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CASPASE-3 ACTIVITY IN PAPILLARY THYROID CARCINOMAS

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Aim: **The aim of this work was to assess caspase-3 activity in the tissue of papillary thyroid carcinomas of patients and analyze the peculiarities of changes in this activity depending upon a number of pathomorphological and clinical features of tumoral process.** ***Methods:*** **Caspase-3 activity was determined by spectrophotometry with regard to acetyl-asp-glu-val-asp-paranitroanilide.** ***Results:*** **At initial stages of tumor development, in the absence of metastases to lymph nodes, blood and lymphatic vessel invasion by tumor cells, extrathyroid spreading of tumor, sclerotic and fibrous changes in tumor stroma, and in the presence of tumor capsule, caspase-3 activity in papillary carcinoma tissue was higher compared to unchanged thyroid tissue of normofollicular structure. In case of a more aggressive behaviour of tumor, enzyme activity in carcinoma tissue did not differ significantly or (in case of extrathyroid spreading of tumor) was decreased compared to that in extratumoral tissue. In combination, this was expressed by a progressive decrease in caspase-3 activity in tumor tissue with increasing T category. Caspase 3 activity was found to be much higher in the tissue of papillary carcinomas of follicular-papillary structure and lower in the tissue of tumors of mixed structure with solid areas, compared to that in the tissue of papillary carcinomas of typical papillary structure.** ***Conclusions:*** **The data obtained in assessing caspase-3 activity suggest that the intensity of spontaneous apoptosis of human papillary thyroid carcinoma cells depends upon the stage and aggressiveness of tumoral process.**

Key Words**: caspase-3, papillary thyroid carcinoma, apoptosis.**

A great number of physiological mechanisms of growth control that regulate cell proliferation and tissue homeostasis are closely related to apoptosis, and, therefore, tumor cell resistance to apoptosis represents undoubtedly a distinctive and important feature of these cells. At the same time, in each tumor, cell population is heterogeneous and contains both cells with a disturbed programme of apoptosis, and cells having preserved this programme. The intensity of spontaneous apoptosis in tumors depends upon their origin, cell type, histological variant, stage of tumor progression, and other factors which, being in interaction with each other, have an impact on the character and intensity of apoptosis.

Data on apoptosis intensity in thyroid tumors are rather contradictory: the highest intensity was reported for medullary, anaplastic, and oxyphilic-cell carcinomas [1, 2], while in papillary and follicular carcinomas apoptosis level appeared to be low [3]. On the contrary, morphologic studies have shown that the index of apoptosis in papillary carcinomas was much higher compared to follicular and medullary carcinomas where it was equal to zero [4]. Properly for papillary thyroid carcinomas, apoptotic activity was shown to vary: in 41% of carcinomas it was at a trace level (which is typical of normal tissue), in 44% it was low, and in 15% apoptotic activity was high [5].

Apoptosis may be initiated either from without (through formation of a ligand-receptor complex), or through an internal mitochondrial pathway, in most of cases this being accompanied by an activation of caspase cascades and caspase translocation from one cell compartment to another. The sequence of interaction of these enzymes also depends on cell type [6]. Among 11 known caspases in humans, a special role belongs to effector ones (caspases 3, 6, 7) that are responsible for cell substrate cleavage, which determines all the range of characteristic biochemical and morphological changes in a dying cell [7, 8].

A study of effector caspase activation, including caspase-3, in tumoral cells allows to assess the pharmacological drug effects aimed at enhancing the apoptosis of transformed cells or decreasing the threshold of their sensitivity to proapoptotic stimuli [9]. Such investigations are also known for thyroid tumors [10–12]; however, data on caspase-3 activity in these tumors as a marker of spontaneous apoptosis are rather limited [13].

The aim of this work was to determine caspase-3 activity in papillary thyroid carcinoma tissue in patients and analyze the peculiarities of changes in this activity depending on a number of pathomorphological and clinical characteristics of tumoral process.

MATERIALS AND METHODS

Patients. We have studied 12 specimens of extratumoral unchanged tissue of normofollicular structure and 15 specimens of papillary thyroid carcinoma tissue from patients having undergone a surgical treatment at the Surgery Department of the Institute of Endocrinology and Metabolism. Patients were informed and gave their consent to using the tissues removed during the operation for research purposes. A permission for study performance was obtained from the Committee for Biomedical Ethics of the Institute. Among 23 patients, there were 21 women and 2 men, aged 30.0 ± 3.4 years and 28.6 ± 2.1 years, respectively, in the above specimen groups.

Caspase-3 activity and protein concentration assays. Preparation of lisates of thyroid and carcinoma tissues, and assessment of caspase-3 activity were car-

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ried out with regard to acetyl-asp-glu-val-asp-paranitroanilide, according to the protocol of the manufacturer of reagent kit for spectrophotometry assay of enzyme activity ("CASP-3", Sigma, U.S.A.). Enzyme activity was expressed in μmol of paranitroaniline/(hour x mg of protein). Protein content was determined using one of the Lowry method modifications [14].

The statistical processing of the data obtained was performed using Student "t" criterium and Wilcoxon — Mann — Whitney non-parametric "U" criterium. The critical level of significance was assumed to be equal to 0.05. In the Tables data are presented as M ± m.

RESULTS

Our studies have shown that in papillary carcinoma tissue the average caspase-3 activity was somewhat higher ($0.1 > P > 0.05$) compared to unchanged tissue of normofollicular structure (Table 1). An analysis of data depending on several clinical characteristics of tumors has shown that caspase-3 activity did not depend on tumor size ($\rho = -0,19$). At the same time, a strong relationship was reported between caspase-3 activity and those characteristics which allow to refer the tumor to one or another T category: with increasing category, caspase-3 activity was gradually decreasing (see Table 1). We had the possibility of studying in the present work only one papillary carcinoma of T_1 category. Caspase-3 activity in tumor tissue (0.512 μmol of paranitroaniline/[hour x mg of protein]) exceeded by 3.2 times the average enzyme activity in unchanged thyroid tissue. This difference for caspase-3 activity in T_2-category tumor tissue was 1.8 times (0.284 ± 0.044 μmol of paranitroaniline/[hour x mg of protein]); for T_3 category tumors 1,2 times; and in T_4-category tumor tissue caspase-3 activity was decreased by 63% (see Table 1). A relationship has also been established between changes in caspase-3 activity and presence of metastases of papillary carcinoma to lymph nodes: the enzyme activity in the tissue of tumors without metastases was higher compared to unchanged tissue, and somewhat higher ($0.1 > P > 0.05$) compared to metastasizing tumor tissue. Besides, caspase-3 activity was found to be much higher in the tissue of papillary carcinomas of follicular-papillary structure, and lower in the tissue of tumors of mixed structure with solid areas, compared to that in the tissue of papillary carcinomas of typical papillary structure (see Table 1).

A subsequent analysis of the results has shown that caspase-3 activity differed in the tissue of carcinomas with a different aggressiveness of biological behaviour. So, in case of tumor invasion to blood vessels, enzyme activity was lower than in the tissue of tumors without invasion; in the latters caspase-3 activity was higher compared to unchanged thyroid tissue (Table 2). In the absence of tumor invasion to lymphatic vessels, an increased enzyme activity was noted as well. Caspase-3 activity was identical in the tissue of papillary carcinomas with or without intrathyroid spreading of tumor, being, however, significantly decreased in the tissue of those carcinomas which spread into soft tissues adjacent to the thyroid, and increased in the tissue of carcinomas without that. The presence of tumor capsule was associated with a higher caspase-3 activity, as was the absence of fibrous-sclerotic changes in tumor stroma. No significant difference was noted in caspase-3 activity levels in tumors with oxyphilic-cell metaplasia or without it, as well as in tumors with a marked necrosis or without it (see Table 2).

Table 1. Caspase-3 activity in extratumoral unchanged thyroid tissue and papillary thyroid carcinoma tissue of patients

Tissue	n	μmol of paranitroaniline/(hour × mg of protein)	P_1	P_2	P_3	P_4
Unchanged tissue of normofollicular structure	12	0.160 ± 0.026				
Papillary carcinoma tissue, including:	15	0.245 ± 0.037				
T_1-T_2 category tumor	8	0.309 ± 0.046	<0.02			
T_3 category tumor	4	0.194 ± 0.046				
T_4 category tumor	3	0.059 ± 0.020	<0.01	<0.001		
N_0 category tumor	9	0.294 ± 0.050	<0.05			
N_1-N_2 category tumor	6	0.171 ± 0.044				
typical papillary structure of tumor	8	0.220 ± 0.032				
follicular-papillary structure of tumor	4	0.414 ± 0.056	<0.01		<0.02	
mixed structure with solid areas	3	0.087 ± 0.030			<0.02	<0.01

Note: P_1 – compared to the activity in extranodular unchanged thyroid tissue of normofollicular structure; P_2 – compared to the activity in the tissue of T_1–T_2 category tumors; P_3 – compared to the activity in the tissue of tumors of typical papillary structure; P_4 – compared to the activity in the tissue of tumors of follicular-papillary structure.

Table 2. Caspase-3 activity in the tissue of papillary thyroid carcinomas-depending on the characteristics of their biological behaviour

Sign under study	μmol of paranitroaniline/(hour × mg of protein)	
	Sign present	Sign absent
Invasion to blood vessels	0.159 ± 0.035 (8)	0.344 ± 0.049[a,b] (7)
Invasion to lymphatic vessels	0.221 ± 0.054 (8)	0.273 ± 0.033[a] (7)
Intrathyroid spreading	0.232 ± 0.040 (11)	0.281 ± 0.087 (4)
Extrathyroid spreading	0.087 ± 0.020[a] (3)	0.285 ± 0.038[a,b] (12)
Presence of capsule	0.327 ± 0.072[a] (5)	0.204 ± 0.039 (10)
Oxyphilic-cell metaplasia	0.247 ± 0.049 (6)	0.244 ± 0.055 (9)
Necrotic changes	0.211 ± 0.061 (3)	0.253 ± 0.045 (12)
Fibrous-sclerotic changes in tumor stroma	0.193 ± 0.047 (8)	0.305 ± 0.055[a] (7)

Notes: [a] – the difference compared to enzyme activity in unchanged tissue of normofollicular structure (0.160 ± 0.026 μmol of paranitroaniline /[hour x mg of protein]) was significant ($P < 0.05$); [b] – the difference compared to enzyme activity in the tissue in the presence of a sign under study is significant ($P < 0.05$); in brackets – number of observations.

DISCUSSION

Today, there is no doubt as to the importance caspases' role in the biochemical mechanisms underlying apoptosis. In spite of the existence of some tissular specificity of the functioning of certain members of caspase family, involvement of effector caspase-3 in the main processes of apoptosis — both in terms of its initiation and cell components' destruction — is recognized by most investigators [7, 15]. The fact of activation of this caspase, along with changes in other apoptosis parameters (decrease in transmembraneous mitochondrial potential, asymmetric redistribution of phosphatidyl serine on plasmatic membrane, internucleosomic DNA fragmentation), is often used

as a marker of spontaneous or stimulated apoptosis [15–17].

We have formerly demonstrated a sharp decrease in caspase-3 activity in thyroid tissue in case of a marked hyperplasia, of microfollicular tissue structure (such an extratumoral tissue is often reported in the thyroid in case of papillary carcinoma), and in the tissue with signs of papillary carcinoma cell invasion — compared to enzyme activity in the tissue of normofollicular structure. Mitochondrial mechanisms of apoptosis initiation in cells of such tissues are also inhibited [18, 19]. Therefore, changes in histological and follicular structure of extratumoral thyroid tissue, associated with a strong inhibition of caspase-dependent mechanisms of apoptosis realization, may represent one of the factors contributing to tumor spreading within the thyroid.

Such an increase in caspase-3 activity in the general group of specimens of papillary thyroid carcinoma tissue agrees with a 3-4-fold increase in the number of apoptotic cells, formerly demonstrated in these tumors [20]. However, there is also evidence in the literature, of a low apoptotic activity in papillary thyroid carcinomas [3, 5]. The results of our studies suggest that changes in caspase-dependent mechanisms of apoptosis in papillary carcinomas have a multidirectional character, which depends upon the stage and aggressiveness of tumoral process. At initial stages of tumor development, in the absence of metastases to lymph nodes, tumoral cell invasion to blood and lymphatic vessels, extrathyroid tumor spreading, sclerotic and fibrous changes in tumor stroma, and in the presence of a capsule, caspase-3 activity was higher than in unchanged tissue of normofollicular structure. In case of a more aggressive character of tumor, the intensity of caspase-dependent processes of apoptosis in papillary carcinoma tissue was not significantly changed or (in case of extrathyroid tumor spreading) it was decreased. In combination, this was expressed by a gradual decrease in caspase-3 activity in tumor tissue with increasing T-category.

Also of interest is the presence of a difference in caspase-3 activity in papillary carcinomas of various histological subtypes: in case of typical papillary structure of tumor, enzyme activity did not differ significantly from that in unchanged thyroid tissue of normofollicular structure, being higher in tumors of follicular-papillary structure and lower in tumors of mixed structure with presence of solid areas. Therefore, inhibition of caspase-dependent mechanisms of apoptosis was reported in cells of less well differentiated tumors having in their structure areas of solid structure, that are characterized to a considerable extent by a loss of orientation of epithelial cells in terms of their location in a follicle, which may have a significant impact on the functional and metabolic activity of thyrocytes. Such carcinomas are characterized by a reduced potential of thyroid hormone synthesis at the expense of a decreased cell differentiation, and by a significant impairment of the process of cellular cycle blocking at the stage of transition to S-phase [21]. In cells of follicular-papillary carcinomas in which abnormalities in follicular tissue structure and, therefore, changes in epithelial cell orientation in a follicle are expressed to a lesser extent, while apoptotic process activity was, on the contrary, higher.

Thus, if we take into account that the magnitude of activity of caspase-3 — which is an important and the most universal caspase in terms of its involvement in apoptosis realization — is considered to be a marker of spontaneous apoptosis, it may be suggested that at initial stages of tumor growth a considerable activation of programmed cell death occurs in the thyroid, which is a response of the body to the malignant transformation of cells. With tumor growth, a decrease in the number of cells is observed, which formerly responded to apoptosis-inducing stimuli, and an increase in the number of cells that are unable to respond to these stimuli due to abnormalities in the mechanisms of initiation, regulation, and realization of apoptosis, which is also corroborated by the results of our previous investigations [19]. It should be noted that the realization of apoptosis programme after effector caspases' activation, may be blocked due to the functioning of proteins from the family of caspase inhibitors IAP, if the activity of the latters will not be inhibited by mitochondrial proteins-inhibitors Smac/DIABLO and Omi/HtrA2. It has been shown that in transformed thyroid cells the level of Smac/DIABLO is significantly decreased [22].

At late stages of carcinoma development, with an increase in tumor aggressiveness, the decrease in caspase-3 activity, found in our study, agrees with an overexpression in papillary carcinoma cells of survivine, an inhibitor of apoptosis, whose important role in carcinogenesis is reported just at advanced stages of tumor development [23], as well as XIAP, one of the proteins of IAP family, whose overexpression (reported in more than 80% of papillary thyroid carcinomas) correlates with an aggressive character of tumor [24].

In the literature, evidence of changes in spontaneous apoptosis intensity depending on the stage of tumor development, is contradictory. Some consider that at initial stages of carcinogenesis tumors are characterized by a low intensity of transformed cells' death, and that the intensity increases along with an enhancement of proliferation activity at a stage of progression [25]. Other investigators report that in the presence of an increase in tumor size the intensity of spontaneous apoptosis is decreasing [26, 27]. Our data, obtained in the process of assessing caspase-3 activity in papillary thyroid carcinomas, appear to confirm the latter. They also agree with the fact that in cells which proliferate actively, a high level of free radicals is observed (that induce mitochondrial mechanisms of apoptosis [28], which, in turn, leads to caspase-3 activation); while at a terminal stage of tumor growth free radical concentration significantly decreases [29], and then this factor of apoptosis activation may not play such an important role. This is corroborated by the lack of response of papillary thyroid carcinoma cells to an excess of active oxygen radicals [30].

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IMMUNOHISTOCHEMICAL ANALYSIS OF BETA-DEFENSIN-2 EXPRESSION IN HUMAN LUNG TUMORS

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***Aim*: The present research was directed on analysis of the expression patterns of human beta-defensin-2 (hBD-2) in human lung tumors. *Materials and Methods*: Specimens of surgically resected human lung tumors (n = 31) of different histological type (1 case of small cell lung cancer, and 30 cases of non-small cell lung cancer (1 case of clear cell carcinoma, 9 cases of squamous cell carcinoma (SCC), and 20 cases of adenocarcinoma (AC)) were analyzed for expression of hBD-2 with the use of immunohistochemical analysis. *Results*: Immunohistochemical analysis has revealed that all lung tumor samples independently on their histological type express hBD-2 peptide, however at different levels (from < 5% to 100% cells). According to our observations, low-differentiated AC differs from moderately differentiated AC by significantly lower hBD-2 expression levels ($p < 0.05$). No correlation between hBD-2 expression patterns and PCNA or Bcl-2 expression has been found. *Conclusion*: Human beta-defensin-2 expression levels may depend on differentiation grade of lung adenocarcinoma.**

***Key Words*: human beta-defensin-2, human lung tumors, lung squamous cell carcinoma, adenocarcinoma, expression.**

Lung cancer is a leading cause of cancer-related death worldwide. That's why numerous studies are directed on estimation of etiology of this disease, search for tumor markers, and development of new strategies for lung cancer treatment. It is well recognized now that the etiology of lung cancer is closely related to smoking habits and is often associated with chronic pulmonary inflammation and underlying immune dysfunction. However, little is known yet about involvement of innate immunity molecules, in particular, defensins, in lung tumorigenesis.

Defensins — cationic antimicrobial peptides — are important components of mucosal immunity which two major functions are thought to be direct antimicrobial action and modulation of innate and adaptive immunity in response to pathogens. According to accumulated evidences, expression of some alpha- or beta-defensins may be altered or deregulated in different tumor types, and these antimicrobials could play a complex and poorly understood role in cancer pathogenesis either promoting or suppressing tumor cell growth [1, 2].

In normalcy, defensins participate in antimicrobial protection of respiratory tract along with other defense molecules [3]. Mailfunction or altered expression of these peptide antibiotics has been well documented in a number of chronic lung pathologies, in particular, cystic fibrosis, reactive airway disease, tuberculosis and many other lung infections [4]. There are some data evidencing on reduced levels of beta-defensin-2 (hBD-2) in sputum and pharyngeal washes of smokers versus nonsmokers with acute pneumonia [5] what points on possible smoking-dependent down-regulation of this peptide in airway epithelium. At the same time, up-to-date there are scarce data for defensin expression patterns in lung cancer. In our recent pilot study [6] we have recorded an altered expression of hBD-1-4 mRNAs in lung cancer samples versus normal lung tissue.

In this regard we aimed to analyze further the expression patterns of hBD-2 in human lung tumor samples using immunohistochemical approach.

MATERIALS AND METHODS

In the study, 31 samples of surgically resected human lung tumors of different histological type were studied. The tissue samples were obtained during the surgical treatment of lung cancer patients cured in the Thoracic Department of National Cancer Institute (Kyiv, Ukraine) in 2001–2008 (Head of the Dept., prof. V.L.Ganul). Immediately after surgical removal, tissue samples were placed in liquid nitrogen and stored at -70 °C until use. The patients did not receive chemo- or radiotherapy prior to the surgery. All patients provided an informed written consent to perform the study, and the present research was approved by Ethic Board of the Institute. Histological type and differentiation grade of lung tumors has been estimated by clinical pathologists (National Cancer Institute, Dept. Pathol. Anat. Dr. E.N. Kovalchuk and Dr. I.N. Troitskaya). From 31 lung tumor samples, 1 tumor was diagnosed as small-cell lung cancer (SCLC), and 30 — as non-small cell lung cancer (NSCLC). The last group of tumors included 1 case of clear cell large cell lung cancer, 9 cases of squamous cell carcinoma (SCC), and 20 cases of adenocarcinoma (AC). The clinico-pathological characteristics of lung cancer cases are presented in Table 1.

Immunohistochemical analysis. Tumor tissue samples were fixed in 4% formaldehyde for 24 h at room temperature, then dehydrated in 50%, 70%, 80%, 90%, 96% spirits, treated with chloroform, saturated with paraffin at 56 °C for 30 min and placed in paraffin

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Abbreviations used: AC – adenocarcinoma; hBD-2 – human beta-defensin-2; HD – high differentiation; LD – low differentiation; MD – moderate differentiation; NSCLC –non-small cell lung cancer; SCC – squamous cell carcinoma; SCLC – small-cell lung cancer.