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TPS, Thymidine Kinase, VEGF and Endostatin in Cytosol of Thyroid Tissue Samples

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Abstract: *The aim of the study was to determine whether VEGF, TPS, TK or Endostatin determination in tissue cytosol may have some additional value in distinguishing among different types of thyroid lesions. These markers were chosen as representatives of the 2 main pathways (angiogenesis and proliferation) involved in thyroid diseases. VEGF is the most potent angiogenic promoter and Endostatin plays an opposing role. Thymidine kinase (TK) is a marker of DNA synthesis and TPS, cytokeratin 18 fragments, is a marker of the rate of proliferation. We determined qualitatively all four markers in tissue extracts: cytosol from 157 tissue specimens (93 goitre,12 Hashimoto's thyroiditis, 39 adenomas and 13 carcinomas). In 6 cases we were able to compare both normal and pathological tissue samples from a single patient. Statistically significant differences were found in the measured markers, but outliers were present in all groups. This fact does not permit their use in differential diagnosis. The highest levels of all markers were reached in adenomas, being higher than in carcinomas, probably explained by the higher overall metabolic rate in adenomas.*

The thyroid gland is well-vascularised, with one of the highest blood flow rates per unit weight of tissue in the body. Pathological conditions such as Grave's Disease, thyroid enlargement and Hashimoto's thyroiditis are accompanied by a markedly increased blood flow. In cancers of the thyroid, microvessel density has been shown to correlate with disease-free survival in papillary carcinoma of the thyroid (1) and in intrathyroid tumours spread in follicular carcinoma (2). Experimental models of goitre have shown that vascularisation is an important factor for further thyroid growth (3). During goitre formation, there is growth of follicular and endothelial cells, and it is probable that this is regulated by the release of cytokines from follicular cells in response to low concentrations of tri-iodothyronine and high concentrations of thyroid stimulating hormone (TSH) (4). Proliferation is an important process involved in goitre formation and an especially high rate and loss of control of proliferation is typical of tumour diseases.

Malignant epithelial cells overexpress cytokeratin 18 and soluble cytokeratin 18 fragments are released into the blood stream. TPS™ is based on the M3 antibody for detection of a well characterised epitope on cytokeratin 18. The TPS™ value is not simply a reflection of tumor mass, but of tumor cell activity.

Thymidine kinase (TK), which catalyses the conversion of deoxythymidine to deoxythymidinemonophosphate (dTMP), is subsequently used in DNA synthesis, after the transformation to dTTP. TK exists as two different isoenzymes in eukaryotic cells. One isoenzyme (foetal, cytoplasmic, TK1) is associated with cell division, the activity being high in the G1/S-phase, while the other (adult, mitochondrial,TK2) remains stable throughout cell division (5). TK1 is not released by normal division of cells, but only after cell membrane disruption, *i.e.* during cancer proliferation, or cytostatic treatment, or else it is produced by non-dividing cells infected by RNA or DNA viruses (6).

Vasculogenesis and angiogenesis are two main processes by which new blood vessels are formed. Vasculogenesis involves *de novo* differentiation of endothelial cells from mesodermal precursors forming the primary plexus. Once

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the new primary plexus has been formed , new capillaries form by sprouting or splitting from pre-existing ones in the process called angiogenesis (7). In 1971, Folkman introduced his sweeping hypothesis (8), which nowadays is generally accepted, that tumor growth is "angiogenesis-dependent" (9). Stated concisely, every increment of tumor growth requires an increment of vascular growth (10).

Numerous factors have been identified which modulate angiogenesis; these include both promoters and inhibitors that interact, control and organise the development of new blood vessels. Vascular endothelial factor (VEGF)is the most potent pro-angiogenic factor compared with Endostatin, a newly discovered anti-angiogenic factor that may block the function of VEGF (11). Several authors have studied VEGF expression in the thyroid gland, mainly using immunohistochemistry or *in situ* hybridisation techniques and, to a lesser extent, the quantification of mRNA (12-14).

The aim of our study was to quantify the levels of proliferation (TPS and thymidine kinase) and angiogenic factors in thyroid tissue extracts (cytosol) using immuno-analytical technique and to prove the potential value of these markers for differential diagnosis, demonstrated in several previous studies.

Materials and Methods

Tissue specimens. Thyroid tissue samples were obtained from patients who underwent surgery in the Department of Surgery, Charles University Hospital in Plzeň and in the Department of Surgery, University Hospital in Hradec Kralové, Czech Republic. Samples were immediately transported to the Department of Pathology and, after necessary pathological assessment, the pieces of the tissue were frozen and stored at –80˚C for further analysis. Histological details are listed in Table I. For the statistical evaluation 4 groups were formed: goitre (n=93), Hashimoto's thyroiditis (n=12), adenomas (n=39) and carcinomas (n=13). In all we examined 157 tissue samples.

Cytosol preparation and protein determination. Tissues were pulverised using a Micro-dismembranator, (Verder, Germany) consisting of teflon capsules, wolfram-carbide balls, cooled by liquid nitrogen. The powder was homogenised with 5-6 volumes of modified EORTC buffer (13) ($Na_2HPO_4x12H_2O$ 1.8 g/L; $NaH_2PO_4x2H_2O$ 0.78 g/L; dithiothreitol 0.154 g/L; K_2EDTA 0.61 g/L; sodium azide 0.195 g/L; glycerol 10% w/v; sodium molybdate 2.42 g/L), and centrifuged at 100,000xg for 60 minutes at 4˚C. Cytosol supernatant was decanted with a Pasteur pipette and it was then divided into several aliquots for immunoanalytical determination.

Protein assay. The total protein in the cytosol was measured on a Hitachi 817 automatic analyser, (Roche, Switzerland). Commercially available assay for protein quantification in the cerebrospinal fluid and urine was used. The principle is the reaction of protein with benzethonium chloride in alkaline pH; generated turbidity is measured at 505nm.

Table I. *Histological types of thyroid tissue samples.*

Histological category	No.	%
Diffuse parenchymatous goitre (DPG)	8	5.2
Colloid goitre (CG)	85	54.8
Hashimoto's thyroiditis (HT)	12	7.7
Follicular adenoma (FA)	31	20.0
Oncocytoma (OA)	8	5.2
Papillary carcinoma (PC)	11	7.1
Follicular carcinoma (FC)	2	1.3
Total	157	100%

Immunoassays. TPS™ was determined using a commercially available IRMA kit from IDL, Sweden and TK by REA from Immunotech, Czech Republic.

VEGF and basic FGF were determined using commercially available kits from R&D Systems, USA, which is a non-competitive sandwich enzyme immunoassay using monoclonal antibodies. Endostatin was determined using kits from Chemicon, USA, which is a competitive enzyme immunoasay using a polyclonal anti rabbit antibody.

For all methods, we evaluated the effect of the protein concentration in the sample in terms of accuracy. We carried out serial dilution tests with modified EORTC buffer and we found no significant interference in these three methods. The results were expressed as ng /g of cytosol protein.

Statistical analysis. The S.A.S. Software, release 8.02, was used for statistical analysis. Basic descriptive parameters (mean, median, SD, minima and maximum) were calculated for particular histological subgroups. Sensitivities and specificities were also calculated. We applied the non-parametric tests (Wilcoxon and Median test) for evaluation of the differences between distributions of particular markers within histological groups. The Spearman correlation test was use to determine possible relations between the variables we studied.

Results

Histological types and numbers of cases are shown in Table I. The most frequent histological types of tissue samples were goitre and follicular adenomas. For further statistical evaluation we formed 4 groups (goitre, thyroiditis, adenomas, carcinomas) according to histological findings. Because of the inadequate amount of tissue, we were not able to do all measurements in all samples. The numbers of specimens (results) are therefore less in the following Tables than in Table II. Table III, Table IV, Table V and Table VI present descriptive statistical parameters of the measured factors in the histological groups which we established. In all groups, the measured factors showed considerable differences in the measured values compared to the levels in goitre, except for VEGF.

Table II. *Grouped histological types of thyroid tissue samples.*

Histological category	No.	%
Goitre (DPG + CG)	93	59.3
Hashimoto's thyroiditis (HT)	12	7.6
Adenomas (FA+OA)	39	24.8
Carcinomas (PC+FC)	13	8.3
Total	157	100%

Table III. *Basic characteristics of the TPS cytosol levels.*

TPS in cytosol levels (U/g of total cytosol protein)						
Histology Group	N	Mean	SD	Median	Min.	Max.
Goitre	79	126120	132978	97029	8952	718831
Thyroiditis	10	205755	133511	145154	70083	425789
Adenomas	31	326004	309246	279808	1517	1363934
Carcinomas	8	245991	251104	130161	33589	657563

Table IV. *Basic characteristics of the thymidine kinase cytosol levels.*

Thymidine kinase (TK) in cytosol levels (U/g of total cytosol protein)						
Histology Group	N	mean	SD	Med.	Min.	Max.
Goitre	87	23.9	42.60	14	1	345
Thyroiditis	11	33.2	18.42	25	12	61
Adenomas	34	175.8	387.67	30	2	1600
Carcinomas	10	46.3	74.46	18	4	248

Table V. *Basic characteristics of the VEGF cytosol levels.*

VEGF in cytosol levels (ng/g of total cytosol protein)						
Histology Group	N	Mean	SD	Med.	Min.	Max.
Goitre	86	665.6	950.75	297	28	7077
Thyroiditis	10	515.3	505.93	421	48	1393
Adenomas	31	749.3	1093.00	167	17	5203
Carcinomas	9	944.2	726.23	524	134	1700

Table VI. *Basic characteristics of the Endostatin cytosol levels.*

Endostatin in cytosol levels (mg/g of total cytosol protein)						
Histology Group	N	Mean	SD	Med.	Min.	Max.
Goitre	80	2.51	4.381	1.0	0.2	25.5
Thyroiditis	10	3.17	2.953	2.6	0.2	9.4
Adenomas	29	5.76	8.467	2.4	0.2	34.3
Carcinomas	8	1.28	1.514	0.4	0.2	4.2

Table VII summarises the statistical evaluation of the significant differences in measured factors between the histological types. The highest levels of all measured markers were reached in adenomas, while carcinomas showed somewhat lower levels. Only TPS cytosol levels differed in all compared groups, followed by thymidine kinase, while VEGF did not differ at all.

The Spearman correlation coefficients and p value were evaluated only in the goitre and adenoma groups. The other groups were not analysed because of the small number of cases. In the goitre group, we found a positive correlation only between TK and TPS ($r=0.548$, $p<0.0001$, $n=87$). In the adenomas group, TPS significantly correlated with TK ($r= 0.465$, $p<0.008$, $n=31$) and VEGF ($r=0.381$, $p<0.034$, $n=31$). In both evaluated groups, no correlation was found with Endostatin.

In only 6 cases did we receive both "normal" and "pathological" tissue. We analysed these samples separately. We found differences in all pairs of all of these 6 cases (Table VIII). Nodules had higher values of both factors than "normal" tissue from their neighbourhood.

Discussion

The determination of such factors in cytosol using quantitative methods (immunoanalysis) has several limitations, especially in the case of thyroid cancer. This method needs a relatively large amount of tissue sample for analysis, *i.e.* approximately 0.5 cm^3 in the case of thyroid gland tissue. Most of the borderline cases are relatively small and the tissue is required for routine pathological assessments. Sample homogenisation gives the factors the average values from the sample, and therefore does not reflect the heterogeneity in tissue samples.

We did not confirm the statistical differences in VEGF values between the groups that have been mentioned by several authors (3, 15-17). Even if there might be some influence of the huge inter-sample variability within the groups and the low number of cases in the thyroiditis and carcinoma groups, the main reason was the amount of blood present in the samples. VEGF could be released in significant amounts from its binding on platelets and leucocytes during

Table VII. *Statistical differences in angiogenic markers between histological groups.*

	ANOVA *p* value	Wilcoxon *p* value	Median *p* value
Goitre *vs* Adenomas			
VEGF	ns	ns	ns
TPS	0.0001	0.0002	0.0016
Thymidine kinase	0.0004	0.0001	0.0001
Endostatin	0.0139	0.0025	0.0213
Goitre *vs* Carcinomas			
VEGF	ns	ns	ns
TPS	0.03	0.094	0.0246
Thymidine kinase	ns	ns	ns
Endostatin	ns	ns	ns
Goitre *vs* Thyroiditis			
VEGF	ns	ns	ns
TPS	0.078	0.031	ns
Thymidine kinase	ns	0.0038	0.0233
Endostatin	ns	ns	ns

ns: statistically non significant

Table VIII. *Cytosol markers in normal and pathological tissue samples from the same individual.*

Histology	VEGF ng/g tp	TPS U/g tp	TK U/g tp	bFGF ng/g tp
Normal tissue around nodule	146	15489	7	40.5
Nodule	1700	38785	17	187.9
Goitre around nodule	87	96515	15	340.4
Goitre nodule	1700	176119	30	361.7
Goitre around nodule	38	mm.	6	58.3
Goitre nodule	1700	124453	10	146.3
Goitre around nodule	87	46273	6	87.4
Goitre nodule	mm.	mm.	51	441.3
Tissue around follicular adenoma	94	68328	13	63.9
Follicular adenoma nodule	330	145000	21	222.3
Tissue around papillary carcinoma	270	mm.	1	38.7
Papillary carcinoma nodule	1700	mm.	6	234.5

tp: tissue protein

thawing of the sample. Therefore the values for VEGF are dependent on the total quantity of leucocytes and platelets present in the sample, *i.e.* on microvessel density and blood flow (18). Even if Table VII clearly shows that the levels of VEGF are higher in nodules in comparison with neighbourhood tissues, these levels are probably caused both by higher production and a higher total amount of blood particles in the tissue sample. We also found a positive, but expected, correlation between TPS and TK levels, both proliferation markers.

Comparison of tissue pairs brings about an improvement in differences between the measured analytes. The highest levels are reached in benign adenomas, which seem to be highly vascularised and have the highest proliferative rate. This discrepancy with the expected highest rate in carcinomas may be caused by a certain heterogeneity in carcinoma tissue samples.

Conclusion

The main advantage of immunoanalysis in cytosol – precise quantitative determination – tends to be lost by imprecise specimen definition. Cytosol determination is a laborious method that does not permit the assessment of small samples. It is necessary to compare both "normal" and "pathological" tissue. This approach avoids the influence of inter-individual variability and allows both comparison of the determined values and confirmation of the proposed relationships. This approach will still not be helpful in differential diagnosis, since adenomas have the highest levels of the measured factors.

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