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ABNORMALITIES OF APOPTOSIS OF THE THYROID GLAND CELLS FROM EXTRATUMORAL MICROFOLLICULAR TISSUE

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***Aim*: The aim of this study was to evaluate the frequency of existence of thyroid extratumoral normo- and microfollicular tissue in patients with thyroid carcinoma and peculiarities of apoptosis in mentioned tissue. *Materials and Methods*: Using samples of normo- and microfollicular thyroid tissue it was determined the content of fragmented DNA and intensity of stimulated internucleosomal DNA fragmentation; activities of caspase-3 and cysteine lysosomal cathepsins. *Results*: It was found that normofollicular tissue is observed more often in patients with nodal euthyroid goiter but microfollicular tissue is more common for patients with carcinoma. Extratumoral microfollicular tissue was found in the thyroid of patients above 50 years old mostly, and more rarely in young ones. The fragmented DNA concentration in microfollicular tissue was lower by a factor of 3.5 and intensity of stimulated internucleosomal DNA fragmentation was also decreased. Activity both of cathepsin B in lisosomes and caspase-3 in lysates of such tissue was also decreased. *Conclusions*: The decrease of intensity of spontaneous apoptosis and the absence of its modulation/induction following proapoptotic factors in extratumoral microfollicular thyroid tissue may be considered as a respond of the thyroid gland tissue to an existence of carcinoma.**

***Key Words:* thyroid extratumoral microfollicular tissue, internucleosomal DNA fragmentation, caspase-3, lysosomal cathepsins, apoptosis.**

A century ago, it was stated by Isaac Levin that tumor development and growth depend not only on host's immune status, but also on local interactions between tumor and host [1]. However, we have meanwhile only a few information concerning metabolic changes in cells of tumor-bearing organs. During last 50–60 years researchers' attention was mostly focused on the study of tumor cell microenvironment that differs from normal cell microenvironment without any doubt [2].

As an example of cell metabolism damage in any organ-bearing tumor may be the data concerning lactate formation in tumor cells due to aerobic glycolysis activation that leads to the pH decrease not only in tumor cells, but also in areas being outside of the tumor by means of proton "leakage" [3]. Moreover more high lactate dehydrogenase activity is registered in extratumor tissue also [4]. Such acid microenvironment is toxic for normal cells that leads to their destruction [3] and well correlated with metastasis.Very often the availability of the tumor is accompanied by various metabolic disturbances even in areas being far from tumor setting [5].

An increased quantity of aberrant thyrocytes is described for tumor-affected thyroid gland (TG) in the all non-affected areas [6]; there are data concerning both increased expression of some molecules taking part in proliferation and apoptosis regulation [7] and increased concentration of fragmented DNA in compare to such in extranodular tissue in patients with euthyroid goiter [8].

The TG activity is known may be changed in physiological conditions or due to the thyroid pathology; the follicular structure of TG tissue may also be changed: the gland may have normo-, micro-, macro-, or heterofollicular structure. During the study of disturbances in mechanisms of apoptosis in patients with benign as well as malignant TG neoplasms we have observed the existence of extratumoral tissue without histological features of pathology in patients with carcinoma that had more often microfollicular structure compare to patients with goiter. The aim of this work was to analyse frequency of existence of thyroid extratumoral normo- and microfollicular tissue of patients with thyroid carcinomas and peculiarities of apoptosis of such tissues.

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MATERIALS AND METHODS

***Tissue*.** It have been investigated extratumoral/extranodular tissues (258 samples) obtained during total or hemithyroidectomy to determine frequency of existence of thyroid tissue of different follicular structures without pathological changes when carcinoma or benign node (e.g., euthyroid goiter) are present. All patients were informed about investigation for scientific purposes. This study has been apptoved by Institute's Committee on Biological & Medical Ethics.

All tissue samples for biochemical investigation (90 samples) had no macro- or microscopical features of pathological changes. Depending on the type of follicular structure all samples were divided into three groups — tissue of normofollicular structure (42 samples, 1st group), tissue of micro- or micronormofollicular structure (30 samples, 2nd group), and tissue of macro- or macro-normofollicular structure (18 samples, 3rd group).

***Fragmented DNA assay*.** Concentration of low molecular weight DNA fragments was analysed in supernatant fluid following cell lysis during tissue homogenization in в 10 mM Na-EDTA-Tris-HCl buffer, pH 7.8, containing 0.5% Triton X-100 and centrifugation (5.000 rpm, 15 min). The pellet containing high molecular weight DNA inside chromatin was suspended in the initial buffer (but without Triton X-100). Quantitative DNA content in both fractions was stimated

by means of a diphenylamine reaction [9] and given as 1 μg/1 g of tissue. The part of fragmented DNA was calculated as percent to the total amount.

The intensity of stimulated internucleosomal DNA fragmentation assay. To determine the intensity of stimulated internucleosomal DNA fragmentation, tissue slices (50–100 mg) were previously incubated during 4 h at 37 ^{0}C in Krebs-Ringer PBS (1 ml, pH 6.0) that permited to increase the quantity of cells on the onset of terminal step of apoptosis under hypoxia. The next steps of DNA isolation and purification were the same as discribed earlier [10]. DNA electrophoresis (1 h, 90 mV) was carried out in 1.7% agarose gel ("Sigma", USA, or "Lachema", Czech Republic) using 10 mM Tris-EDTA buffer, pH 7.0–7.2 containing ethidium bromide (1 μg/ml). The markers as large as 100–1000 base pairs (bps) that have been used corresponded to DNA fragments. Quantitative analysis of low molecular weight DNA fragments (200, 400, 600, and 800 bps) was carried out using the program "Photo Capt Mw". Relative nucleosome contents were calculated from total area of all DNA peaks taken as 100%.

Caspase-3 and cysteine lysosomal cathepsin activity assay. Obtaining of tissue lysates and determination of caspase-3 activity (acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide being as a substrate) were provided using kit for estimation of caspase activity ("CASP-3", Sigma, USA). Activity of cysteine lysosomal cathepsins was estimated both in cytosol and lysosomes according to [11] using synthetic substrates N_2-benzoyl-DL-arginine-4-nitroanilide (for the cathepsin B) and L-leucine-4-nitroanilide (for the cathepsin H) or azocasein being previously denatured by 6 M urea solution (for the cathepsin L). Activity of caspase-3, cathepsin B, and cathepsin H was given as μmoles of *p*-nitroaniline that are cleaved from the substrate during 1 h of incubation per 1 mg of protein. Activity of cathepsin L is given in conventional units of low molecular weight peptide (non-precipitated by trichloracetic acid) following 1 h of incubation per 1 mg of protein.

The allocation of lysosomal and cytosol fractions, and protein concentration assay. Lysosomal and cytosol fractions were isolated by means of differential centrifugation and protein content was estimated according to Lowry modification [12].

Statistical analysis. Data were given as mean ± standard error (S.E.) and analyzed statistically using a one-tail analysis of the Student's t-test and χ^2-test for comparison between groups. Differences were considered significant at $p < 0.05$.

RESULTS

Among 258 extranodal TG samples studied, different features of pathological changes (lymphoid infiltration, invasion of tumor cells, chronic thyroiditis, autoimmune thyroiditis, sclerotic changes of stroma tissue, marked hyperplasia, cysts) were detected in 168 ones (65,1%). Pathological events in extranodal tissue were found mostly in patients with carcinomas (131 samples/191; 68.5%) comparing to patients with euthyroid goiter (37 samples/67; 55.2% p=0,0483, χ^2-test). Among the intact tissue samples with normal follicular structure were mostly detected in nodular euthyroid goiter (Table 1, p= 0.0071, χ^2-test) and tissue with microfollicular structure has been mainly found at carcinomas (p = 0.00003). In such pathological conditions extratumoral/extranodular tissue of macrofollicular structure was seen with the same frequency (p = 0.576).

Table 1. Existence of an extratumoral/extranodular tissue of a different follicular structure in a TG of patients with carcinoma and a nodular euthyroid goiter

Patients	1st group	2nd group	3rd group
patients with carcinoma (n=60)	26 (43.3%)	26 (43.3%)	8 (13.4%)
patients with nodular goiter (n=30)	20 (66.7%)*	3 (10.0%)*	7 (23.3%)

Note: * — significant difference in the 1st and the 2nd group of patients, $p<0.05$, χ^2-test; n – number of observations.

Such differences in distributions of normo- and microfollicular tissue in TG affected with carcinoma do not depend on patients' sex (p=0.182, χ^2-test), on tumor metastatic potential (p = 0.281), tumor invasive properties (p = 0.943), or tumor location — tumor-containing lobe or contralateral one (p=0.111). At the same time, it may be clearly detected the dependence of existence of tissue of different follicular structure on patients' age (extratumor microfollicular tissue is mostly present in TG of patients above 50 years and significantly rarer in young patients). On the contrary, it was found an opposite situation for distribution of normofollicular tissue (Table 2). No dependence has been noted in the distribution of extranodal tissue with different follicular structures with age of patients having euthyroid goiter. The differences in existence of tissue with different follicular structures are statistically significant in 30–50 years old patients as well as above 50 years (p=0.0295 and 0.0183, respectively, χ^2-text).

Table 2. Existence of an extratumoral/extranodular tissue of a different follicular structure in a TG of different age patients

Age	Cancer			Goiter		
	n	1st group	2nd group	n	1st group	2nd group
Under 30 year	18	13 (72.2%)	5 (27.7%)	6	5 (83.3%)	1 (16.6%)
31–50 years	21	12 (57.1%)	9 (42.9%)	14	13 (92.9%)	1 (7.1%)
Up 50 year	13	1 (7.7%)*#	12 (92.3%)*#	3	2 (66.7%)	1 (33.3%)

Note: * – significantly different from group under 30 year; # – from group 31–50 years, $p<0.05$, χ^2-test, n – number of observations.

Concentration of fragmented DNA in tissue of microfollicular structure was by a 3.5 fold lower, its part in total DNA content was by a 2.4 fold also lower comparing to tissue of normofollicular structure (Table 3). It has been found that mononucleosome content in samples of DNA from microfollicular tissue has been decreased by 55%, total oligonucleosome content (the nucleosome size was 200, 400, 600, and 800 bps) has been by 23% lower than in samples of DNA isolated from normofollicular tissues (Table 4).

Table 3. Concentration of the DNA in an extratumoral thyroid tissue of a different follicular structure

Parameters	1st group (n=6)	2nd group (n=4)
High-molecular DNA (μg/g)	1.00 ± 0.09	0.94 ± 0.01
Low-molecular DNA (μg/g)	0.57 ± 0.07	0.16 ± 0.01*
Part of fragmented DNA (%)	35.81 ± 2.03	14.92 ± 0.51*

Note: * — significantly different from the 1st group, $p < 0.05$, Student's t- test; n – number of observations.

Table 4. Level of oligonucleosoms in DNA extracted from an extratumoral thyroid tissue of a different follicular structure

Size of nucleosomes	1st group (n=6)	2nd group (n=4)
200 bp. (%)	8.77 ± 0.64	3.94 ± 0.94*
400 bp. (%)	5.45 ± 0.48	3.49 ± 1.34
600 bp. (%)	8.17 ± 0.41	7.19 ± 1.24
800 bp. (%)	6.63 ± 0.65	6.89 ± 1.12
Total amount (200–800) bps. (%)	29.0 ± 0.39	22.3 ± 2.87*

Note: * – significantly different from the 1st group, $p < 0.05$, Student's t- test, n – number of observations.

Caspase-3 activity in lysates of microfollicular tissue was significantly lower than in tissue of normofollicular structure (0.038±0.022 versus 0.156±0.028 μmoles *p*-nitroaniline/h/mg of protein, respectively, $p<0.05$). Activity both of cathepsins H and L in lysosomal and cytosol fractions isolated from normo- or microfollicular tissues do not differ significantly (data not shown). Cathepsin B activity was drastically lower in lysosomal fraction isolated from microfollicular tissue (22.45±3.29 versus 11.1±2.89 μmoles *p*-nitroaniline/h/mg of protein, respectively, $p<0.05$).

DISCUSSION

While studying both intensity and mechanisms of regulation of apoptosis under thyroid pathology it was detected the prevalence of extratumor microfollicular tissue in presence of carcinoma that was confirmed statistically. The extratumor microfollicular tissue was seen more often (in a factor of 4.5) than extranodular tissue of the similar structure in case of euthyroid goiter. As it is known the microfollicular structure of TG of healthy persons is characteristic for children TG**.** Following aging, the TG becomes more homogenous, follicles of average size are predominant. However, in healthy grown-up and older persons it was observed the microfollicular changes of TG architectonics more oft [13]. Such changes are thought to be correlated with physiological TG changes stipulated by aging. The increase of microfollicular TG elements and the decrease of normofollicular ones in extratumoral tissue of patients with carcinoma may be related to their age. However, such dependence is absent in patients with euthyroid goiter. That is why the age cannot be the only cause of more oft occurrence of microfollicular extratumoral tissue in TG with carcinoma. Other factors, such as patients' sex, tumor aggressiveness, location of tumor in TG are not crucial.

As it is known the decrease of follicle sizes takes place due to the factors stimulating the TG function. It is shown that level of serum thyrotropin (TSH) in patients with carcinoma may be increased, being, however, not significantly higher than the upper normal level [14–16]. Some authors think that such increase is observed only in patients with papillary but not with follicular carcinomas [15]. Other investigators have found the increase of serum TSH concentration in patients with papillary, follicular, and undifferentiated carcinomas [16]. Consequently, despite of the absence of clinical symptoms indicating the damage of thyroid function in patients with carcinoma level of thyroid hormones remains within normal ranges. It is impossible to deny a possibility of definite stimulation TG by the TSH (in a case of subclinical hypothyroidism) that resulted in the decrease of follicle volume.

In extratumoral TG areas of microfollicular structure, it was not found any significant changes of lysosomal cysteine cathepsins H and L activity under such stimulation. Taking into account their role in thyroglobulin processing (cathepsin L takes part in all steps, but cathepsin H is active during complete protein degradation [17]), it may be an indirect evidence that there is absent significant quantitative shifts in synthesis of thyroid hormones. At the same time, a drastical droping of cathepsin B activity in lysosomes isolated from microfollicular tissue may lead to small decrease of content of thyroid hormones in blood: cathepsin B takes part in this process at steps both of protein solubilization and thyroxine formation [17]. It has been earlier shown that thyroxine, but not triiodothyronine content is something lower in blood of patients with carcinoma [16]. Such decrease of circulating thyroxine (something lower than normal range) is proposed to be as a cause of a little increase of TSH secretion and, consequently, diminished follicle volume.

On the other hand, as it was ascertained earlier the release of lysosomal proteases into the cytosol due to permeability of lysosomal membrane is dominant intracellular phenomenon but not as a part of the following later stages of cell death throughout the apoptosis. Releasing of proteases including cathepsins from lysosomes causes the mitochondrial disfunction followed by activation of caspases and cell death [18]. In this work, no cathepsin release into the cytosol from microfollicular tissue has been registered. This fact and our data obtained concerning the transmembrane potential as well as intensity of swelling of mitochondria isolated from such tissue [19] suggest that there are absent activation of apoptosis in thyroid extratumoral microfollicular tissue.

However, the spontaneous apoptosis in such tissue may by inhibited. Such assumption is confirmed by a drastic drop of caspase-3 (whose participation in apoptosis has been confirmed of numerous researchers [20, 21]), as well as by low cathepsin B activity (its role in regulation of apoptosis has been already established [18]). The decrease of activity of these proteases in microfollicular thyroid tissue coincides also with the data concerning decreased level of fragmented DNA (Table 3) and gradual delay of swelling of mitochondria [19]. All mentioned above and significant decline of mitochondrial transmembrane potential [19] may be an evidence of the change of membrane permeability and decrease of proapoptic molecule releasing from mitochondria.

In addition, some disturbances of mitochondrial function in response to apoptosis modulating factors in thyroid tissue have been shown earlier. It may be important for realization of cell death following proapoptosis signals. First of all, mitochondria in microfollicular tissue becomes to be resistant to the affect of calcium ions (pore opening inducers) and antioxidants that can modulate this process. Intensity of mitochondria

swelling (the most adequate parameter permitting to estimate the nonspecific mitochondrial membrane permeability) has not been changed following calcium ions, melatonin or α-tocopherol addition to the incubation medium that is inherent for normofollicular tissue. The transmembrane potential is an integral parameter describing energetic potential of mitochondria. Its decrease causes the release of proapoptic factors from mitochondria into the cytosol. However calcium ions promoted the increase of transmembrane potential in some tissue samples [19]. The resistance of microfollicular cells to proapoptic signals is confirmed by our data obtained: decrease of the intensity of terminal stage of apoptosis — stimulated internucleosomic DNA fragmentation.

It has been derermined the decrease of spontaneous apoptosis in extratumoral microfollicular thyroid tissue and its resistance to proapoptotic factors may be as a result of the tumor influence. It may be promoted further tumor progression, as well as thyroid tissue stimulation by means of TSH.

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