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ORIGINAL ARTICLE

Calcitonin gene-related peptide (CGRP) - microadenomas of the thyroid gland induced by cadmium toxicity. Experimental study

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Summary

Purpose: *The objective of our study was to make morphological and functional analysis of thyroid C cells in rats chronically exposed to cadmium (Cd).*

Methods: *The study was carried out on female albino Wistar rats (n=22, age=35-37 days, body mass 120-140 g), divided in control (n=11) and experimental group (n=11). The rats of the experimental group were treated with 15 mg/kg Cd dissolved (as $CdCl_2$) in drinking water. The animals were sacrificed 3 months later. The thyroid glands were removed and macroscopic, histological and immunocytochemical examinations were done. Monoclonal antibodies for chromogranin A (Chr A), neuron specific enolase (NSE), calcitonin (CT), somatostatin (SST) and calcitonin gene related peptide (CGRP) were used for immunocytochemical examinations.*

Results: *No pathological changes were found in parafollicular cells of the animals of the control group. All the animals of the experimental group showed bilateral diffuse C cell hyperplasia, mostly in the middle and upper thirds of the lobes. Strong immunoreactivity was present to all tested polypeptides (Chr A, CT, NSE, CGRP and SST). In 5 (45%) of the animals of the experimental group, C cell microadenomas were exclusively made of CGRP-secreting cells.*

Conclusion: *Chronic Cd exposure causes preneoplastic changes and functional differentiation of parafollicular cells of the thyroid gland, the first cell type being present in the area of diffuse hyperplasia, and the other C cell type being the constituent of microadenomas secreting CGRP exclusively. The results of this study indicate that chronic Cd exposure disturbs the structure and function of C cells of the thyroid gland.*

Key words: cadmium toxicity, immunocytochemistry, microadenomas, parafollicular cells, thyroid

Introduction

Cadmium is a soft, silvery-white metal which is usually found in nature as a minor component in most zinc, lead and copper ores. Cadmium and its compounds have multiple applications. It is used for the production of alkaline batteries, nickel-cadmium batteries, for the production of copper alloys and as a pigment or stabilizer in plastic industry. Cadmium emissions arise from natural sources (weathering and erosion of parent rocks, volcanic activity, forest fires) and anthropogenic sources (steel industry and incineration of cadmium-containing solid waste products). The diffuse cadmium pollution of soil is caused by intensive use of phosphate fertilizers. Drinking water normally contains low concentrations of cadmium (0.1 to 2 μg per liter). Cigarettes may contain 0.3-0.5 μg of cadmium. Food is the most common source of cadmium for non smokers [1-4].

Cadmium is classified as one of 126 priority pollutants by the US Environmental Protection Agency. It can cause chronic obstructive lung disease and emphysema, tobacco-related lung disease, chronic rhinitis, destruction of the olfactory epithelium with subsequent anosmia, cadmium nephropathy, osteoporosis and osteomalacia [5,6]. Many of the epidemiological studies have revealed that exposure to cadmium causes tumors at multiple tissue sites [2,4,7].

Reports about the effects of cadmium on C cells of the thyroid gland are scarce. The aim of our study was to carry out micromorphological and functional investigation of C cells of thyroid glands in rats, chronically treated with cadmium.

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Methods

Animals and experimental design

The study was carried out on female albino Wistar rats (n=22, age=35-37 days, body mass 120 g ± 10 g), divided in control (n=11) and experimental group (n=11). The rats of the experimental group were treated with 15 mg/kg Cd dissolved (as $CdCl_2$) in drinking water. The animals were raised in controlled laboratory conditions (in an animal room with a 12 h light/12 h dark cycle, at 22± 2° C) and were provided with standard laboratory rat food and tap water *ad libitum*. The animals were sacrificed 3 months later.

All procedures on animals followed the Guideline for Work on Experimental Animals approved by the Ethics Committee of the Faculty of Medicine in Kragujevac.

Histopathology and immunocytochemistry

Both thyroid lobes were fixed in Bouin's fluid for 24 h, routinely processed, and were embedded in paraffin. 5 μm paraffin sections were stained with hematoxylin and eosin (H&E) method for lesion verification, and immunocytochemical ABC technique using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Immunocytochemical reactions were performed with the use of antibodies against chromogranin A (Chr A 1:3000, DAKO, Denmark), neuron specific enolase (NSE, 1:1600, DAKO, Denmark), calcitonin (CT, 1:2000, Milab, Sweden), calcitonin gene-related peptide (CGRP, 1:2000, Milab, Sweden) and somatostatin (SST, 1:1600, DAKO).

Immunocytochemical staining was done with controlled quality and specificity, using positive and negative controls according to the propositions of the UK NEQAS (UK National External Quality Assessment for Immunocytochemistry). Samples of medullary thyroid gland carcinoma that had previously undergone multiple testing and were certain to contain the studied antigens were used as positive controls. Tissue samples treated with non-immune serum instead of the primary antibody were used as negative controls.

Image analysis

The assessment of C cells' expression was based on the number of 10 HPF positive cells and staining intensity of the examined hormones deposits (Chr A, NSE, calcitonin, CGRP and somatostatin; Tables 1 and 2).

Table 1. Assessment of C cell expression

Grade	*No. of cells/10HPF*
I	Up to 50 cells
II	50 - 200 cells
III	More than 200 cells

Table 2. Intensity of hormone deposit

Intensity	*Staining*
+	Faint
++	Mild
+++	Intensive

Results

Histopathology

No histopathological changes were found by micromorphological examination of the thyroid glands of animals in the control group. Thyroid gland lobes were made of spherical follicles. The wall of the follicles consisted of basal membrane and thyreocytes, which were cubical, with oval nuclei, where chromatin was moderately present. The follicles were filled with colloid. On their periphery, between the basal membrane and thyreocytes, there were individual, paired or grouped oval parafollicular C cells (small groups, 3-5 at the most). Using H & E, their cytoplasm was clearer than the cytoplasm of the follicular cells; as a result they are also called light cells. The nuclei were large, oval and took up the middle part of the cytoplasm. In the cytoplasm there were numerous secretory granules that became visible after anticalcitonin staining (Figure 1).

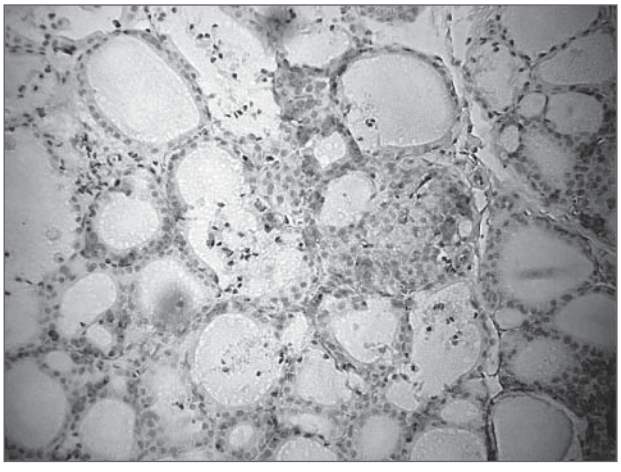

Figure 1. Calcitonin-immunoreactive C cells in animals of the control group (ABC ×200).

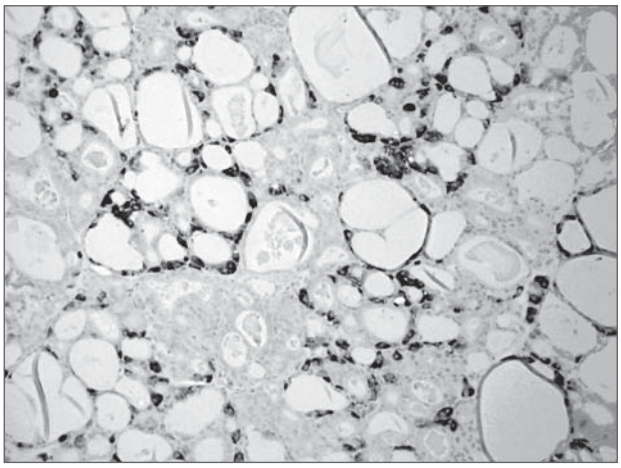

Figure 2. C cells' hyperplasia in cadmium-exposed rats shows a week reaction to CHR A antibody (ABC ×200).

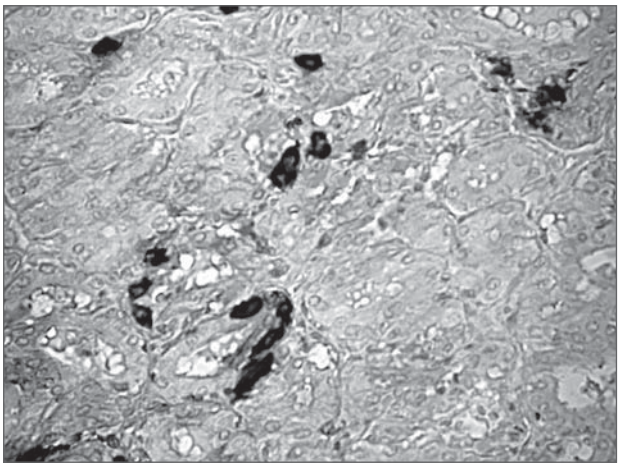

Figure 4. Few C cells showing strong immunopositive reaction with anti-SST antibody (ABC ×400).

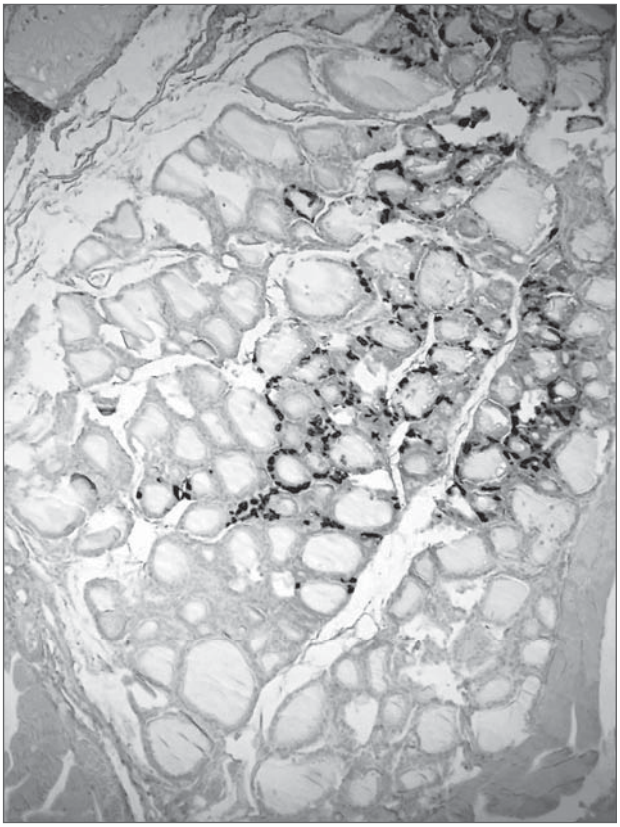

Figure 3. C cells' hyperplasia in the upper and middle third of the lobe, showing strong CT expression (ABC ×100).

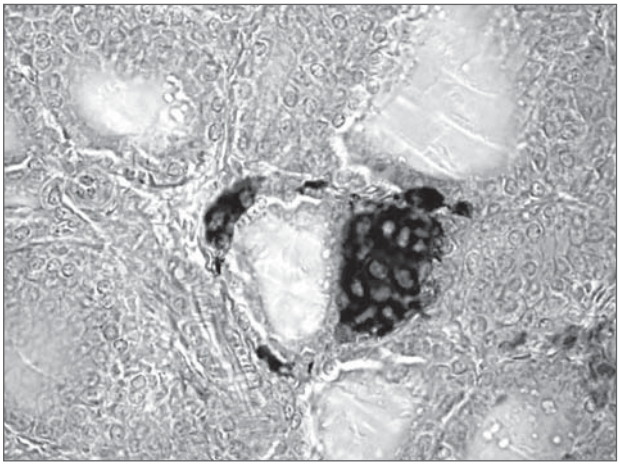

Figure 5. Strong immunoreactivity of CGRP deposit in C cell microadenoma (ABC ×400).

Immunocytochemistry

Bilateral, strong, diffuse C cells' hyperplasia (CCH) was verified in all the animals of the experimental group (Figure 2). The highest C cells' density was present in the middle and upper thirds of lateral lobes (Figure 3). In cells constituents of diffuse hyperplasia strong immunoreactivity was verified to NSE, Chr A (Figure 2), CT (Figure 3) and CGRP. SST was found only in rare, polymorphic C cells with cytoplasmic extentions (Figure 4, Table 3).

Nodular hyperplasia of C cells/C cell microadenoma was found in 5 animals (45%) along with diffuse C cells' hyperplasia. Microadenomas were made by groups of 10-20 cells. These were large, oval or polygonal and secreted CGRP alone (Figure 5). Presence of other neuropeptides was not verified in them.

Discussion

Occupational exposure to cadmium, a potent human carcinogen, is associated with increased incidence of cancers of lung, prostate, pancreas and kidney [8-10]. Association of chronic cadmium exposure with cancers of the breast and urinary bladder is also suggested by epidemiological studies and cadmium is classified as a

Table 3. C cell hyperplasia quantification

No. of rats	*Assessment of C cell expression (G) and intensity of hormone deposit*				
	CHR A	*NSE B*	*CT*	*CGRP*	*SST*
2/1	G II ++	G II ++	G II +++	G II ++	G I +++
2/2	G III ++	G III +	G III ++	G III +++	G I +++
2/3	G II+	G II +	G II +++	G II ++	G I ++
2/4	G III++	G III +	G III +	G III +	G II++
2/5	G III+	G I+	G II++	G III +	G I+
2/6	G II++	G II+	G II +++	G II+	G II++
2/7	G II++	G II+	G III ++	G II ++	G I+
2/8	G II+	G I +	G III +++	G II ++	G I+
2/9	G I++	G I+	G II +++	G II+	G I++
2/10	G II+	G I++	G III +++	G II ++	G I+
2/11	G III++	G III +	G III +++	G III ++	G I+++

CHR A: chromogranin A, NSE: neuron-specific enolase, CT: calcitonin, CGRP: calcitonin-gene related peptide, SST: somatostatin
+ = 10-30% of cells stained; ++ =30-60% of cells stained; +++ = 60-90% of cells stained

category I human carcinogen by the International Agency for Research on Cancer and the National Toxicology Program of the USA [7,11,12].

Experimental studies in animals have shown that cadmium causes tumors and/or preneoplastic (hyperplastic) lesions of the prostate in rats. Benign interstitial cell (Leydig) tumors in rats are induced by high doses of cadmium. Subcutaneous or intramuscular injections of cadmium salts can induce mesenchymal tumors (typically fibrosarcomas) at the site of application in rodents. The other rodents' organs targeted by cadmium are liver, adrenal, pancreas, pituitary, hematopoietic system, heart and vascular system [4,13,14].

In our study preneoplastic changes, manifested as diffuse C cell hyperplasia and nodular C cell hyperplasia/C cell microadenoma, were verified in thyroid glands of experimental animals after chronic cadmium exposure.

The term C cell hyperplasia (CCH) is used for two different biological processes. Neoplastic CCH is used to describe the precursor of familial medullary thyroid carcinoma (FMTC) associated with multiple endocrine neoplasias (MEN) IIa, MEN IIb and FMTC without other events [15-17]. Neoplastic CCH in MEN II syndrome is caused by a germ-line mutation of the RET proto-oncogene. Some authors have proposed to call CCH "*in situ* MTC" because it is a neoplastic condition [15,18].

The second form of CCH is reactive or physiologic C cell proliferation which has been recognized in neonates, in elderly, in patients with hyperparathyroidism or hypothyroidism, Hashimoto's thyroiditis, previous hemithyroidectomy, follicular thyroid neoplasms (nodular and diffuse goiter) and in a patient with primary thyroidal non-Hodgkin's lymphoma [19-22].

Physiologic and neoplastic CCH are two completely distinct entities not only biologically but also morphologically. Neoplastic CCH is characterized with cytological atypia which is a qualitative change. Physiologic CCH is believed to be caused by external stimuli on the C cell, and its premalignant potential is not documented [15-17].

The mechanisms responsible for the development of physiologic CCH are not well recognized yet. Because physiologic CCH has been induced via TSH overstimulation in laboratory animals it is suspected that physiologic CCH in neonates and in some cases of hypothyroidism is caused in the same way. However, other factors could also be important. The fact that most patients with physiologic CCH do not have elevated serum TSH levels indicates that it may be caused by some other pathogenetic factors. Interactions between C cells and follicular cells could explain great variations in the number of C cells under physiologic and pathologic conditions. It has been suggested that C cells may play a role in the modulation of thyroid function by paracrine mechanisms in experimental animals [16, 18].

Two functionally different types of C cells have been verified by immunoenzyme reactions in our experiment. Micronodules in the thyroid glands of the animals with nodular CCH are made exclusively of CGRP-secreting C cells. On the other hand, almost all calcitonin-positive C cells in diffuse CCH perform co-expression of CGRP, whereas the co-expression of somatostatin is present in a small number of calcitonin positive C cells. DeLellis et al [23] have performed ultrastructural analysis and also found two major cell types of CCH. The first cell type was rich with secretory granules (type I) with diameter size of 280 nm. This cell type is predominant in areas of diffuse CCH and it is also found in normal thyroid glands. The relative lack of develop-

ment of granular endoplasmic reticulum and Golgi regions is a sign that these cells are in the storage phase of their secretory cycle. The second cell type has fewer secretory granules (type II) that are 130 nm in diameter. This cell type is located in areas of nodular CCH. Cytologic evidence of active protein synthesis and secretion are found in cells with type II granules. Differences in granule morphology and cell ultrastructure reflect functional variations in C cell populations [23].

Spontaneous occurrence of thyroid C cell adenoma in rodents was reported in the literature. Formation of parafollicular adenoma is found in 3.6% male and in 2.9% female Sprague-Dawley rats [24]. Other authors reported occurrence of spontaneous C cell adenoma in Sprague-Dawley rats in 6.5% [25]. Spontaneous tumors of thyroid gland in Wistar rats are verified in 9.3% males and 8.7% females [26]. Thyroid C cell adenoma in F334/DuCrj rats is found in 5% of the animals. The incidence of spontaneous tumors is proportional to animal age. The majority of spontaneous tumors are formed between the 85th and 97th week of life in males and between the 98th and 110th week of life in females [27,28]. We used young rats (35-37 days old) for our experiment so as to avoid spontaneous tumor occurrence. Hypothetically, in C cell carcinogenesis 3 specific stages of neoplastic progression can be distinguished: diffuse C cell hyperplasia, focal C cell hyperplasia and genuine C cell tumors [29].

It is supposed that the key event in cadmium carcinogenesis could be cell defects accumulation, probably by a combination of increased proliferation and blocked apoptosis. Disturbance of DNA repair caused by cadmium, along with increased proliferation, could also result in tumor formation [28,29].

The basic mechanisms involved in cadmium carcinogenesis are modulation of gene expression and signal transduction by oxidative stress, induction of apoptosis, inhibition of DNA repair processes, and disruption of E-cadherin-mediated cell - cell adhesion [10,30-33].

Multiple mechanisms are involved in the carcinogenicity of cadmium and further investigations are needed to identify a single molecular event.

Conclusions

Chronic Cd exposure causes preneoplastic changes and functional differentiation of parafollicular cells of the thyroid gland. The functional differentiation is manifested by the ability of the C cell type present in areas of diffuse hyperplasia to secrete simultaneously a variety of regulatory peptides (CT, CGRP, Chr A, NSE and SST), and the ability of another C cell type, constituent of microadenomas, to secrete CGRP exclusively.

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