT (Fig. 4) and CGRP (Fig. 5) were strong in the majority of cells, and in the remaining cells these reactions were distinctly weaker, or only slight. After chronic treatment with PTU, the CT (Fig. 6) and CGRP (Fig. 7) immunoreactivity were weaker in the most of cells in comparison to the controls. The average optical density of immunocytochemical reactions for both peptides, evaluated using Olympus Soft, were significantly increased in comparison to the control thyroid glands (Fig. 2 and 3).

Results of ultrastructural study of thyroid gland

The ultrastructure of the follicular cells of control thyroids was similar to that of the epithelial cells of other secretory organs, i.e. microvilli at the apical margin, well developed endoplasmic reticulum and Golgi apparatus, a heterogenous population of secretory granules, nucleus with nucleolus and numerous mitochondria (Fig. 8). The round or oval nuclei were usually placed centrally. Mitochondria with noticeable cristae were distributed throughout the whole cytoplasm. The ergastoplasmic reticulum was studded by numerous ribosomes.

The oval or ellipsoid parafollicular cells in the control thyroids were located in follicles and were surrounded with a common follicular membrane (Fig. 9). Their ultrastructure was characterized by the existence of numerous granules, covered by a single membrane. Most of them were accumulated in the region of cells neighboring with capillaries. The number and electron density of the secretory granules was similar in all of rats from control group with predominance of granules with high electron density.

After chronic treatment with PTU evident changes were noted in follicular and parafolicular cells. The hyperplasia of the follicular cells, expansion of the endoplasmic reticulum and the alteration of the nuclei pattern were observed (Fig. 10 and 12). At higher magnifications, the apical margin presented numerous elongated, hyperthrophic microvilli that penetrated into the follicular lumen. The nuclei were large and had irregular shape. The chromatin was adhered to the membrane in the form of hyperchromatic blocks. The dilated ergastoplasmic sacs had a pleomorpheus aspect and were ringed by cytomembrane studded with a small number of ribosomes. Many ribosomes were free, arranged in rosettes in the cytoplasm. The mitochondria were numerous, oval and most of them were hypertrophic. Golgi complex was well developed, hypertrophic and distributed in clumps. In the basal zone marked expansion of the follicular basement membrane formed a series of lamelliforms that penetrated into the cytoplasm.

After treatment with PTU the quantity and the electron density of secretory granules were decreased and some of the granules were even empty (Fig. 11). Ultrastructural pattern of parafollicular cells showed irregular shape of nuclei with a periphery located chromatin. In the majority of mitochondria irregular cristae were seen. Moreover, also local damage of the mitochondria was present. The short and thin ergastoplasmic sacs with a small number of ribosomes on the their external surface were observed.

Discussion

The main finding of the present study was the attenuation of C cells activity observed in the experimental model of hypothyroidism in rats, evoked by chronic treatment with PTU, expressed by decrease of CT and CGRP-immunoreactivity, accompanied by CT plasma level diminution. Immunocytochemical changes were confirmed by ultrastructural pattern of parafollicular cells hypoactivity. There are several studies supporting the hypothesis concerning the mutual cooperation between parafollicular and follicular cells in physiological and also in pathological conditions [2,4,5,6].

In an attempt to explain observed effects some possibilities could be taken under consideration, like a direct influence of PTU, direct or indirect influence of TSH, as well as, thyroid hormones on parafollicular cells activity.

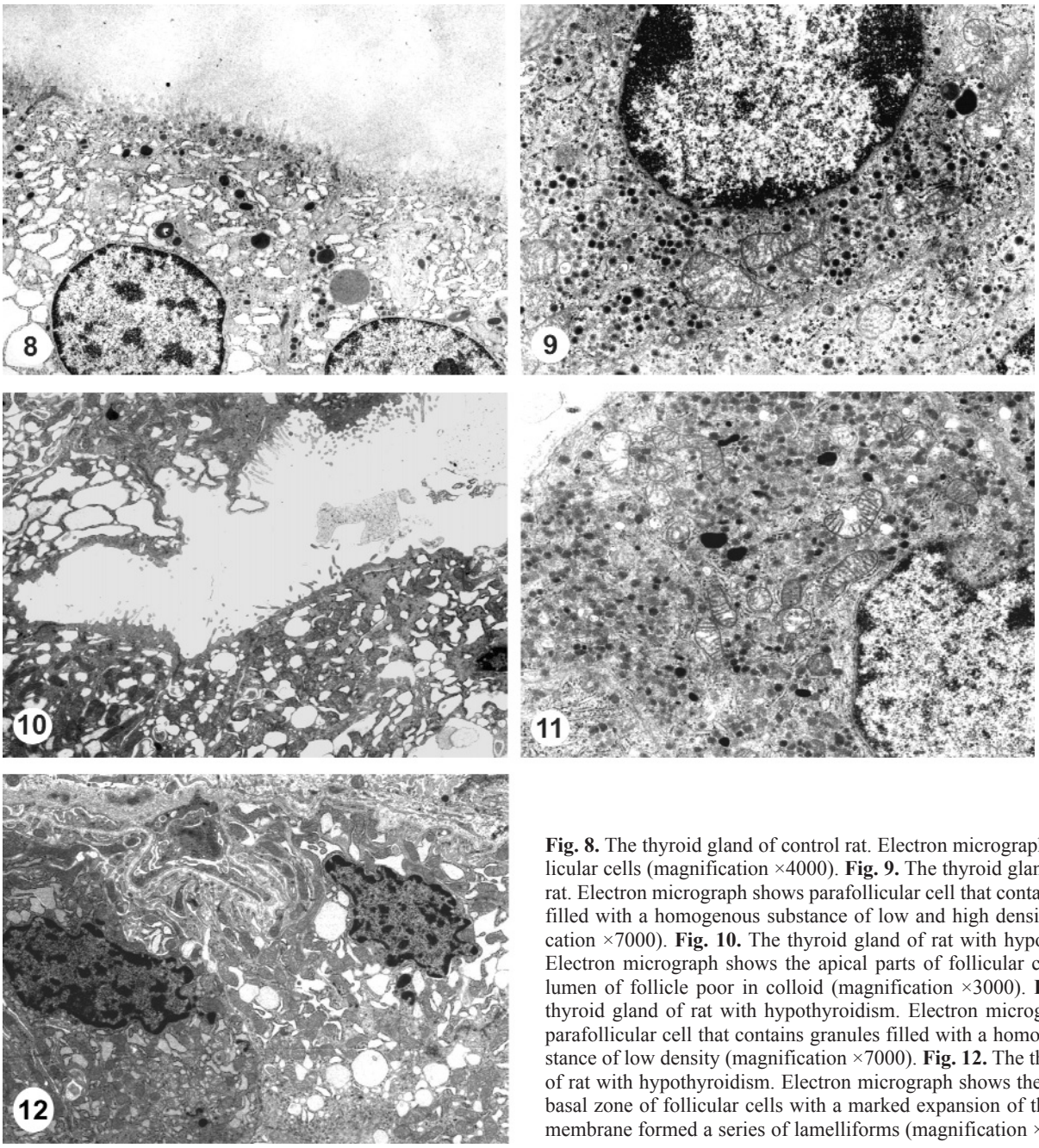

Fig. 8. The thyroid gland of control rat. Electron micrograph shows follicular cells (magnification ×4000). **Fig. 9.** The thyroid gland of control rat. Electron micrograph shows parafollicular cell that contains granules filled with a homogenous substance of low and high density (magnification ×7000). **Fig. 10.** The thyroid gland of rat with hypothyroidism. Electron micrograph shows the apical parts of follicular cells and the lumen of follicle poor in colloid (magnification ×3000). **Fig. 11**. The thyroid gland of rat with hypothyroidism. Electron micrograph shows parafollicular cell that contains granules filled with a homogenous substance of low density (magnification ×7000). **Fig. 12.** The thyroid gland of rat with hypothyroidism. Electron micrograph shows the central and basal zone of follicular cells with a marked expansion of the basement membrane formed a series of lamelliforms (magnification ×3000).

Thyroid hormones concentrations are controlled by a feedback mechanism that involves the hypothalamus, anterior pituitary and the thyroid gland [22]. The prolonged treatment with goitrogens *e.g.* PTU is followed by an increased blood TSH level via a negative feedback at the pituitary-thyroid axis [14,16]. The thyroid hyperplasia or neoplasia in some cases was observed as a consequence of the TSH stimulation of the follicular epithelium [10,18,27,28]. The microfollicular hyperplasia was also observed in the present study after chronic treatment of rats with PTU. Morosini *et al.* [18] have reported that changes in the thyroid following long-term TSH stimulation in the normal rats progress in three different stages, i.e.: an initial phase sustained of several days, a period of rapid thyroid growth with drastic increases in both mitotic activity and the number of follicular cells, and a phase of plateau with the declining of growth rate, as compared to the previous one.

TSH is well known to be the most important modulator of thyroid follicular cell growth, but recently other growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) have also been proven to be involved [19,26]. Recently, it was

demonstrated that thyroid hyperplasia induced by PTU in rats was associated with the increased density of thyroid receptors for IGF [19]. Moreover, it has been shown that in the absence of other growth factors TSH cannot stimulate DNA synthesis in isolated thyroid follicular cells [26]. This finding indicates that the responsiveness of follicular cells to TSH stimulation is probably mediated by other factors. It has been also reported that TSH enhances the expression of TGF-β_1, a potent growth inhibitor of many epithelial cell types including thyroid follicular cells [15,16]. Moreover, it has been shown that PTU and methimazole treatments increase serum TSH level and expression of TGF-β_1 mRNA in thyroid follicular cells [14].

There is no data concerning the direct influence of mentioned growth factors on C cells activity. However, in the present study after PTU administration the activity and density of C cells in thyroid gland was attenuated in comparison to the control group. It seems to be possible that observed in the present study attenuation of C cells activity, accompanying follicular cells hypertrophy, might be connected with the direct influence of follicular cells on C cells mediated by TGF-β_1 which increased expression was observed in follicular cells after PTU treatment [14]. The observation published by Zabel *et al.* [30], demonstrating an enhancement of CT mRNA expression in TT line cell culture conducted together with follicular cells, confirms the possibility of direct influence of follicular cells on TT cells, derived from C cells.

Moreover, the enhancement of CT plasma concentration observed in 19.2% of patients with Graves disease, together with a weak immunoreactivity for CT found in our earlier study [5], supports the functional relation between follicular and parafollicular cells.

In our previous study, performed on experimental model of hyperthyroidism evoked by L-thyroxine a strong CT and CGRP- immunoreactivity accompanied by a significant diminution of CT plasma concentration was observed [6]. The ultrastructural picture of both, follicular and parafollicular cells revealed features of their hypoactivity, what indicates that the strong reactions for CT and CGRP, were due to a release inhibition [4]. Since in our present study the attenuation of plasma CT level was also observed despite the thyroid hormones synthesis inhibition evoked by PTU, one could presume that observed changes in C cell activity were independent of direct influence of circulating TSH and thyroid hormones.

Taken together, since in both experimental models of hyperthyroidism [4,6] and hypothyroidism, described in the present study, hypoactivity of follicular cells confirmed by histological and ultrastructural studies was observed together with the diminution of CT plasma level, while in patients with Graves disease the enhancement of plasma CT was accompanied by hyperactivity of follicular cells [5], it could indicate that parafollicular cells activity is closely related to the activity of follicular cells.

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