

endocrine regulations

VOLUME 38

JUNE 2004

NUMBER 2

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REAL TIME RT-PCR ANALYSIS OF THYROGLOBULIN mRNA IN PERIPHERAL BLOOD IN PATIENTS WITH CONGENITAL ATHYREOSIS AND WITH DIFFERENTIATED THYROID CARCINOMA AFTER STIMULATION WITH RECOMBINANT HUMAN THYROTROPIN

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Objective. Thyroglobulin (Tg), measured by immunometric assay, is the most sensitive and widely used clinical marker for thyroid cancer progression and relapse. However, these Tg determinations are of limited sensitivity and susceptible to interference by Tg autoantibodies. As a possible diagnostic alternative, we tested a real time RT-PCR protocol to determine Tg mRNA levels in peripheral blood.

Methods. Tg mRNA was determined by real-time RT-PCR using total RNA from peripheral blood. Tg mRNA blood levels were calibrated to the mRNA encoding the housekeeping enzyme glyceraldehyde phosphate dehydrogenase (GAPDH); pooled blood from ten healthy subjects served as a RT-PCR positive control.

Results. Tg mRNA and serum Tg were detected in twelve patients with differentiated thyroid cancer (DTC) after thyroidectomy and radioiodine therapy, however, there was no correlation with the clinical stage. An increase in Tg mRNA and protein was observed after application of recombinant human thyrotropin (rhTSH) in four patients with DTC stimulated with rhTSH for postoperative follow up. Tg mRNA and protein were also detected in four congenital athyreotic patients. Analysis of Tg mRNA levels using a commercial multiple tissue Northern blot revealed Tg hybridization signals in several extrathyroidal tissues (salivary gland, trachea, kidney, pancreas, adrenal gland, etc.).

Conclusions. Our data suggests that RT-PCR detects Tg mRNA of extrathyroidal origin, from leukocytes or from metastasizing carcinoma cells under basal conditions or after TSH stimulation. However, considering the marked and highly variable individual Tg mRNA backgrounds, interpretation of real time PCR results requires caution. This limits the clinical use of Tg mRNA determination by real time PCR to an individual tumor progression marker in follow-up.

Key words: Tumor progression - Ectopic transcription - Molecular diagnostics - Follow-up

Differentiated thyroid carcinomas (DTC), although the most frequent endocrine neoplasm, are rare and account for about 1 % of all human cancers with a higher prevalence in women than in men (MAZZAFERRI and KLOOS 2001; SCHLUMBERGER and TORLANTANO 2000).

Usually, DTC is a curable disease with a good prognosis and an overall 10-year survival rate of 70-95% (GIMM 2001). Current treatment protocols include thyroidectomy, I¹³¹ therapy and TSH-suppressive T4 application. However, recurrent disease is observed in 5-30 % of

the cases and metastases develop in 10-15% (MAZZA-FERRI and KLOOS 2001; SCHLUMBERGER and TORLANTANO 2000), with the problem of further dedifferentiation of the neoplastic tissue in 30 % (GORETZKI et al. 1993). As the prognosis is better if a relapse is recognized and treated as early as possible, sensitive postoperative monitoring is crucial. In the follow up of DTC, determination of serum Tg (S-Tg) levels - complemented by ultrasonographic imaging and whole-body radioiodine scans (WBS) - is the most important and sensitive technique and its sensitivity is further increased following TSH stimulation. As Tg expression is retained also in advanced thyroid carcinomas (PACINI and LIPPI 1999), any increase in S-Tg indicates metastases, recurrent or persistent disease.

S-Tg is measured by immunometric (IRMA, ILMA) or radioimmuno (RIA) assays. Such antibody based procedures, however, present some pitfalls, such as suboptimal interassay precision, limited sensitivity, interference by Tg autoantibodies, or the so-called Hook effect. Besides, there are a limited number of tumors or tumor recurrences without any elevation of S-Tg (SPENCER 2000; WESTBURY et al. 2000). Nevertheless the sensitivity of immunochemical Tg measurement for early cancer recognition has certain limits. The detection of circulating tumor cells, minimal residual disease and micrometastases may, therefore, have important therapeutic and prognostic implications. Several studies recently demonstrated the utility of RT-PCR based assays in the detection, staging and monitoring of minimal residual tumor burden in malignant melanoma, prostate, breast, gastrointestinal, neuroblastoma and thyroid cancer (BURCHILL and SELBY 2000; GOSSEIN et al. 1999; ZIPPELIUS and PANTEL 2000; FENTON et al. 2001; RINGEL et al. 2001; TALLINI et al. 1998). PCR was shown to be superior to conventional techniques in detecting occult tumor cells, allowing the identification of one malignant cell mixed with one to ten million normal cells (GOSSEIN et al. 1995). These findings suggest, that this might also hold true for thyroid cancer and that the measurement of Tg mRNA in peripheral blood by real time PCR might represent a more sensitive diagnostic alternative in post-operative monitoring of DTC. In the present study we established a real time PCR protocol and examined the sensitivity and specificity of real time PCR for the detection of Tg mRNA in peripheral blood in a limited number of patients. We show that relevant changes in Tg mRNA levels are detectable by RNA-based real time PCR e.g. in rhTSH stimulated patients.

Patients and Methods

Patients. S-Tg and Tg mRNA levels were measured in peripheral blood of four congenital athyreotic patients with negative I¹²³ or Tc^{99m} scans. We also examined four patients treated for DTC by total thyroidectomy and I¹³¹ ablation, twice stimulated with 0.9 mg/d recombinant human TSH (rhTSH) on two consecutive days (LUSTER et al. 2000). These patients were scheduled for diagnostic whole body scanning in the follow up of thyroid carcinomas. Two of them presented with current and two without metastases as diagnosed by imaging techniques (scintigraphy, MRT, CT) or S-Tg determination; three had papillary carcinomas (PTC) and one a follicular carcinoma (FTC). Blood was obtained from three patients after the first and after the second rhTSH intramuscular injection (i.m.) and from one patient before the first and after the second rhTSH i.m. injection. In addition Tg mRNA and protein levels of twelve arbitrarily selected DTC patients were measured. Five of them had current metastases as diagnosed by imaging and S-Tg measurement and seven had not. A pool of total RNA from three resected goiters was used to generate a standard curve for real time PCR. Total RNA prepared from blood of ten healthy subjects was pooled and used as a control. All patients were treated at the Department of Nuclear Medicine of the University of Wuerzburg. Informed written consent was obtained from rhTSH and athyreotic patients and the healthy subjects.

S-Tg and thyrotropin measurements. Serum thyroglobulin levels were determined by immunoradiometric IRMA with DYNtest® Tg-S and Tg-pluS Assay (B.R.A.H.M.S Diagnostica GmbH, Biotechnology Center, Henningsdorf/Berlin, Germany) according to the manufacturer's protocol. The lower detection limit of the DYNtest® Tg-S was 0.3 ng/ml and of DYNtest® Tg-pluS Assay 0.04 ng/ml. The functional assay sensitivity (FAS) with the interassay coefficient of variation < 20 % was 0.1 ng/ml (MORGENTHALER et al. 2002). Thyrotropin (TSH) measurement was carried out by DYN TSH 1 (B.R.A.H.M.S Diagnostica GmbH, Biotechnology Center, Henningsdorf/Berlin, Germany) according to the manufacturer's protocol with a detection limit of 0.03 mU/l.

RNA extraction. 2.7 ml venous blood was collected from patients in EDTA tubes (Sarstedt®, Nümbrecht, Germany) at routine appointment. From 1 ml venous EDTA blood, total RNA was extracted with QIAamp RNA blood mini kit with a DNase I treatment

(Qiagen GmbH, Hilden, Germany). Total RNAs were resuspended in 50 µl of RNase free water and stored at -80°C. RNA integrity was controlled for representative examples by electrophoresis on denaturing formaldehyde gels.

cDNA synthesis. Quantification of isolated RNA was achieved by spectrophotometric determination at 260 nm. 2.5 µg of total RNA were reverse transcribed in a final volume of 20 µl with 2.5 µM oligo(dT)₁₂₋₁₈ primers; and 200 units Superscript TM II in first strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 0.1 M DTT, 0.5 mM each dATP, dTTP, dGTP and dCTP; 40 units RNaseOut™ according to the manufacturer's protocol [Lifetechnologies, Karlsruhe, Germany]). These samples were designated "RT+"; samples incubated without reverse transcriptase "RT-" were used as negative controls. Complementary DNA (cDNA) was resuspended in nuclease-free water at a final volume of 100 µl and stored at -20°C.

Polymerase chain reaction (PCR) - conventional PCR and agarose gel analysis. In conventional PCR an intron-spanning GAPDH primer set (Stratagene, Amsterdam, Netherlands) with the following nucleotide sequences was used: GAPDH_{sense} (exon 3) 5'-CCACCCATGGCAAATTCATGGCA-3' and GAPDH_{antisense} (exon 7) 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. These primers were used at a final concentration of 500 nM. Tg primers for conventional PCR were the same as used in the real time PCR assay (see below). PCR was performed in a final volume of 50 µl, containing 10 µl of the cDNA solution (approximately 0.125 µg cDNA), 200 µM of each dNTP (Amersham Pharmacia Biotech GmbH, Freiburg, Germany), forward and reverse primer and 1.25 units Taq DNA polymerase in a PCR buffer supplied by the manufacturer (QIAGEN GmbH, Hilden, Germany) on a thermal cycler, PTC-200 (Biozym Diagnostics, Oldendorf, Germany). One sample containing water instead of cDNA served as no template control (NTC).

After initial denaturation, 40 amplification cycles were performed for Tg and 25 for GAPDH (consisting of denaturation at 92°C/15 s, annealing at 60°C/15 s and extension at 72°C/30 s). Reaction products were analyzed on an ethidium bromide-stained agarose gel.

Real time PCR. Tg intron-spanning primers (TAKANO et al. 2000) for real time PCR were designed to amplify a 167 bp product in the cDNA sequence as follows: Tg_{sense} 5'-GAGAAGAGCCTGTCGCTGAA-3' (exon 46; 7942- 7960 bp (VAN DE GRAAF et al. 2001)), Tg_{antisense} 5'-CAGCTCACTGAACTCCTTGT-3' (exon

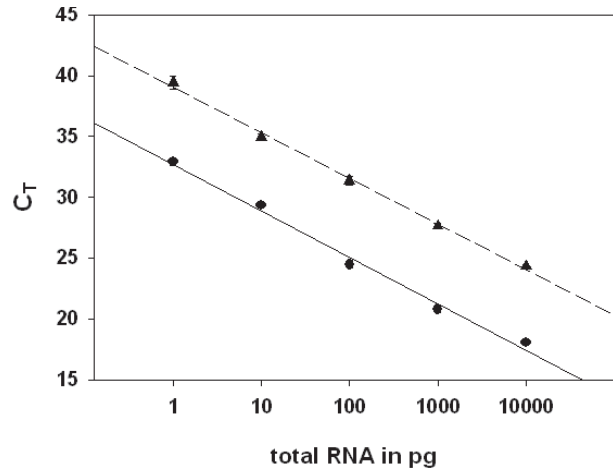


Fig. 1 Typical Standard curve of GAPDH (▲) and Tg (●) mRNA established with TaqMan PCR standard analysis software. The standard curve plots the C_T (described in "Material and methods") versus the log of input amount of total RNA (pg) of three pooled goitre samples. The slope was between -3.5 and -3.8 and the correlation was -0.99 in all experiments (n = 8).

47; 8090- 8111 bp (VAN DE GRAAF et al. 2001)), Tg_{probe} 5'FAM-TGAGTTCTCACGGAAAGTACCCA-3'TAM-RA. Real time PCR for Tg mRNA determination was done in a final volume of 50 µl containing 10 µl cDNA solution (approximately 0.125 µg), 300 nM sense and 900 nM antisense primer and 200 nM probe in TaqMan Universal Master Mix; GAPDH mRNA was measured using the Predeveloped TaqMan Assay. All primers, probes and solutions were from P.E.-Applied Biosystems. Real time PCR reactions were performed on an ABI Prism 5700 (P.E.-Applied Biosystems) according to standard protocols of the manufacturer. "RT+" samples were measured in triplicates and "RT-" samples were assayed in duplicates. Tg and GAPDH mRNA contents of each clinical sample were simultaneously determined in a 96 well plate. The threshold cycle (C_T) represents the PCR cycle at which an increase of the reporter fluorescence (DRn) above the baseline is first detected. No increase in DRn was observed in the "RT-" or in NTC samples.

Standard curve. Tg mRNA was normalized to the mRNA coding for the housekeeping enzyme GAPDH. For this purpose, a standard curve comprising 1-10000 pg of total RNA (in 5 successive steps of 10-fold dilutions in water) isolated in water from three pooled goiter samples was generated (Fig. 1) to mimic a biological sample work up for DTC cells. Based on this plot the amounts of GAPDH and Tg mRNA of each test

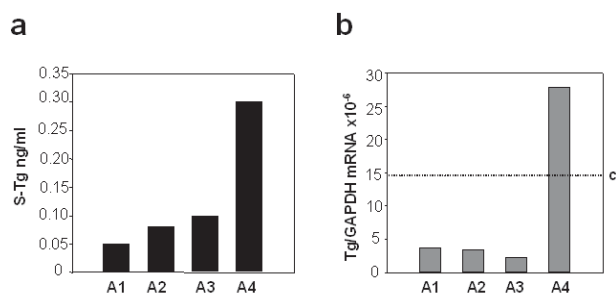


Fig. 2 Serum Tg levels and GAPDH/Tg mRNA ratios of congenital athyreotic patients (A1-4). **A** Serum Tg measurement with the DYNOTest Tg-pluS assay. **B** Tg/GAPDH mRNA ratio. TSH levels are in all subjects in the normal range (0.3-4 mU/l). **c** (reference line) = control of ten pooled blood samples of ten healthy subjects. In three patients Tg mRNA levels were lower than in the control, except for A 4.

sample were calculated. Quantity of Tg mRNA was divided by GAPDH mRNA content, and the ratio is shown.

Northern blot. To examine the expression of Tg mRNA, the Human Multiple Tissue Expression (MTE™) Array 2 from BD Biosciences Clontech (Heidelberg, Germany) was hybridized to a [α^{32} P]dTCP- labeled 167 bp Tg PCR product (see above real time PCR). Hybridization was performed according to the manufacturer's protocol, with a final washing step using 0.3 SSPE/ 0.3% SDS at 65°C. The blot was analyzed with the Phosphoimager Cyclone™ (Packard, Meriden, USA). Signal intensity is expressed as **digital light units per unit area (DLU/mm²)** and is defined as total signal intensity divided by the area of the hybridizing region in mm² after subtraction of the background signal.

Results

Detection of Tg mRNA and S-Tg in patients with thyroid agenesis. Tg mRNA levels were determined in peripheral blood of four patients with congenital thyroid agenesis (Fig. 2). Imaging techniques, ultrasound and I^{123/131}- or Tc^{99m}-scintigraphy, gave no clue for ectopic or hypoplastic thyroid tissue. TSH levels in all subjects were in the normal range (0.3 - 4 mU/l) under thyroxine supplementation. As shown in Fig. 3, a 167 bp product was amplified from the cDNA of all four athyreotic patients demonstrating the presence of Tg mRNA in their peripheral blood. Similar results were obtained using two other Tg primer sets (data not shown). This was not due to contamination by genom-

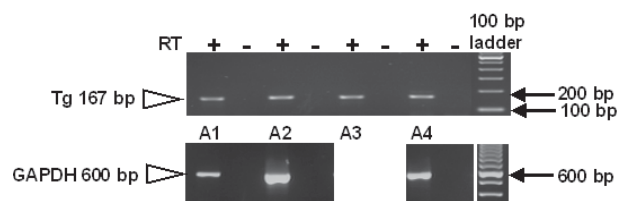


Fig. 3 Conventional RT-PCR amplification of GAPDH (A1, A2, A4) and Tg (A1- 4) mRNA from subjects with thyroid agenesis. RT +/-: cDNA synthesis reaction with/without reverse transcriptase. Expected PCR products of 660 bp for GAPDH and of 167 bp for Tg are visualized by agarose gel electrophoresis and staining with ethidiumbromide.

ic DNA, as on the one hand, primers were intron-spanning and on the other hand, neither GAPDH- nor Tg-specific amplicons (Fig. 3) were detected when "RT-" samples were used as templates for conventional PCR.

The expression levels of Tg and GAPDH mRNA were also measured by real time PCR. Tg/GAPDH mRNA ratios in athyreotic patients ranged from 3.66×10^{-6} to 27.8×10^{-6} . They were lower in three of the athyreotic patients than in the pooled normal control (14.9×10^{-6}) except for patient A4 (Fig. 2B), who also had the highest S-Tg level. No amplification was observed in "RT-" or in "NTC" control samples. S-Tg was not detectable in the four athyreotic subjects with DYNOTest® Tg-S assay by IRMA. When the more sensitive DYNOTest® Tg plus assay was used, S-Tg levels between 0.05 (A1) and 0.33 (A4) ng/ml were determined (Fig. 2A).

Northern blot. To find a potential source for the observed Tg mRNA in the athyreotic patients, we carried out Northern blot analysis of mRNA using a commercial multiple tissue mRNA array. A 167 bp Tg-specific, radioactively labeled PCR product was hybridized to a human Multiple Tissue Expression Array, and hybridization signals were analyzed by phosphoimaging (Fig. 4). As expected, the most intense signal (800 DLU/mm²) was obtained in the thyroid gland. However, the salivary gland and the trachea also show prominent hybridization (around 20 DLU/mm²), and a couple of other extrathyroidal tissues - kidney, pancreas, adrenal gland, liver and heart - still gave significant signals between 7 and 10 DLU/mm². Peripheral blood leukocytes and bone marrow demonstrate signals between 3.5 and 4.5 DLU/mm². These data suggest illegitimate and/or ectopic Tg gene transcription in extrathyroidal tissues, which may contribute to the observed Tg mRNA in the serum of athyreotic patients.

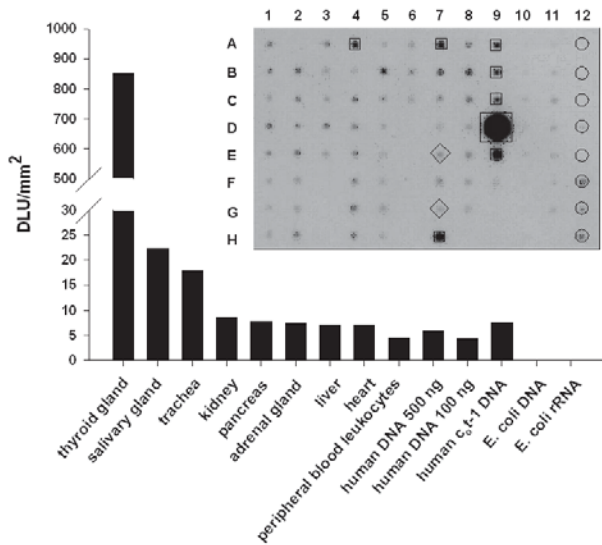


Fig. 4 Densitometric evaluation of the Multiple Tissue Expression array. The bars represent the intensity of the hybridization signals. All signals with intensities of > 7 DLU/mm² are shown. DLU: Digital Light Units. **Insert:** Multiple Tissue Expression array hybridized with a Tg cDNA probe (167 bp). Signals with an intensity higher than 7 DLU/mm² are marked by squares (□): A4 = heart, A7 = kidney, A9 = liver, B9 = pancreas, C9 = adrenal gland, D9 = thyroid gland, E9 = salivary gland, H7 = trachea. Peripheral blood leukocytes (E7) and bone marrow (G7) are marked by rhombes (◊). The controls are marked by circles (○): A - H 12: yeast total RNA, yeast tRNA, E.coli rRNA, E.coli DNA, Poly r(A), human C₀t-DNA, human DNA 100 ng, human DNA 500 ng.

Detection of S-Tg and thyroglobulin mRNA in rhTSH treated patients. Tg mRNA expression was also examined in four patients with DTC (Table 1) who were treated with rhTSH before diagnostic radioiodine scintigraphy during postoperative follow up (LUSTER et al. 2000). Peripheral blood samples were obtained on two consecutive days after they (R1-R4) had received 0.9 mg/d i.m. rhTSH. Blood was obtained from R1, R2 and R3 before and after the second rhTSH application; from R4 before and after the first rhTSH stimulation. S-Tg was detectable in patient R1, R2 and R4 at low or high serum TSH levels caused by exogenous rhTSH stimulation. In patient R3 S-Tg was measurable only after rhTSH stimulation. In all patients, rhTSH stimulation caused a rise in S-Tg. In the two patients with metastases (R1, R4), S-Tg levels were higher than in the metastasis-free subjects (R2, R3) (Fig. 5a). Tg mRNA was detectable in all of the individuals and was increased after rhTSH stimulation in all 4 patients (Fig. 5b); Tg/GAPDH mRNA ratios were between 1.8 x 10⁻⁶ and 16.9 x 10⁻⁶, i. e. below

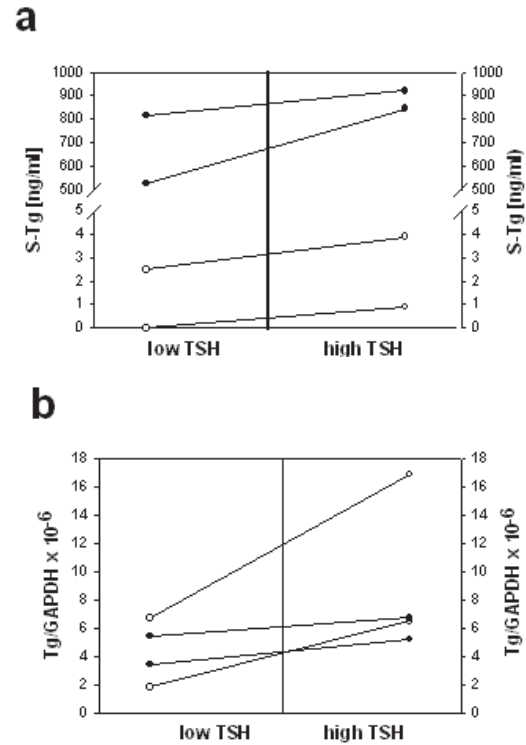


Fig. 5 A S-Tg levels (ng/ml) and B Tg/GAPDH mRNA ratios (x 10⁻⁶) at low and high serum TSH levels after rhTSH application in 4 DTC patients.

or in the range of the value observed in the healthy control.

Detection of thyroglobulin mRNA by RT – PCR in DTC patients during the follow up. Finally, we examined a heterogeneous set of 12 randomly selected patients with DTC after thyroidectomy and ablative radioiodine therapy in the follow up (Table 2). Five (M1 – M5) of them had known metastases diagnosed by imaging and /or by increased S-Tg levels, seven (N1 – N7) showed no evidence of metastatic disease. The median of the Tg/GAPDH mRNA ratios was 2.9 in the first group (M1 – M5) and 5.5 in the second group (N1 – N7). No obvious correlation was found between S-Tg, clinical stage (TNM, MACIS) and mRNA ratios. Interestingly, three patients of the second group developed local recurrence (N3, N7) or increased S-Tg (N4) half a year after Tg mRNA measurement

Discussion

We developed a RNA based real time PCR assay to measure Tg mRNA in peripheral blood. This assay was

Table 1
Patients treated with rhTSH during follow-up

Patient	DYNtest Tg-plus (ng/ml)		TSH (mU/l)		Tg/GAPDH $\times 10^{-6}$		TNM	Histology	Imaging WBS ¹⁾
	low	high	low	high	low	high			
R 1	815	921	125	136	5.44	6.67	pT ₃ N ₀ M ₀	FTC ²⁾	distant metastases ⁴⁾
R 4	525	845	0.03	138	3.40	5.18	pT ₃ N ₀ M ₁	PTC ³⁾	distant metastases ⁴⁾
R 2	2.5	3.9	17.4	113.7	1.84	6.49	pT ₂ N ₀ M ₀	PTC ³⁾	NED ⁵⁾
R 3	0	0.9	87.3	93.5	6.69	16.9	pT ₂ N ₀ M ₀	PTC ³⁾	NED ⁵⁾

¹⁾ WBS: whole-body radioiodine scan; ²⁾ FTC: Follicular Thyroid Carcinoma; ³⁾ PTC: Papillary Thyroid Carcinoma; ⁴⁾ pulmonal, osseous metastases; ⁵⁾ NED: No Evidence of Disease

Table 2
DTC patients after thyroidectomy and ablative radioiodine therapy in the follow up

Patient	TSH (mU/l)	DYNtest Tg-S (ng/ml)	Tg/GAPDH $\times 10^{-6}$	TNM	MACIS	Histology	Imaging			
							Ultra- sound	WBS ⁷⁾	CT	PET
M1¹⁾	0.04	352	1.0	pT ₃ N ₀ M ₀	10.9	FTC ⁴⁾	local	distant uptake	Ø ⁸⁾	
M2	>80	39	2.6	pT ₃ N ₀ M ₀	7.06	FOTC ⁵⁾		NED ³⁾		
M3	>80	108	2.9	pT _{4a} N _{1b} M ₀	7.1	PTC ⁶⁾		NED ³⁾		
M4	0.4	32	5.1	pT ₃ N ₀ M ₁	12.09	PTC ⁶⁾	local	distant uptake	Ø ⁸⁾	
M5	< 0.3	63358	5.6	pT _{4a} N ₀ M ₁	8.75	FOTC ⁵⁾	local	distant uptake	Ø ⁸⁾	
N1²⁾	< 0.03	NED ³⁾	0.04	pT _{2a} N ₀ M ₀	3.7	FOTC ⁵⁾		NED ³⁾		
N2	< 0.03		0.1	pT _{1b} N ₀ M ₀	4.3	PTC ⁶⁾				
N3	< 0.03		4.4	pT ₄ N ₀ M ₀	6.08	FTC ⁴⁾				
N4	< 0.03		5.5	pT ₂ N _{1a} M ₀	4.52	PTC ⁶⁾				
N5	0.2		8.7	pT _{4b} N ₁ M ₀	4.49	FTC ⁴⁾				
N6	< 0.03		9.4	pT ₄ N ₀ M ₀	6.6	PTC ⁶⁾				
N7	< 0.03		44.0	pT _{4a} N _{1b} M ₁	7.85	PTC ⁶⁾				

¹⁾M1 – M5: with current metastases or local recurrency; ²⁾N1 –N7: No metastases; ³⁾NED: no evidence of disease; ⁴⁾FTC: Follicular Thyroid Carcinoma; ⁵⁾FOTC: Follicular- Oncocytic Thyroid Carcinoma; ⁶⁾PTC: Papillary Thyroid Carcinoma; ⁷⁾WBS: Whole- Body radioiodine Scan; ⁸⁾ Ø: PET: Positron-Emission Tomography not performed

applied to healthy individuals, congenital athyreotic patients and patients with treated DTC. The real time PCR protocol was sensitive enough to quantify Tg mRNA in peripheral blood of healthy subjects. By examining congenital athyreotic patients we had intended to create truly negative controls, especially by avoiding any interference with thyroid remnant tissue after surgical therapy. Surprisingly, we detected Tg mRNA in the peripheral blood of these congenital athyreotic patients. Imaging techniques gave no indication for hypoplastic or ectopic thyroid tissue in these athyreotic patients, and contamination by ge-

nomnic DNA in our PCR assays was also excluded by appropriate controls. These results suggested that there must be an extrathyroidal source for Tg mRNA in the sense of illegitimate (CHELLEY et al. 1989) or ectopic transcription (SARKAR and SOMMER 1989), where expression levels of one transcript in 500 to 1000 cells (CHELLEY et al. 1989; COOPER et al. 1994) may be expected.

Indeed, using a multiple tissue expression array screened with high stringency, we detected Tg signals also in non-thyroid tissue, e.g. salivary gland, trachea, kidney, pancreas, adrenal gland, liver and heart. These

results are in line with similar data obtained by other experimental approaches: SPITZWEG et al. (1999) reported that, according to RT-PCR data, normal thymus tissue expresses thyroid-related genes and proteins like sodium iodide symporter, thyrotropin receptor, thyroid peroxidase and thyroglobulin. SELLITI et al. (2000) amplified by RT-PCR thyroglobulin mRNA from mesangium cells associated with glomerular basal membrane of the kidney. BOJUNGA et al. (2000) found Tg mRNA in thymus, suprarenal gland, hypophysis, lung, testis and vermiform appendix after 40 cycles of PCR. KIMOTO et al. (1998) published that lymphocytes express virtually all human mRNAs. CHELLY et al. (1989) postulated illegitimate transcription in any cell type and anticipated that all promoters could be minimally active when ubiquitous transcriptional factors reach their DNA binding site, leading to very low, but not null, gene transcription. Recently, BUGALHO et al. (2001) published expression of Tg mRNA in lymphocytes and granulocytes. Thus, Tg mRNA observed in athyreotic patients most likely results from an ectopic or illegitimate transcription of the Tg gene in extrathyroidal tissues including blood cells. Basal levels of Tg mRNA strongly vary from patient to patient. With respect to a diagnostic application of real time PCR, this means that Tg mRNA levels cannot be used as absolute markers for the status of thyroid cancers. However, this problem also arises with the new and more sensitive immunological Tg-pluS assay, which also detected the low levels of Tg protein in the peripheral blood of athyreotic patients given that the ectopically expressed mRNA is faithfully spliced and translated. In conclusion, both assays might be interference-prone and given the high sensitivity of the two methods, there seems to be no advantage in any of them regarding the point of specificity.

Several groups (HAUGEN et al. 1999; LADENSON 2000; McDUGALL and WEIGEL 2001) reported that rhTSH stimulates the radioiodine uptake and S-Tg production as measured by immunological methods in cancer patients undergoing DTC monitoring for thyroid remnant and/or tumor recurrence. We here report on an increase in S-Tg in four rhTSH-treated DTC patients. Furthermore, according to our real time PCR assay, Tg/GAPDH mRNA ratios rose in all four patients after rhTSH stimulation. This sensitivity to alterations in Tg mRNA levels due to rhTSH stimulation shows that the assay should also be able to measure increases in Tg mRNA due to tumor progression, relapse or metastases independent of any high background levels caused by ectopic Tg gene transcription. If Tg mRNA levels are regarded as an individual tumor pro-

gression marker, i.e. if they are compared with one or more reference S-Tg values obtained from the same patient at earlier time points, then real time PCR determinations of Tg mRNA may serve as a useful tool for monitoring DTC patients during follow up.

Tg mRNA was also observed by real time PCR in all DTC patients. However Tg/GAPDH ratios and S-Tg levels did not correlate in this limited number of patients. This is not unexpected, because on the one hand the exact amount of neoplastic tissue required to increase serum Tg levels in DTC patients is unknown and probably varies with the biological characteristics of the tumor (VAN HERLE and VAN HERLE 1997), on the other hand JOHNSON et al. (1995) reported that every tumor cell synthesizes different amounts of mRNA. Also, we did not observe a correlation of Tg mRNA levels with the TNM staging in 16 patients. TANAKA et al. (2000) reported that Tg mRNA levels positively correlate with the prognostic MACIS score for papillary thyroid cancer. FENTON et al. (2001) published a correlation between Tg mRNA levels with total body ¹³¹I uptake and S-Tg in children with previously treated papillary thyroid cancer. In 12 DTC patients, we did not find such correlations either. Several studies have been performed trying to establish the clinical value of Tg mRNA measurement for the follow-up of thyroid cancer patients. The results are controversial, as some of them demonstrate a correlation between Tg mRNA levels in peripheral blood and the progress of thyroid malignancies, some of them do not (RINGEL et al. 1999; BISCOLLA et al. 2000; GRAMMATOPOULOS et al. 2003; TAKANO et al. 2001; ESZLINGER et al. 2002; SPAN et al. 2003). One study confines such a correlation to a subtype of thyroid carcinomas, i. e. PTC (BELLANTONE et al. 2001), one recommends the combined evaluation of circulating Tg mRNA and serum Tg by means of an immunoassay (FUGAZZOLA 2002), and one allowed the definition of a positive cutoff point at 1 pg/ μ g total RNA which produced fewer false negative results than those obtained with S-Tg assays (SAVAGNER et al. 2002). Altogether, this data leave the diagnostic value of Tg mRNA quantification in peripheral blood still open.

In conclusion, our assay demonstrated no direct correlation between Tg mRNA levels and S-Tg as well as between Tg mRNA and protein levels. However increased Tg mRNA levels after exogenous stimulation with rhTSH were observed. It may be assumed that the additional amount of Tg mRNA derives from residual thyroid tumor or from their metastases and not from non-thyroidal sources, as the extrathyroidal tissues are not supposed

to react to stimulation by TSH. This indicates, that real time PCR should also be able to show rising Tg mRNA levels accompanying thyroid tumor progression, relapse or metastasis, underscoring the diagnostic potential of real time PCR assays of Tg mRNA as an individual tumor marker. Further studies are needed to confirm whether stimulation with rhTSH will amplify circulating Tg

mRNA signals originating from TSH receptor-positive (residual) thyroid tissue and metastases.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft, grant number: Kö 922/8-1/2 and by Wilhelm Sander Foundation.

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IN VITRO EFFECTS OF INHIBIN ON APOPTOSIS AND APOPTOSIS RELATED PROTEINS IN HUMAN OVARIAN GRANULOSA CELLS

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Objective. To clarify the in vitro effects of inhibin A (I) on apoptotic cell death and its mechanisms in ovarian granulosa cells the immunoexpression patterns of the apoptosis markers caspase-3 and pro- and anti-apoptotic proteins (Bcl-2, Bcl-xl, Bak) were evaluated in ovarian granulosa cells collected from women with different hormonal status.

Materials and Methods. Granulosa cells were isolated from follicles of women participating in an in vitro fertilization (IVF) program, normally cyclic (NC) and premenopausal women (PrM). The obtained cells were cultured for 72 h with inhibin A (Sigma, USA) – 10 ng/ml. The concentration of estradiol in the culture medium was determined by radioimmunoassay using the Coat-A-Count kit (Nippon, Japan), whose intra- and interassay coefficients of variations were 6,8 % and 6,2 % respectively. The expression of caspase-3, Bak, Bcl-2, Bcl-xl was investigated immunohistochemically. The percentages of immunopositive cells were calculated and Student's t-test was used for statistical analysis.

Results. Addition of inhibin A (10 ng/ml) to granulosa cells cultures resulted in increased estradiol production. Maximal stimulation was observed in granulosa cells collected from women participating in IVF whereas minimal effect of inhibin treatment on estradiol production was demonstrated in premenopausal women. Inhibin A exposition enhanced the immunoexpression of pro-oncogenes (Bcl-2, Bcl-xl) and reduced the expression of caspase-3 and pro-apoptotic protein Bak in ovarian granulosa cells from the three experimental groups.

Conclusions. Our findings suggest that inhibin A in vitro stimulates the estradiol secretion by granulosa cells dependently of the woman hormonal status, while it inhibits apoptotic process in granulosa cells independently of the hormonal status.

Key words: Human ovarian granulosa cells – Inhibin – Apoptosis – Apoptosis-related proteins H

During ovarian follicle growth and development the follicular atresia is a negatively selective degenerative process which involves granulosa cell (GC) death by apoptosis (TILLY et al. 1991; HUGES et al. 1991; VASKIVUO et al. 2002). Apoptosis (programmed cell death) is a distinct physiological form of cell death of characteristic morphology and biochemistry. When the cells proceed to apoptosis, the death cascade leads to activation

of caspases, such as caspase-3, which break down the cells (OTALA et al. 2002). The balance between proliferation and apoptosis of granulosa cells is crucial for the growth, development and differentiation of ovarian follicles both before birth and during the reproductive life. Diverse stimuli are known to compromise this balance leading to either degenerative diseases or via uncontrolled cell proliferation to cancerogenesis (TAL et

al. 1995; COATES et al. 1996; OTALA et al. 2002; CATZ and JOHNSON 2002). The cyclic proliferation, differentiation and programmed cell death of ovarian granulosa cells are under the complicated and still obscure control of ovarian (steroid) and pituitary hormones, paracrine and/or autocrine regulators and growth factors. In order to clarify whether inhibin (a member of transforming growth factors- β super family) is involved in the control of programmed cell death, *in vitro* studies were undertaken to characterize the effects of this growth factor on the apoptosis and the immunoppression patterns of apoptosis-related proteins in human ovarian granulosa cells from women of different age and hormonal status.

Materials and Methods

Granulosa cells were isolated by laparoscopy via the non-enzymatic needle puncture method from ovarian antral follicles of: 1. women participating in an *in vitro* fertilization program (age: 27-31 years, $n=18$); 2. young normally cycling women (age: 21-26 years, $n=6$) during sterilization via salpingo-oophorectomy or hysterectomy for benign indications (myoma uteri, bleeding disorders), 3. premenopausal women (without any hormonal therapy for at least one year; age: 45-51 years, $n=12$) at the Institute of Sterility and Assisted Reproduction Technologies in Sofia. All patients have given their informed consent to the study.

Granulosa cells were cultured in Dulbecco minimal essential medium (DMEM, Sigma, USA) in the presence of 10% fetal calf serum (FCS, Sigma) either with or without Inhibin A (Sigma, 10 ng/ml) for 72 hours. The cells grown as monolayer on microscopic slides were then washed in 0.1M PBS, fixed in 4% formaldehyde, freshly prepared from paraformaldehyde, in 0.1M phosphate buffer with 7.5 sucrose added for 30 min, washed again in 0.1M PBS and preserved until use at -20°C in a preserving medium. The cell monolayers were conditioned for 30 min in a blocking solution, consisting of 1% immunoglobulin-free bovine serum albumin (BSA Fraction V, Sigma, USA) in 0.1% PBS and incubated overnight at 4°C in a humidified chamber with one of the primary monoclonal antibodies: Caspase-3 (CPP32) (diluted 1:500, Serotec), Bcl-2 (diluted 1:5, BioGenx, CA, USA), Bcl-x1 (diluted 1:500, Serotec), Bak (diluted 1:400, Dako, Denmark). To calculate the percentage of immunopositive cells by light microscopy at 400 X magnification, positive cells were

counted out of 450 randomly selected granulosa cells from three slides per patient (3 patients per group).

Statistical evaluation. Results reported as mean \pm SD throughout the study were statistically analyzed by Student's t-test.

Results

The results shown in Fig.1 illustrated the effect of inhibin on estradiol production by granulosa cells collected from women with different hormonal status, while progesterone secretion remained unaffected. The amount of estradiol, secreted by granulosa cells was highest in women taking part in IVF, whereas the amount of estradiol, secreted by granulosa cells of PrM women was lowest as compared to controls (C) – granulosa cells cultured in the absence of inhibin. Apoptotic granulosa cells in the cultures were identified by their condensed chromatin and darkly stained fragmented nuclei using light microscopically evaluation. Taken into consideration the activity of caspase-3 (Fig.2) the smallest amount of apoptotic cells was noted in IVF women (7%), larger amount in normally cyclic women (16%) and the largest in premenopausal patients (27%). The obtained values of the percentages of caspase-3 positive cells in granulosa cells cultures after inhibin exposure pointed out decrease of the number of enzyme positive cells as compared to controls (IVF-5%; NC-11%; PrM-25%) (Fig.3). The number of Bak positive cultured granulosa cells (Fig.4) varied from 8% for women undergoing IVF to 16% for premenopausal women. The applied inhibin provoked depletion of the values of Bak positive cells in all experimental groups (3%; 4%; 12%) for women after IVF, normally cyclic and premenopausal women, respectively (Fig.5). The number of cultured granulosa cells with pro-oncogene Bcl-2 expression was almost similar in both groups of normally cycling patients (23%) and patients participating in an IVF (24%) and was lower in premenopausal women (16%). After inhibin application some changes in the percentages of positive cells were observed (IVF-27%; NC-26%; PrM-15%) (Fig.6). The reaction for Bcl-x1 in granulosa cells cytoplasm showed numerous positive cells in women after IVF (26%) and in normally cycling patients (25%), but almost 2 fold less in granulosa cells cultures of premenopausal women (14%). The applied inhibin changed slightly the proportions of Bcl-x1 stained granulosa cells (IVF-28%; NC-27%; PrM-15%) (Fig.7).

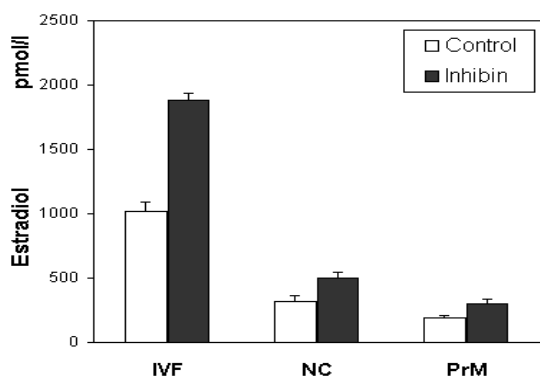


Fig.1 In vitro inhibin A action on estradiol secretion by cultured human ovarian granulosa cells. Values are means \pm SD of 9 cultures from 3 experiments, $p < 0.001$ when compared to control. IVF = in vitro fertilisation group, NC = normally cycling group, PrM =premenopausal group

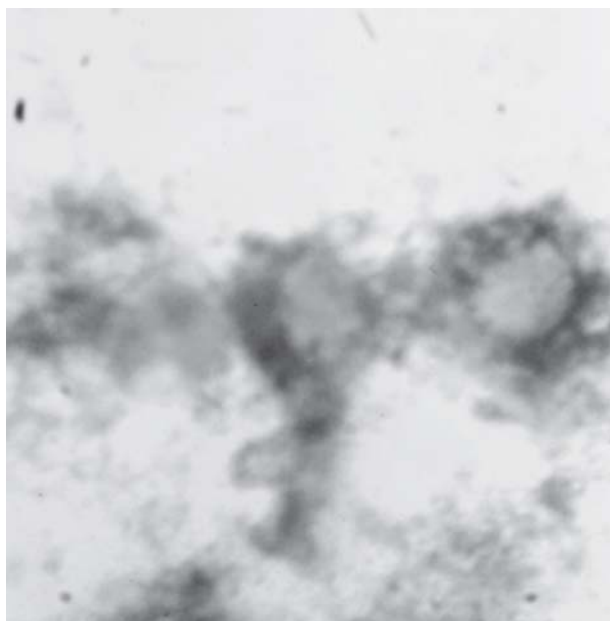


Fig.2 Caspase-3 immunopositive ovarian granulosa cells. X400

Discussion

With regards to the inhibin A action we established that this growth factor reduced apoptosis in granulosa cells isolated from women with different hormonal status, suggesting its apoptotic effect. Granulosa cell death in vitro through apoptosis was shown by the detection of caspase-3 enzyme activity (one of the specific cell human death enzyme after GALLAHAR et al. 2001) and by the immunopositive expression of pro- and anti-apoptotic

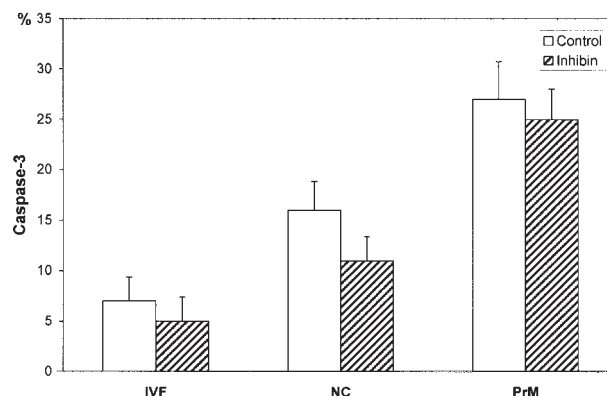


Fig.3 Percentage of caspase-3 immunopositive ovarian granulosa cells from women with different hormonal status (IVF, NC and PrM) after inhibin treatment. Values are mean \pm SD of 12 cultures from 3 experiments, IVF, PrM ($p < 0.01$ vs.control); NC ($p < 0.05$ vs. control). IVF = in vitro fertilisation group, NC = normally cycling group, PrM =premenopausal group

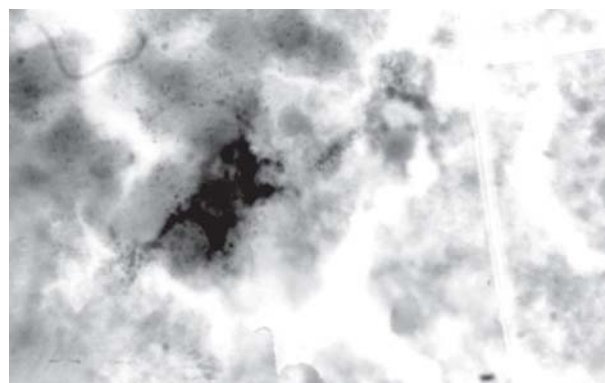


Fig.4 Bak immunopositive ovarian granulosa cells. X100

proteins respectively. The enhancement of granulosa cells apoptosis in our study was paralleled by up-regulated expression of proapoptotic protein Bak and down regulated expression of anti-apoptotic Bcl-2 and Bcl-x1 proteins. Immunohistochemical and biochemical analyses of BOONE and TSANG 1998; KUGH et al. 1998; SAKAMAKI 2003; GABRIEL et al. 2003; ELISEEV et al. 2003 have also shown apoptosis in animal and human granulosa cells to be regulated by caspases and Bcl-2 gene family members. The role of apoptosis in human reproduction has been discussed repeatedly (BENIFLA et al. 2002; HSUEH 2002; TAKAGIMORISHITA et al. 2003). In our study inhibin has been shown to enhance estradiol secretion by cultured granulosa cells. Based on the

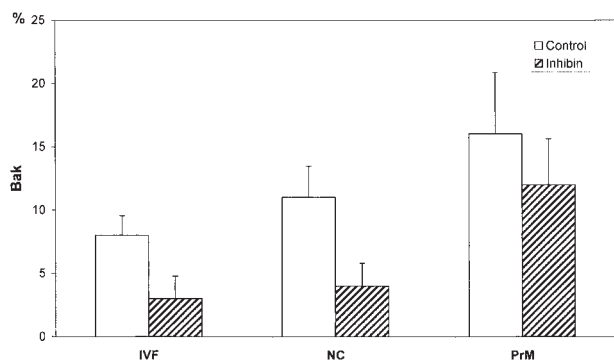


Fig.5 Percentage of Bak immunopositive granulosa cells from IVF, NC and PrM women after inhibin treatment. Values are mean \pm SD of 12 cultures from 3 experiments, $p < 0.001$ when compared to control. IVF = in vitro fertilisation group, NC = normally cycling group, PrM =premenopausal group

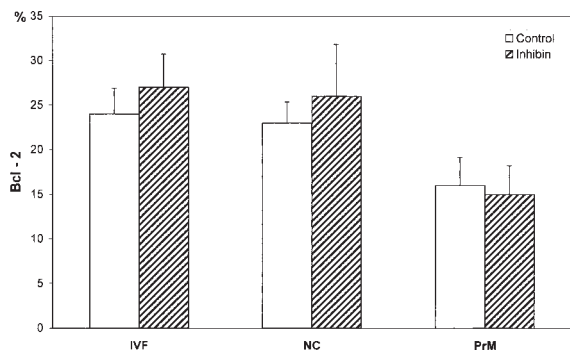


Fig.6 Percentage of Bcl-2 immunopositive granulosa cells from IVF, NC and PrM women after inhibin treatment. Value are mean \pm SD of 12 cultures from 3 experiments, $p < 0.01$ when compared to control. IVF = in vitro fertilisation group, NC = normally cycling group, PrM =premenopausal group

obtained radioimmunoassay of estradiol levels in granulosa cells culture we found that the enhanced production of estradiol by cultured granulosa cells depend on the woman hormonal status (the most intensive stimulation of the basal estradiol production was observed in IVF patients and the less one in premenopausal patients). The enhanced estradiol secretion correlate positively with the apoptosis suppression. The number of granulosa cells immunopositive for caspase-3 revealed highest number in cultures from premenopausal wom-

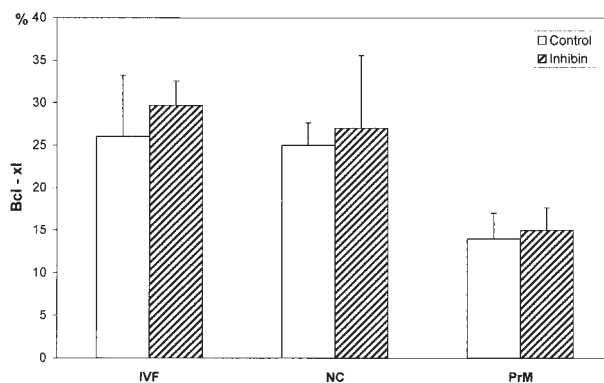


Fig.7 Percentage of Bcl-xl immunopositive granulosa cells of IVF, Y and PrM women after inhibin treatment. Values are mean \pm SD of 12 cultures from 3 experiments, $p < 0.05$ when compared to control.

IVF = in vitro fertilisation group, NC = normally cycling group, PrM =premenopausal group

en, contrary to the small number of the enzyme positive granulosa cells in women undergoing IVF, when the estrogen in the follicular fluid is the highest. The obtained data revealed parallel alterations of the increased estradiol production after inhibin treatment, reduced apoptotic process and weakly expressed proapoptotic proteins. This is in agreement with the data of BILLIG et al. 1993; CAMPBELL and BAIRD 2001; SONG and SANTEN 2003) who considered estrogen as an inhibitor of ovarian granulosa cells apoptosis and in contrast to the negative effect of inhibin on estradiol production established by JIMENEZ-KRASSEL et al. (2003). According to these authors it is possible that the variable results among the laboratories that examined the direct effect of inhibin treatments on granulosa cell function were attributed to the length of cultures and hormonal or growth additives during culture, causing a different basal inhibin production. It is interesting that the inhibitory effect of inhibin on cultured granulosa cell apoptosis was uniform intense regardless to the hormonal status of the investigated women. Our findings provide some evidence that inhibin A has a key local autocrine role on stimulating estradiol secretion by granulosa cells and inhibitory effects on granulosa cells apoptotic process in addition to its endocrine role.

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CONCENTRATION OF METALLOPROTEINASE-2 AND TISSUE INHIBITOR OF METALLOPROTEINASE-2 IN THE SERUM OF PATIENTS WITH BENIGN AND MALIGNANT THYROID TUMOURS TREATED SURGICALLY

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Objectives. Neoplastic angiogenesis is an essential stage of growth, progression and invasion of solid tumours. The process of basement membrane degradation and remodelling of the extracellular matrix (ECM) involves proteolytic enzymes called metalloproteinases. Among the numerous proteolytic enzymes of this group the key role is played by metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase 2 (TIMP-2). Tissue expression and concentration of these compounds in body fluids have been used in early diagnostics of tumours development, assessment of tumours advancement and treatment results monitoring. The aim of the study was to evaluate the concentration of MMP-2 and TIMP-2 in blood serum of patients with benign and malignant thyroid tumours and the effect of surgical treatment on these parameters in the postoperative period as well as assessment whether to MMP-2 and TIMP-2 serum concentration in patients with thyroid cancer positively correlates with the clinical staging classification of the International Union Against Cancer (UICC).

Patients and methods. The study group consisted of 53 patients with various types of thyroid cancer and 23 patients with benign thyroid tumours, while 26 healthy adults served as controls. According to clinical staging classification of thyroid cancer the 32 patients were classified with stage I, 6 with stage II, 8 with stage III and 7 with stage IV. We have found higher mean concentration of MMP-2 in 53 patients with thyroid cancer as compared to the control group and the group of 23 patients with benign thyroid tumours. All patients were treated operatively. Additionally, a significant effect of radical surgical treatment on mean concentration of MMP-2 and TIMP-2 in patients with papillary and follicular thyroid cancer was demonstrated.

Conclusions. MMP-2 and its tissue inhibitor TIMP-2 apparently play a significant role in the pathogenesis of thyroid cancer. Evaluation of their concentration in peripheral blood serum may be useful for the differentiation between benign and malignant thyroid tumours. Serum MMP-2 and TIMP-2 concentrations in patients with thyroid cancer did not significantly correlate with the clinical staging of thyroid cancer.

Key words: metalloproteinase 2 - tissue inhibitor of metalloproteinase 2 - thyroid cancer - thyroidectomy - clinical staging of cancer

Formation of new blood vessels in the process of angiogenesis is connected with proliferation and migration of endothelial cells. Endothelial cells first invade the surrounding extracellular matrix (ECM). As observed by INGBERG and FOLKMAN (1989) the process

involves numerous interrelated interactions, and many proangiogenic factors. Main mediators of this process include: growth factors, proteins of extracellular matrix, cellular adhesive molecules and numerous proteolytic enzymes. Neoplastic angiogenesis, the process

of formation of blood vessels within solid tumours, is an essential stage of growth, progression and invasion of these neoplasms. This complex process is initiated by damage to the basement membrane of a vessel and remodelling of the extracellular matrix, which enables migration of endothelial cells towards the source of angiogenic signal, i.e. neoplastic cells. The process of basement membrane degradation and ECM remodelling involves proteolytic enzymes called metalloproteinases (MMPs). The first metalloproteinase extracted by GROSS and LAPIERE (1962) was interstitial collagenase. DOCHERTY et al. (1985) identified further seven metalloproteinases and STETLER-STEVENSON et al. (1989) identified two tissue inhibitors of these metalloproteinases (TIMP). Presently, over 20 endopeptidases have been described, showing activity against the majority of macromolecules of the extracellular matrix and their 4 tissue inhibitors. KOHN et al. (1994) found that metalloproteinases are endopeptidases connected with a zinc atom, active in neutral pH and in the presence of calcium ions. Depending on the structure, localisation within the cell and type of substrate, metalloproteinases can be divided into 5 main groups: collagenases, gelatinases, stromelysins, matrylisins and membrane metalloproteinases (MT-MMPs). MMPs are released as an inactive proenzyme (zymogene), in which catalytic zinc ion is bonded to the cysteine rest. The majority of proMMPs are activated by plasmin, formed from inactive plasminogen by urokinase plasminogen activator (uPA). Natural inhibitors of MMPs are tissue inhibitors of metalloproteinases (TIMPs). There are four types of TIMPs (TIMP-1,-2,-3,-4). TIMPs inhibit the activity of MMPs forming non-covalent complexes resistant to proteolysis. Lack of balance between the proteolytic activity of MMPs and the activity of their inhibitors (TIMPs) is one of the elements necessary for the growth and progression of solid tumours as observed by TALBOT et al. (1996). HANEMAAIJER et al. (1993) found that endothelial cells are able to produce some MMPs, including: MMP-1,-2,-3,-9. HOFMANN A et al. (1988) observed that most enzymes of the MT-MMP/MMP class of proteases facilitating invasion of thyroid tumor cells seem to have been produced by fibroblasts, not by the tumor cells themselves. Among these metalloproteinases, the constitutional one showing the greatest expression in endothelial cells is MMP-2, also called gelatinase A. TIMP-2 is its natural tissue inhibitor, at the same time necessary for the activation of MMP-2.

Important role played by MMP-2 and TIMP-2 in the pathogenesis of solid tumours led to attempts of applying the evaluation of their tissue expression and con-

centration in body fluids in early diagnostics of tumours, assessment of tumours advancement and treatment results monitoring.

The aim of this study was to evaluate the concentration of MMP-2 and TIMP-2 in blood serum of patients with benign and malignant thyroid tumours, the effect of surgical treatment on these parameters in the postoperative period and to answer the question whether concentration of the above mentioned factors in serum correlate with the clinical staging of thyroid cancer. We used the clinical staging of thyroid cancer, introduced by the International Union Against Cancer (UICC) in 2002. Apart from such parameters as tumor size (T), regional lymph node status (N) and the presence of distant metastases (M), this classification also takes patient's age into consideration. Criteria, worked out by the International Union Against Cancer, permit a precise assessment of the clinical staging of thyroid cancer.

Patients and Methods

Patients. The study comprised patients operated on at the Clinic of General and Endocrinological Surgery, Institute of Endocrinology, Medical University of Lodz for benign and malignant thyroid tumours. The first group comprised 53 patients (40 F, 13 M), mean age 45.96 ± 11.5 years, in whom preoperative cytological study of fine needle aspiration biopsy specimens led to the diagnosis of thyroid cancer. The material obtained during operation, subjected to routine histopathologic examination, was used for the verification of the diagnosis. The final diagnosis was as follows: 36 cases of papillary thyroid cancer (PTC), 7 follicular thyroid cancers (FTC), 5 medullary thyroid cancers (MTC) and 5 cases of anaplastic thyroid cancer (ATC). Intraoperative evaluation and postoperative histopathology was the basis for division of all studied thyroid cancers according to the clinical staging of thyroid cancer, introduced by the International Union Against Cancer (UICC). According to the clinical staging, we qualified patients with thyroid cancer as stages I (32 patients), II (6 patients), III (8 patients) and IV (7 patients). The second group consisted of 23 patients (18 females, 8 males), mean age 40.19 ± 19.3 years with diagnosed simple nodular goiter (SNN). Cytological evaluation of material from thin needle aspiration biopsy revealed the presence of follicular nodule *neoplasma folliculare* (NF). In postoperative histopathologic examination the diagnosis was verified as benign tumour – follicular adenoma. The control group comprised 26

healthy volunteers (20 females, 6 males), mean age 41.03 ± 13.7 years, in whom clinical examination, evaluation of thyroid hormones concentration (fT_3 ; fT_4), concentration of TSH and ultrasound examination excluded thyroid diseases.

Treatment. The studied patients were operatively treated. In cases of differentiated thyroid cancer complete struma resection was done with modified lymphadenectomy (removal of central cervical lymph nodes, lateral cervical lymph nodes without jugular vein, sternocleidomastoid muscle and nerve XI). The completeness of operative treatment was based on post-operative ultrasound, iodine uptake in cervical scintigraphy after stimulation with endogenous TSH and histopathologic examination. In patients with anaplastic tumours, due to the advancement of the process, the operation was limited to reduction of the tumour mass. In patients with simple nodular goiter a lobectomy and subtotal resection of other lobe was performed.

The concentration of MMP-2 and TIMP-2 was measured in blood serum sampled in aseptic conditions from a peripheral vein on the day preceding the operation, and 4 weeks after the operation. The study was approved of by the Ethical Committee of the Medical University of Lodz. Determinations of MMP-2 were done using commercial kits of ELISA test from Amersham Biosciences (inter-assay CV – 5.6 %; intra-assay CV – 10 %), and TIMP2 from Amersham Pharmacia Biotech (inter-assay CV – 3.9 %; intra-assay CV – 4.8 %). The results were presented as mean values \pm mean standard error (SEM), and statistical analysis was done between the measured parameters using Student's t-test. The values were statistically significant at $p < 0.05$. We examined a correlation between the staging of thyroid cancer and mean values of investigated factor concentrations. Pearson correlation coefficient r was determined and regression line was plotted.

Results

We have found statistically significantly higher concentrations of MMP-2 in blood serum of patients with thyroid cancer as compared with the control group (1346.79 ± 22.11 vs. 1231.73 ± 59.85 ng/ml; $p < 0.05$). Within particular thyroid cancer groups higher mean concentrations of MMP-2 were also seen when compared with the control group. Statistical significance was noted in the papillary thyroid cancer (PTC) group (1363.19 ± 27.58 vs. 1231.73 ± 59.85 ; ng/mL $p < 0.05$). In patients with benign tumours the concentration of MMP-2 was significantly lower than in the control

group (1071.73 ± 41.49 vs. 1231.73 ± 59.85 ng/mL; $p < 0.05$) (Figure 1). Mean concentration of TIMP-2 in blood serum of thyroid cancer patients was higher than in the control group, but the difference was not significant (155.75 ± 24.55 vs. 115.62 ± 6.28 ng/mL; $p > 0.05$). In particular cancer types mean TIMP was higher than in the control group, but this difference also was not significant. In patients with benign thyroid tumours the concentration of TIMP2 was lower than that in the control group, but with no statistical significance (Figure 2).

When compared between thyroid cancer group and that with benign tumours, mean concentration of MMP-2 was significantly higher in thyroid cancer patients (e.g. 1346.79 ± 22.11 vs. 1071.73 ± 41.49 ng/mL; $p < 0.001$). In particular thyroid cancer types mean concentration of MMP-2 was significantly higher than that in the group with SNN(NF): in the PTC group (1363.19 ± 27.58 vs. 1071.73 ± 41.49 ng/mL; $p < 0.001$), follicular thyroid cancer (FTC) group (1281.43 ± 67.57 vs. 1071.73 ± 41.49 ng/mL; $p < 0.05$), ATC group (1320.00 ± 44.02 vs. 1071.73 ± 41.49 ng/mL; $p < 0.05$), medullary thyroid cancer (MTC) group (1347.00 ± 75.87 vs. 1071.73 ± 41.49 ng/mL; $p < 0.05$) (Figure 3). Mean blood serum concentration of TIMP-2 was higher in the thyroid cancer group than that in benign tumours, but the difference was not significant statistically (155.75 ± 24.55 vs. 105.02 ± 18.50 ng/mL; $p > 0.05$). In particular types of cancer mean concentration of TIMP-2 was higher than in the SNN(NF) group, but the differences were also not significant statistically (Figure 4). We compared the blood serum concentration of MMP-2 and TIMP2 before and after the operation. After radical thyroidectomy the level of MMP-2 decreased. Statistically significant decrease of this parameter was noted after thyroidectomy for PTC (1363.19 ± 27.58 vs. 1128.19 ± 45.51 ng/mL; $p < 0.001$); FTC (1281.43 ± 67.57 vs. 1096.43 ± 66.38 ng/mL; $p < 0.005$) (Figure 5). Mean concentration of TIMP2 after thyroidectomy significantly decreased in patients with PTC (169.74 ± 35.97 vs. 94.25 ± 9.53 ng/mL; $p < 0.05$) and FTC (121.66 ± 10.72 vs. 104.74 ± 11.63 ng/mL; $p < 0.05$). In the MTC group, however, it did not change significantly after the operation (Figure 6). After palliative procedure for ATC an increase of both studied parameters in the postoperative period was observed, but only the concentration of MMP-2 was statistically significantly higher after the operation than before (1320.00 ± 44.02 vs. 1609.00 ± 57.69 ng/mL; $p < 0.05$) (Figure 5). After partial struma resection in SNN(NF) a significant increase of MMP-2 was noted in the postoperative period

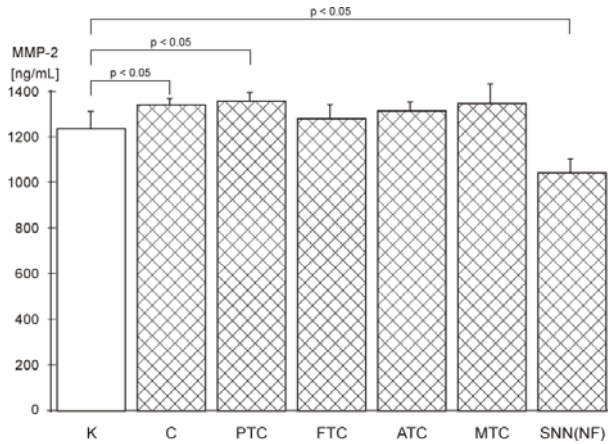


Fig 1 Mean concentration of MMP2 \pm SEM in the group of patients with thyroid cancer (C) and its particular types (PTC – papillary thyroid cancer, FTC – follicular thyroid cancer, ATC – anaplastic thyroid cancer, MTC – medullary thyroid cancer) and in the group of patients with benign thyroid tumours (SNN NF) vs. control group (K)

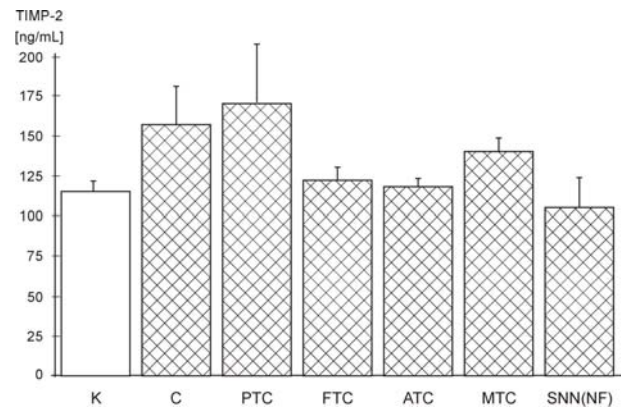


Fig 2 Mean concentration of TIMP2 \pm SEM in the group of patients with thyroid cancer (C) and its particular types (for legend see Fig. 1), and in the group of patients with benign thyroid tumours (SNN NF) vs. control group (K).

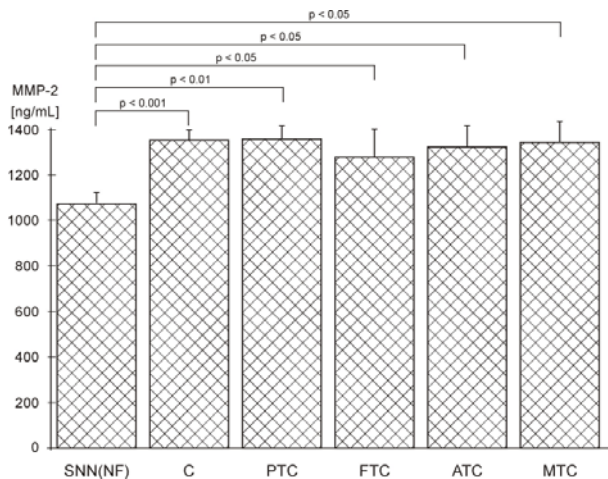


Fig 3 Mean concentration of MMP2 \pm SEM in the group of patients with thyroid cancer (C) and its particular types (for legend see Fig. 1) vs. patients with benign thyroid tumours (SNN NF)

(1071.73 \pm 41.49 vs. 1203.85 \pm 57.21 ng/mL; $p < 0.01$) (Figure 5) and statistically insignificant decrease of the concentration of TIMP-2 (Figure 6).

Table 1 presents mean examined MMP-2 and TIMP-2 concentrations \pm standard error of mean (SEM) of the respective stages of thyroid cancer.

We investigated if correlation existed between mean analyzed neoangiogenesis mediator concentrations and the staging of thyroid cancer. To achieve this objective, we determined Pearson correlation coefficient r and regression line was plotted. However, no significant correlation has been found between the staging of thy-

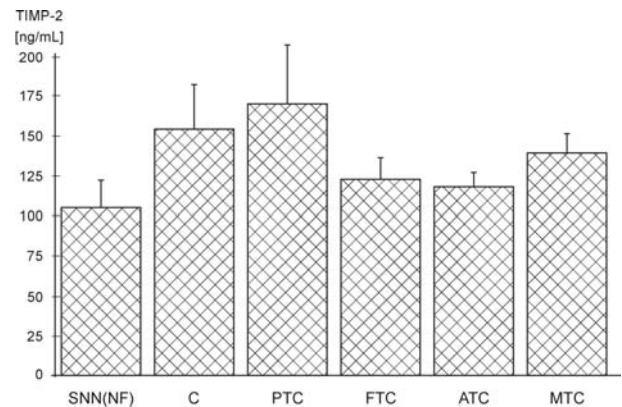


Fig 4 Mean concentration of TIMP2 \pm SEM in the group of patients with thyroid cancer (C) and its particular types (for legend see Fig. 1) vs. patients with benign thyroid tumours (SNN NF)

roid cancer and mean MMP-2 concentration ($y = 12.22x + 1324.7$; $r = 0.0856$; $p > 0.05$) and between the staging of thyroid cancer and mean VEGF concentration ($y = -8.0227x + 170.27$; $r = -0.0506$, $p > 0.05$).

Discussion

For a long time the biological function of MMPs was thought to be limited to their participation in the decomposition and degradation of extracellular matrix components and basal membrane in various physiological and pathological processes. The concept that

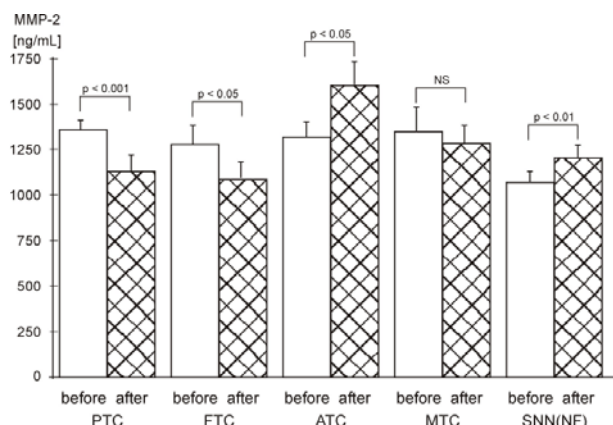


Fig 5 Mean concentration of MMP2± SEM in the group of patients with various types of thyroid cancer (for legend see Fig. 1), and in the group of patients with benign thyroid tumours (SNN NF) before and after surgery. NS – statistically not significant

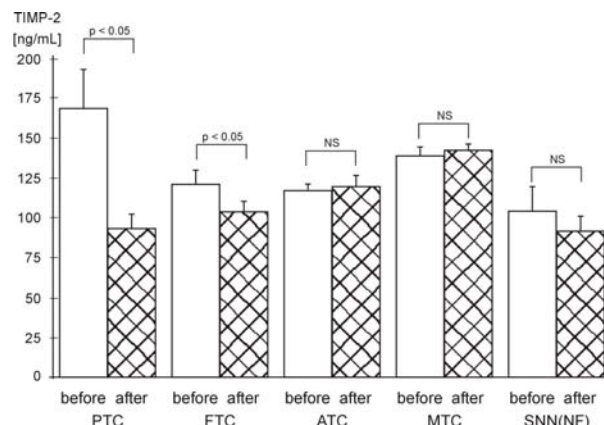


Fig 6 Mean concentration of TIMP2± SEM in the group of patients with various types of thyroid cancer (for legend see Fig. 1) and in the group of patients with benign thyroid tumours (SNN NF) before and after surgery. NS – statistically not significant

Table 1.
Mean MMP-2 and TIMP-2 concentrations ± standard error of mean (SEM) of the respective stages of thyroid cancer

stages of cancer	MMP-2 [ng/ml]	TIMP-2 [ng/ml]
I	1327.7±33,6	148.4±27.7
II	1410.0±45,8	280.1±161.4
III	1380.6±35.5	123.8±9.8
IV	1341.4±33.8	119.3±2.1

MMPs activity in the process of neogenesis is limited only to the invasion of basal membrane and destruction of ECM elements in distant metastases has been modified and nowadays various effects of this group of proteolytic enzymes in tumour progression are stressed. MMPs play a key role in tumour development and growth, in neoangiogenesis, penetration and migration of cancer cells into and out of blood vessels and colonisation of distant tissues. KRAIEM et al. (2000) found that the role of tissue inhibitors of metalloproteinases (TIMP) is not limited to anti-invasive properties and inhibition of MMPs, but they also participate in the activation of MMP and tumour growth. Among metalloproteinases constitutively produced by endothelial cells the highest expression is demonstrated by MMP-2. Therefore, evaluation of its concentration in body fluids and tissue expression of this enzyme and its tissue inhibitor TIMP2 are thought to have a potential in the assessment of advancement of solid tumours. Numerous researchers have used various techniques to confirm the prognostic value of eval-

uation of the increase of tissue expression of MMP-2 and lack of balance between MMP-2 and TIMP-2 in various forms of solid tumours. Several authors (MORI et al. 1997; THERET et al. 1997; TALVENSAARI-MATTILA et al. 1998; GONG et al. 2000; SILLETTI and CHERESH 1999; YAMAMURA et al. 2002; YOUNG and al. 1966) observed a significant correlation between the increase in MMP-2 expression and poor prognosis in stomach cancer, colon cancer, breast, pancreas, prostate, urinary bladder, lung and ovarian cancer. Numerous authors reported increased MMP concentration in such body fluids as: serum, peripheral blood plasma and urine. ZUCKER et al. (1999) reported high concentration of MMP in blood plasma in patients with colonic, breast, prostate and urinary bladder cancer. Several authors (SASAKI et al. 2002a; SASAKI et al. 2002b; KOREM et al. 2002) reported significantly increased concentration of MMP-2 in blood serum of patients with lung cancer, thymoma and adrenal cancer. There are numerous reports on the inhibitory effect of TIMP2 on the growth of solid tumours and distant metastasing. BRAMHALL et al. (1996) confirm close correlation between the reduction of tissue expression of TIMP-2 and increase in the aggressiveness of pancreatic cancer. Several authors (MURASHIGE et al. 1996; REE et al. 1997; FONG et al. 1996) observed positive correlation between an increase of TIMP expression and poor prognosis of colonic cancer, breast and lung cancer. There are few reports on the application of metalloproteinases (and their tissue inhibitors) levels evaluation in the assessment of endocrine glands tumours advancement and monitoring treatment results. The

aim of the study was evaluation of the concentration of MMP-2 and TIMP-2 in blood serum of patients with benign and malignant thyroid tumours and the effect of surgical treatment on these parameters in the post-operative period. We found increased mean concentration of MMP-2 in all types of thyroid cancer, as compared with the control group. However, statistical significance was present only in papillary thyroid cancer. This fact is due to higher number of patients in this group than in other thyroid cancer types.

It is also noteworthy that mean concentration of MMP-2 and TIMP-2 in blood serum of patients with thyroid cancer was higher than in benign thyroid tumours group. However, the differences were statistically significant only for MMP-2. This fact justifies the application of the assessment of MMP-2 and TIMP-2 concentration in differentiation between benign and malignant thyroid tumours. There was no significant correlation between the clinical staging of thyroid cancer and mean MMP-2 and TIMP-2 concentration.

Our results confirm significant role of MMP-2 and TIMP-2 in the process of thyroid cancer development, their usefulness in the diagnostics and differentiation of benign and malignant thyroid tumours. The results are consistent with MAETA et al. (2001) observation on increased tissue expression of MMP-2 and TIMP-2 in thyroid cancer. KOREM et al. (2002) demonstrated the diagnostic value of tissue MMP-2 expression evaluation but lack of its prognostic value. This is consistent with our findings on the usefulness of MMP-2 serum concentration evaluation in differentiation between benign and malignant thyroid tumours. The fact of significant decrease of mean MMP-2 and TIMP-2 concentration after radical surgery confirms reports on their constitutive expression in thyroid cancer cells. Evaluation of MMP-2 blood serum concentration in long post-operative period may become an additional parameter in the monitoring of operative treatment radicality and early detection of recurrences.

Acknowledgements

The study has been financed form the internal grant of the Medical University of Łódź, Poland. Nr 502-11-727.

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HORMONAL CONTROL OF PROTEIN GLYCOSYLATION: ROLE OF STEROIDS AND RELATED LIPOPHILIC LIGANDS

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Abstract. Glycosylation represents one of the most frequent and certainly the most variable co- and post-translational modification of proteins. Carbohydrate moieties of glycoproteins are known to provide important prerequisites for various biological functions, and their structural diversity can serve as ideal candidate to carry also biological information. Production and/or function of various glycoproteins is under control of steroids and other ligands of nuclear receptors which influence synthesis, glycosylation, storage or usage of target proteins. It appears that among small lipophilic hormonal compounds the steroids are chiefly involved in regulation of protein glycosylation. There is no apparent difference between ability of these hormones to regulate *N*- or *O*-glycosylation, but majority of documented cases deals with terminal modifications involving sialylation or fucosylation of *N*-linked carbohydrate moieties. In spite of the knowledge on glycosylation in general, published results offers only a glimpse of data on the hormonal control of glycosidase activity which is equally required for carbohydrate chain elongation as is the activity of various glycosyltransferases. The significance of this research is daily growing owing to the fact that changes in glycosylation pattern of various intracellular or secretory proteins not only reflect developmental or differentiation stage but also serve as well established markers in invasiveness or regression of numerous cancers responding to hormonal stimuli. Combination of classical methods and more complex approach in genetically well defined model systems can not only increase recent surge of interest in glycosylation but also provide a formidable amount of qualitatively new type of data on the mechanism how hormones control glycosyltransferases and glycosidases and how their activity is interconnected to the synthesis of substrates, posttranslational maturation, and final destination or function of target proteins.

Key words: Hormones - Steroids - Thyroid hormones - Retinoids - *N*-glycosylation - *O*-glycosylation - Glycosidases - Glycosyltransferases

Introduction

Understanding the specific molecular interactions that govern correct functions of the cells is of pivotal scientific and clinical relevance. When considering these molecular interactions and their regulation, there is still a strong prejudice towards thinking only in terms of proteins. In recent past, however, it is starting to be appreciated that the carbohydrate moieties of glycoproteins provide important prerequisites for various biological functions. By their potential for structural diversity they can serve as ideal candidates to carry

also biological information. Thus, it is not surprising to unravel programmed and strictly regulated changes in carbohydrate composition and sequence in glycoconjugates within the course of complex processes such as development and differentiation. Consequently, strong changes in presentation of cellular glycoproteins have also been detected upon malignant transformation and tumorigenesis (SCHAUER et al. 1988; SHARON 1991; HAKOMORI 1996). In addition, mevalonate-sensitive *N*-linked glycosylation of proteins is necessary for cell growth, including initiation and propagation of DNA synthesis in normal and tumor-transformed cells

(CARLBERG and LARSSON 1993; WEJDE et al. 1993; CARLBERG et al. 1996; CHAN et al. 2001).

Glycoproteins as prominent and most variable group of glycoconjugates play a prominent part (*e.g.* as mucins) in the secretion. The biosynthesis of the glycan chains was demonstrated to be controlled by various exogenous (such as nutritional) or endogenous (such as developmental) factors. Even though the metabolic regulation by dietary variations seems to be less apparent as they cause mostly quantitative changes, on the other hand, modifications in glycosylation during animal development control the quality of the glycan chains of mucins and other glycoproteins (TAKASAKI et al. 1991; HIRAYAMA and WRIGHT 1992; VAN BEERS et al. 1995; THORENS 1996). Developmental changes including tissue differentiation are accompanied by changes in glycosylation patterns, *e.g.* like a shift from sialylation to fucosylation, depending on coordinate changes in glycosyltransferase activities, in sugar-nucleotide breakdown or synthesis and activity of regulatory proteins (BIOL-N'GARAGBA and LOUISOT 2003). These activities are largely sensitive to developmental stimulations involving hormonal triggers (SOMMER and COWLEY 2001). Small lipophilic hormones including steroids, retinoids, thyroids, vitamin D₃ as well as large variety of ligands for peroxisome proliferator activated receptors (PPARs), liver X receptor (LXR), farnesoid X receptor (FXR) and other nuclear receptors are known to affect protein synthesis at translational and transcriptional levels. However, there is very fragmentary information on the specific hormonal control of glycoprotein production with special emphasis on secretion and whole machinery involved in protein glycosylation preceding process of secretion. With the advent of completing genomes of several eukaryotes including yeasts, *Caenorhabditis*, *Drosophila*, mouse and humans it become possible to undertake comparative studies for conservation or evolutionary divergence of proteins involved in glycosylation and secretion, but the role of particular hormones in developmental and/or metabolic regulation of enzymes participating in protein glycosylation remains fragmentary and obscurely defined. In this paper we try to review our present knowledge on mechanisms how steroid hormones and other related small lipophilic ligands regulate protein glycosylation and thus influence subsequent function of these proteins.

General effects of hormones on secretory process and glycoprotein formation

The earliest studies of SPIRO and SPIRO (1968, 1971) have suggested that thyroid hormone has pronounced

effect on thyroglobulin biosynthesis and glycosylation, as well as on the subcellular distribution of skin, thyroid, kidney and spleen glycosyltransferases in rats. Glycosyltransferase distribution and activity was also affected by pregnancy when hormonal milieu of females have drastically changed. Subcellular distribution of enzyme activities and cell fractionation provides some indication that one of the targets of the hormone action and glycosyltransferase activity might be collagen. Complex approach of SPIRO and SPIRO (1971) has provided also some clues that galactose, glucosamine and glucose moieties are being predominantly utilized under high glycosyltransferase activities in mentioned organs and tissues. VONDERHAAR (1975) concluded that thyroxine and hydrocortisone principally affect differentiation of mouse mammary gland by influencing lactalbumin synthesis and maturation via changes in galactosyltransferase activity. TADOLINI et al. (1976) have found causal relationship between rat testicular and prostate *N*-acetylglucosaminylglycopeptide- β -1,4-galactosyltransferase activity, prostate secretory capabilities and levels of plasma testosterone. Similar conclusions were reached also for human testicular and prostate glycosyltransferase with potential diagnostic significance. Both the formation and size of Golgi apparatus and the cytoplasmic dense bodies in pig female pinealocytes are dependent on continuous presence of ovarian steroid hormones and their secretory activity become strongly diminished upon castration (PRZYBYLSKA et al. 1993). However, readministration of estradiol or combination of estradiol and progesterone rescues secretory features of the cells and also lead to decrease in relative volume of lysosomes whereas failure in supplementing steroids lead to enlarged lysosomes and spontaneous cessation. In this case, continuous presence of the steroid provides for antiapoptotic or survival signal which is sufficient to maintain secretory, probably main, physiological program of the tissue, and thus hormones act at very general and non-specific level.

Among the first serious indications that small lipophilic hormones acting through nuclear receptors may be involved in the control of glycoprotein synthesis and/or modification comes from the observation that thyroxine, hydrocortisone and testosterone influence the activity of the glycosyltransferases responsible for the sequential transfer of xylose and galactose from UDP-xylose and UDP-galactose, respectively, in the formation of first two monosaccharide units of the chondroitin sulfate-protein linkage region during cartilage deposition in young rats (SCHILLER 1976). Another important

observation was that protein galactosyltransferase of the ventral prostate of hypophysectomized rats is markedly enhanced by testosterone and potentially down-regulated by androgens (REDDY et al. 1976). Subsequently it was described that cortisol affects collagen biosynthesis and the activities of collagen galactosyltransferase and collagen glucosyltransferase in chick embryo tendon cells (OIKARINEN 1977). On the other hand, it was observed that testosterone administration causes rat pituitary gland cells to become ovoid and their Golgi complex enlarged, displaying dilated cisternae with increased numbers of immature secretory granules followed by increased levels of pituitary and serum prolactin (HERBERT et al. 1977). The work of INGENBLEEK and DE VISSCHER (1979) on endemic goiter has indicated that thyroid hormone can be involved in the control of secretory activities and protein glycosylation in the liver. Quite interesting was study of IP (1980) who reported that changes in sialyltransferase activity are not only related to proliferation and involution of the rat mammary gland but it was possible to upregulate this enzyme by administration of estradiol and progesterone. In addition, testosterone was found to be crucial for induction and maintenance of large areas of Golgi cisternae and production of secretory granules in mouse epididymal epithelial cells (YAMAOKA et al. 1983). Very similar general effect of testosterone on enlargement of rough endoplasmic reticulum and Golgi areas has been described for rat male seminal vesicles which secrete several tissue-specific glycoproteins. The mRNA for two of these glycoproteins that have been analyzed, was 1000-fold induced during androgen treatment, suggesting relatively complex action of the steroid hormone on secretory process (FAWELL and HIGGINS 1984). Production and secretion of rabbit uteroglobin, the primary secretory protein of uterine epithelium, appeared to be dependent on 17β -estradiol and progesterone (SHROYER et al. 1987). Progesterone appears to regulate uteroglobin synthesis at the transcriptional level and to regulate also mode of its secretion by the induction of a different pathway, compared with the one used when 17β -estradiol is administered alone, showing that two different steroid hormones acting on the same target tissue utilize particular mechanisms to provide full and complex control over its secretory process. On the contrary, TSENG et al. (1987) described synergistic action of corticosterone and thyroxine on formation of secretory granules and mucus production in rat gastric chief cells, in which thyroxine alone failed to induce any positive response and corticosterone alone was insufficient to provide full induction. Several other authors

came to similar conclusion regarding overall control of secretion and secretory material production by hormones and feed-back mechanism under hormonal control including requirements of estradiol and corticosteroids for normal secretory function of exocrine pancreas (DAVIS et al. 1984; TSUKADA et al. 1985; BEAUDOIN et al. 1986; FERREIRA and CELIS 1986; MOOR and CROSBY 1987). Explanted guinea pig endometrial glandular cells grown in primary culture if treated with 17β -estradiol plus progesterone, or with oestrone sulphate alone become stimulated for development of large endoplasmic reticulum and Golgi system, displaying secretory activity (ALKHALAF et al. 1989). Likely, the maturation of the secretory apparatus, including formation of granules of varying electron density, in the uterine glandular epithelium of the ovariectomized sheep continued only under estrogen treatment (MURRAY 1992). Similar situation has been observed in estrogen-primed and progesterone treated sheeps indicating cooperative action of both steroid hormones on the same process.

Nevertheless, study of CAPONY et al. (1987) was one of the first which indicated for very complex role of a steroid hormone in the control of secretory process in mammalian cells. An estrogen-induced 52 kD autocrine mitogen of MCF7 human breast cancer cell line requires proteolysis, glycosylation and phosphorylation for its maturation, secretion and function. These authors provided evidence that at least synthesis, high mannose *N*-glycosylation and proteolytic cleavage of the 52 kD mitogenic protein, which very probably is cathepsin-like lysosomal protease, depend on the estrogen action. They also concluded that mature form of this 52 kD protease facilitates proliferation, migration and metastatic activity of mammary cancer cells, which therefore may be controlled by manipulating estrogen levels. Natural and synthetic glucocorticoids are known to affect collagen metabolism at the level of its production and also posttranslational modifications including hydroxylation of proline and lysine residues followed by glycosylation of hydroxylysine residues on procollagen. Particularly, hydroxylation of lysine and glycosylation on hydroxylysine are depressed by glucocorticoids which is caused via downregulation of the activity of specific enzymes involved in these posttranslational modifications (KUCCHARZ 1988). These effects of glucocorticoids has important pathophysiological aspect due to the role of collagen metabolism in hepatic and pulmonary fibrosis as well as in keloids.

Hormonal regulation of the secretion or of the activity of secretory tissue may take place at another and rather

unusual level. Widespread secretory components, chromogranin B and secretogranin II, which are normally present in almost every type of mammalian secretory vesicle are considered to be also responsible for aggregation of regular secretory proteins in trans-Golgi network (TGN). Several hormones, including corticosteroids, increase formation of pituitary secretory granules via upregulation of granins synthesis rather than affecting synthesis of own secretory proteins (CHANAT and HUTTNER 1991). The granins preferentially exclude glycosaminoglycan chains containing polypeptides from TGN towards constitutive secretory bulk and concentrate regular secretory proteins into vesicles of regulated pathway; hormonally upregulated pool of granins then increases the rate of aggregation of regular secretory proteins and formation of vesicles. Mineralocorticoids are thought to decrease intracellular pH and mobilize TGN pools of calcium, two other factors facilitating aggregation of granins and formation of secretory vesicles.

Good and rather interesting example of steroid hormone effects on glycoprotein formation and secretion is in glucocorticoid regulation of the transport and processing of mouse mammary tumor virus (MMTV) glycoproteins in viral-infected HTC rat hepatoma cells. GOODMAN and FIRESTONE (1993) have documented that administration of synthetic glucocorticoid, dexamethasone, resulted in a 5-fold increase in the steady state level of the intracellular and cell surface MMTV glycoproteins. Regardless of the fact that terminal ends of MMTV DNA contain well defined glucocorticoid responsive elements, under these conditions, dexamethasone did not alter the level of MMTV glycoprotein transcripts or translatability of the messages as incorporation of ^{35}S -labelled methionine has not been increased. Data these authors provided strongly suggest that glucocorticoids are able increase the stability of MMTV glycoproteins by a posttranslational mechanism and that this effect is occurring relatively early within the exocytotic pathway.

Great chapter of the hormone involvement in glycoprotein production deals with regulation of mRNA synthesis at transcriptional level. For example, Muc-1, a major mucin glycoprotein expressed on the surface of mammary epithelial cells, has attracted considerable attention as it is expressed in an aberrant form on many breast tumor cells. Under continuous supply of insulin mouse Muc-1 mRNA levels are increased by both hydrocortisone and prolactin or their combination leading thus to increased rate of Muc-1 protein maturation and its secretion (PARRY et al. 1992). Additionally, Muc-1 displays developmentally-linked variations in its gly-

cosylation as it become increasingly sialylated during the course of pregnancy and lactation indicating role of estrogens in this specific step just preceding secretion. Subsequently, level of Muc-1 glycosylation affect its intracellular localization being tightly polarized to the apical surface of the epithelium of lactating and pregnant mice whereas showing non-polarized distribution in ductal cells of virgin mice. Similar transcriptional mechanism for the control of secretory protein production has been observed also in amphibians. In *Xenopus laevis* liver, two out of four major estrogen-regulated (inducible) proteins are secreted vitellogenin and serpin Ep45 (HOLLAND et al. 1992). The mRNA levels of both of them are almost undetectable in absence of estrogen, and become more than 100-fold increased upon its administration, and encoded proteins secreted shortly thereafter. An opposite effect the steroids can be seen on corticosteroid-binding globulin (CBG), an acidic glycoprotein produced primarily by the liver which is the major glucocorticoid transport protein in mammalian blood. While estrone, prednisolone or corticosterone produced no significant response of this protein and its mRNA, both protein and RNA were significantly reduced (14-fold) after administration of dexamethasone or estradiol (SMITH and HAMMOND 1992). More complex level of the protein glycosylation has been found in the regulation of 2,6-sialyltransferase mRNA by thyroid hormone in the rat thyroid cell line FRTL-5. GROLLMAN et al. (1993) have demonstrated that both the cell surface membrane and the thyroglobulin secreted by cells grown in the presence of the hormone exhibit a marked decrease in the level of α -2,6-bound sialic acid with little or no change in the number of α -2,3-sialic acid residues. Steady-state levels of the thyroid hormone tend to decrease 2,6-sialyltransferase mRNA. An additional, and unexpected, sequel is the finding of a coordinated decrease in all of the core monosaccharide constituents of the secreted thyroglobulin. This shows invariable and tight feed-back mechanism for the control of thyroglobulin secretion under particular hormonal milieu. Merely different but to mammals may be a common way of thyroglobulin synthesis and secretion operates in porcine thyroid cells. Steroid hormone hydrocortisone alone did not modify total RNA or thyroglobulin mRNA content but the hormone amplified total RNA and thyroglobulin mRNA when insulin and pituitary thyroid stimulating hormone (TSH) were present together. The basal level of thyroglobulin secreted into the apical medium was increased threefold by insulin and fourfold by TSH. However, when the three hormones were added together, the hor-

monal response was amplified even more. These hormones were able also to increase both incorporation of ^3H -mannose into thyroglobulin and the content of the anionic residues in the thyroglobulin molecule. Thus, the effects of the hydrocortisone and two hormones supplemented together appeared to be additive (DESRUISSEAU et al. 1994). In accordance with above mentioned data of GROLLMAN et al. (1993) in the rat, HELTON and MAGNER (1994a, 1995) have found that α -2,6-sialyltransferase mRNA increases in thyrotrophs of hypothyroid mice, as a consequence of thyroid hormone withdrawal indicating that this glycosyltransferase is under negative thyroid control at transcriptional level, and may partially explain the increased sialylation of TSH during hypothyroidism. Thyroid hormone, in cooperation with TSH, pro-opiomelanocortin (POMC) and lutropin (LH), another pituitary hormones, can control glycosylation process also in nonendocrine target tissue. Their action on single product, carbonic anhydrase VI, in parotid and submaxillary glands results in distinct glycoforms of the enzyme (HOOPER et al. 1995). These authors have shown that while the glycoprotein:GalNAc-transferase and the GalNAc-4-sulfotransferase are coordinately expressed in bovine submaxillary gland, the GalNAc-transferase is expressed in the parotid gland in the absence of the GalNAc-4-sulfotransferase. The relative expression of these two transferases in submaxillary and parotid glands correlates with the presence of unique Asn-linked oligosaccharides on carbonic anhydrase VI synthesized in each of these tissues, and reflects tissue-specific response to the hormones. The majority of Asn-linked oligosaccharides on carbonic anhydrase VI synthesized in submaxillary gland terminate with GalNAc-4-SO₄. In contrast, carbonic anhydrase VI which is synthesized in bovine parotid gland bears oligosaccharides which terminate predominantly with β -1,4-linked GalNAc which is not sulfated. These results document that hormones can mediate tissue-specific differential expression of glycosyltransferases which has the potential to generate functionally distinct glycoforms of otherwise identical proteins.

One of the most abundant protein products of human secretory endometrium is glycodeilin, which is believed to affect immunomodulatory activity during human embryonic implantation and inhibition of sperm-egg binding after ovulation. Glycodeilin protein production *in vivo* by endometrial epithelial cells is directly up-regulated 4- to 9-fold by progesterone. Transcriptional regulation of the glycodeilin gene promoter expressed in HeLa cells is progesterone

receptor-dependent. As it was observed in the primary endometrial cells, progestins and antiprogestins both act as agonists on the *in vitro* expression of the glycodeilin gene at the mRNA level (TAYLOR et al. 1998).

In spite of the wealth of data which provide evidence that steroid hormones and sibling lipophilic hormonal ligands control production of glycoproteins and in several cases also their secretion, among vertebrate models there is a general lack of information on exact molecular mechanism by which these effects are implemented. Nevertheless, it should be stressed that very important notion about complex control of glycoprotein production and secretion by steroid hormone comes from an invertebrate model, *Drosophila melanogaster*, and its initial insights are dated as early as mid 1970s. Larval salivary glands of *Drosophila melanogaster* are unique in their production of small group of secretory glycoproteins, referred to as SGS (salivary glue secretion), which are synthesized only once a life time during short period between mid and late 3rd instar. After the finding that insect steroid hormone ecdysone induces in salivary gland nuclei a set of specific chromosomal puffs representing transcriptionally active genetic loci (CLEVER and KARLSON 1960; ASHBURNER 1971, 1972a,b), it was soon found that specific portion of puffing active during interecdysial period, *i.e.* when endogenous levels of ecdysone are constantly low, is tightly related to the synthesis of SGS proteins which are subsequently utilized in the production of electron-dense granules (for review see BERENDES and ASHBURNER 1978). At the time of metamorphosis upon the burst of high ecdysone levels the granules are secreted into the lumen and the secretion is expectorated from the gland to be used as a glue to affix the newly formed puparium to the substrate (FRAENKEL and BROOKS 1953; BOYD and ASHBURNER 1977). The huge amount of SGS made an isolation of discharged secretion from the salivary gland lumen easy and facilitated their initial electrophoretic characterization which revealed presence of 6 to 7 proteins (KORGE 1975; BECKENDORF and KAFATOS 1976). The variable size of these proteins as well as incorporation of radioactive glucose (KRESS 1979; ENGHOFER and KRESS 1980) indicated that some of them, if not all, are glycosylated. Activity of a small group of chromosomal puffs at the time of SGS synthesis gave an opportunity to link some SGS proteins to corresponding genetic loci (KORGE 1977a,b; BERENDES and ASHBURNER 1978; VELISSARIOU and ASHBURNER 1980, 1981). These studies were shortly followed by cloning of 68C chromosomal cluster encoding *Sgs-3*, *Sgs-7* and *Sgs-8* genes (MEYEROWITZ and HOGNESS 1982; MEYEROWITZ et

al. 1985, 1987; CROWLEY et al. 1983, 1984; CROWLEY and MEYEROWITZ 1984; CROSBY and MEYEROWITZ 1986), of 3C locus responsible for transcription of *Sgs-4* (MUSKAVITCH and HOGNESS 1980, 1982; CHEN et al. 1987), cloning of 95B locus harboring *Sgs-5* gene (GUILD 1984; GUILD and SHORE 1984), and cloning of *Sgs-1* from 25B puff (ROTH et al. 1999).

Furthermore, regulation of expression of *Sgs-3* and *Sgs-4* has been extensively studied as synthesis of SGS proteins was considered to be a "textbook" example of tissue-specific gene expression. These studies revealed valuable information on redundant tissue-specific enhancers upstream of 5' transcription start site of both *Sgs-3* (GIANGRANDE et al. 1987, 1989) and *Sgs-4* (KRUMM et al. 1985; HOFMANN et al. 1987; JONGENS et al. 1988). Besides specific binding sites, these two promoters are commonly bound by several transcription factors including ecdysone receptor (*EcR/USP* complex), broad-complex factors (*BR-C*) and fork head (*fkh*) (LEHMANN 1996; LEHMANN et al. 1997). In addition, the *Sgs-4* promoter was found to bind also secretion enhancer binding proteins SEBP2 and SEBP3 factors the binding sites of which are in the vicinity or overlap with those for *EcR/USP* and *BR-C* within so-called ecdysone response unit (LEHMANN and KORGE 1995, 1996). Taken together, levels of the regulation of *Sgs* system in *Drosophila* reveal tremendous complex of functions related to steroid signalling which encompass positive as well as negative control of *Sgs* transcription, possibly control of SGS protein glycosylation and granule formation, and definite control of exocytosis in which ecdysone plays the role of ultimate trigger.

Small lipophilic hormones can control both *N*- and *O*-glycosylation

An important insight in specific hormonal control of glycosylation has been brought about by paper of PHILIPP and SHAPIRO (1981) who demonstrated that estrogen regulates hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGcR) in *Xenopus* frogs. In addition to its main function being a major rate-limiting enzyme in the cholesterol biosynthesis, the HMGcR is also a key regulator of all other isoprenoids synthesis including dolichol phosphate, a hydrophobic lipid carrier, required for *N*-glycosylation of proteins by attaching core oligosaccharide to asparagine residues on target polypeptide skeleton. Besides general effects of estrogen on enlargement of endoplasmic reticulum and the Golgi apparatus, it controls also synthesis of the egg yolk precursor, vitellogenin, which is highly *N*-

glycosylated. Another and very interesting level of the control of protein *N*-glycosylation by steroid hormone testosterone has been presented by IUSEM et al. (1984) studying secretory glycoproteins of rat epididymis which are known to interact with spermatozoa in order to facilitate their maturation. In this case dolichol phosphate pathway was not affected, excluding HMGcR from the androgen action, but rather glucosyl and mannosyl transferase activities in rat epididymal microsomes were drastically decreased upon castration, and restored by testosterone readministration. The restoration of glucosyl and mannosyl transferase activities was blocked by simultaneous application of cyproterone, indicating involvement of androgen receptor. An analogical case was soon observed in rabbit mammary gland where cortisol along with insulin and prolactin control mannosyl transferase activity associated with transferrin secretion (BRADSHAW et al. 1985). Thyroid hormones were shown to be involved in the control of *N*-glycosylation of thyroid gland proteins by regulating utilization of UDP-glucosyl-, -galactosyl and -mannosyl precursors for synthesis of dolichol-P-P-sugar moieties (SPIRO and SPIRO 1985) which may represent tissue-specific mechanism depending also on feed back action of the hormone. Human chorionic gonadotropin of normal pregnant women contains four Asn-linked sugar chains and four mucin-type sugar chains. The structures of asparagine-linked sugar chains of this hormone are constant and site-specific. Chorionic gonadotropins obtained from the patients with invasive mole or choriocarcinoma, which show significantly lowered progesterone and estrogen levels, have quite different sets of oligosaccharides although the primary structures of the polypeptides and the numbers of the sugar chains are the same as those of normal gonadotropin. The extent of sialylation of oligosaccharides in the this gonadotropin reflects its hormonal activity where complete desialylation induced remarkable loss of full activity (AMANO et al. 1990). Results of these authors indicated not only that the structures of the neutral oligosaccharide portion are important for full hormonal activity of gonadotropins, but also that level of this *N*-linked glycosylation can be under progesterone and/or estrogen control. As discussed in more detail below, one aspect of hormonal modulation of Asn-linked glycoprotein biosynthesis is to regulate the synthesis of glucosidase I, endoplasmic reticulum enzyme involved in glucose trimming from *N*-linked glucosyl moieties, by coordinate action of hydrocortisone, insulin and prolactin on rat mammary gland, where they all have lactogenic effect (SHAILUBHAI et al. 1990a,b). In

addition, thyroid hormone has been shown to control degree of sialylation of Asn-linked carbohydrate chains in the circulating thyrotropin (PERSANI et al. 1998). These modifications have a major impact on TSH biological properties. In particular, highly sialylated TSH is characterized by impaired intrinsic bioactivity and prolonged half-life, whereas lower degree of TSH sialylation is associated with higher biological activity and shorter half-life. The impaired thyroid hormone action induces an expression of α -2,6-sialyltransferase activity and results in the secretion of high amounts of sialylated TSH, while normally acting thyroid hormone downregulates α -2,6-sialyltransferase expression.

In addition to steroid and thyroid hormones, also vitamin A derivatives, retinoids, can influence *N*-glycosylation of proteins. *All-trans* retinoic acid (atRA) applied to mouse P19 teratocarcinoma cells caused a dose-dependent and protein synthesis-dependent induction of UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase (GPT) enzyme activity, and an increased transcription rate of the *GPT* gene. GPT is the first enzyme in the dolichol pathway of protein *N*-glycosylation, and is implicated in the developmental programs of a variety of eukaryotes (MEISSNER et al. 1999). The atRA was found to be capable affecting protein *N*-glycosylation also by altering ratio of the utilization between mannose and glucosamine in Vero cells and thus causing extremely potent antiviral effect against herpes simplex virus infection (ISAACS et al. 2000). Decreasing levels of viral envelope protein *N*-glycosylation was sufficient to inhibit virus propagation 10,000-fold.

On the other hand, BOLANDER and TOPPER (1981) presented evidence about complex hormonal control of mouse mammary lactalbumin, lactose synthetase and galactosyltransferase by insulin, prolactin, cortisol and triiodothyronine. Both, cortisol and triiodothyronine are required for maintaining production and secretion of matured *O*-glycosylated lactalbumin. Role of steroid hormones in the control of specific protein *O*-glycosylation was further substantiated by DEIS and DELOUIS (1983) who observed significant downregulation of rat lactalbumin galactosyl transferase upon progesterone administration, while no apparent effect was obtained by estrogen. The ontogenic profile of sialyltransferase activity in mouse brains and its positive response to triiodothyronine in myelogenic embryonic brain cells indicated that it is at least under partial thyroid hormone control which may be shared with some other humoral factors required for brain differentiation (SHANKER and PIERINGER 1983). Mouse uterus en-

dometrium responds to high estrogen levels or during pregnancy by increased activity of α -(1-2)-fucosyltransferase (FUT1) and upregulation of FUT1 mRNA upregulation, and this is accompanied by concomitant upregulation of polypeptide-Gal β -(1-3)-*N*-acetyl-galactosyltransferase (SIDHU and KIMBER 1999). Although, targets of these *O*-glycosylation enzymes remain unknown so far, there is a good reason to believe that those may be proteins co-regulated by estrogen at the same time. Control of *O*-glycosylation machinery by estrogens may have also another, feed-back regulatory function. Significant population of mouse estrogen receptors- α (ER α) is modified by *O*-GlcNAc moiety at C-terminal Thr575 and Ser576 as well as N-terminal Ser10 and Thr50 residues, modulating thus ER α turnover (JIANG and HART 1997; CHENG and HART 2000). In amphibian testis, androgens seem to support presentation of GalNAc and Neu5Ac(a2,6)Gal/GalNAc *O*-glycosylated moieties on the interstitial tissue, and suppress synthesis of *N*-glycosylation machinery, while do not affect significantly ratio of *N*- and *O*-linked oligosaccharides in the surrounding lobules with spermatozoa bundles (SAEZ et al. 2001).

Control of specific glycosyltransferases

Protein glycosylation is a multistep modification process which, in general, requires several sequential reactions, some of which consist of adding and some of trimming glycomoiety. Addition of mono- or oligosaccharide residues to amino acids is performed by glycosyltransferases, whereas trimming off glycomoiety is performed by glycosidases. One of the earliest reports on hormonal control of glycosyltransferases comes from the work of LEDINKO and FAZELY (1989) who found that retinoic acid and retinol acetate downregulate sialyltransferase activity in human lung carcinoma cells, A549, suppressing their invasiveness by reducing cell surface contents of sialylated glycoproteins. BIOL et al. (1992) reported interesting case of dual control of fucosyltransferase activity in suckling rats. While the enzyme did not respond to thyroxine or insulin, its expression was strongly induced by glucocorticoids as a function of the duration of treatment. This responsiveness of suckling rats to hydrocortisone or cortisone disappeared at the time of weaning. The effects of glucocorticoids were prevented by RU-38486 antagonist, showing that hormonal responsiveness is mediated by glucocorticoid receptor. However, RU-38486 did not prevent the developmental rise of the fucosyltransferase activity when administered in the

time of weaning, suggesting that the normal developmental rise of the fucosyltransferase activity is independent of glucocorticoids and must be under control of other factors which are capable of preventing glucocorticoid inducibility. As already mentioned under general effects above, GROLLMAN et al. (1993) have demonstrated that steady-state levels of the thyroid hormone tend to decrease β -galactoside- α -2,6-sialyltransferase mRNA in rat thyroid cell line FRTL-5 secreting thyroglobulin, and thus affecting degree of its glycosylation. Furthermore, the work of THOTAKURA et al. (1994) indicates that regulated levels of 2,6-sialyltransferase mRNA and protein have strong impact on thyrotropin (TSH) sialylation and subsequently on TSH biological activity. It seems that continuous 2,6-sialyltransferase activity is required for maintaining majority of TSH molecules to be sialylated and thus prevented from sequential deglycosylation, mainly removal of galactose or *N*-acetylglucosamine, the process which transform stored form of TSH into biologically highly active hormone with short half-life. Under reinitiation of 2,6-sialyltransferase expression, the deglycosylated unreleased TSH can be resialylated. From the point of view of glycosylation process it is necessary to note that sialic acid most probably, even in the case of TSH, represents terminal glycosidic residues and thus sialylation is the last glycosylation step. Sialic acid is added upon galactosyl or glucosyl residues which must be attached first due to activity of galactosyltransferase or glucosyltransferase. HELTON and MAGNER (1994b) used experimentally-induced hypothyroid mice as a model to study TSH glycosylation further, and thus found that low or undetectable levels of thyroid hormone lead to rapid 5-fold increase in pituitary β -1,4-galactosyltransferase mRNA expression and higher rate of galactosyl residues in secreted TSH molecules. Interestingly, HELTON and MAGNER (1994a, 1995) also found that this physiological conditions subsequently resulted in more highly sialylated form of TSH than in their euthyroid counterparts, also due to 1.5-fold increased β -galactoside- α -2,6-sialyltransferase and almost 3-fold increased β -galactoside- α -2,3-sialyltransferase mRNA expression. Thus, final oligosaccharide modification of TSH is achieved by coordinate, albeit not the same upregulation in expression of three key glycosylating enzymes. Both discussed enzymes, β -galactoside- α -2,3-sialyltransferase and β -galactoside- α -2,6-sialyltransferase, were found to be upregulated not by thyroid hormones but by corticosteroid in several other target tissues like rat jejunum (KOLINSKA et al. 1996) and brain, liver and kidney (COUGHLAN et al. 1996). Each enzyme respond-

ed to dexamethasone differentially and also various portions of the brain including cortex, cerebellum, and brainstem have shown variable response. Unfortunately, we lack data on target proteins of these two sialyltransferases, and therefore, we cannot clearly relate their upregulation to specific function. Such an objection is reflected in the fact that activity of neither 2,3-sialyltransferase nor 2,6-sialyltransferase must necessarily be associated with concomitant presentation of sialyl residues in intracellular or extracellular proteins as is the case of rat and human hepatocytes, neurons and gastrointestinal epithelia in which despite relatively high enzyme activities authors failed to detect 2,3- or 2,6-linked sialoglycoconjugates (KANEKO et al. 1995). In contrast to so far described stimulating effects of steroids on sialyltransferase activity and expression, HAMR et al. (1997) have studied developmentally-linked loss of sialic acid presentation on the brush-border membrane glycoproteins of the rat small intestine during the transition from suckling to weaning. This loss of sialic residues is one of the major biochemical changes which occur during mentioned postembryonic phase of rat development, and this process is speeded up by an injection of exogenous glucocorticoids. As authors found, loss of detectable sialic acid residues on the brush-border membrane is due to hydrocortisone-driven downregulation of β -galactoside- α -2,6-sialyltransferase activity and mRNA expression, and mediated by glucocorticoid receptor because hydrocortisone effects are preventable by mifepristone, a glucocorticoid receptor antagonist. Further studies of BIOL-N'GARAGBA et al. (2003) on this model revealed that hydrocortisone also acts on increasing activity of *O*-glycan:galacto-sialyltransferase and of an α -1,2-fucosyltransferase, through transcriptional regulation of the *FTB* gene, thus substantiating developmental shift from sialylation towards fucosylation. Moreover, ANIC and MESARIC (1998) obtained evidence that sex steroid hormones can regulate activity of both sialyltransferases in the rat kidney. Castration of male rats resulted in significant increase of kidney 2,3-sialyltransferase and 2,6-sialyltransferase activities, but this effect was reversed by subsequent administration of testosterone. While testosterone was able to downregulate both enzymes, estradiol and progesterone decreased only activity of 2,6-sialyltransferase but not 2,3-sialyltransferase. In the hormone-dependent MCF-7 human breast cancer cell line estradiol induces a statistically significant increase in expression of CMP-Neu5Ac:Gal β 1-3(4)GlcNAc- α -2,3-sialyltransferase and a decrease in CMP-Neu5Ac:Gal β 1-4GlcNAc- α -2,6-sialyltransferase,

whereas the three other enzymes, CMP-Neu5Ac:Gal β 1-4GlcNAc- α -2,3-sialyltransferase, CMP-Neu5Ac:Gal β 1-3GalNAc- α -2,3-sialyltransferase, and CMP-Neu5Ac:Gal β 1-3GalNAc- α -2,3-sialyltransferase are not modified in their expression (PEYRAT et al. 2000). Estradiol effects were dose dependent and completely antagonized by tamoxifen, and did not show any direct relation between sialyltransferase expression and proliferation of breast cancer cells, although in some cancer types was established apparent connection between proliferation-related invasiveness and deregulated sialyltransferase activity. Potentially more widespread but so far only little studied system of complex control of protein glycosylation can be found in estrogen-induced phasic remodelling of synapses in the adult rat female arcuate nucleus. HOYK et al. (2001) by studying synaptic plasticity came to the conclusion that estradiol reduces GABAergic axo-somatic synapses by decreasing polysialylation of neural adhesion molecule (NCAM) via reduced sialyltransferase activity/expression as well as NCAM expression. In addition, active desialylation of existing NCAM molecules could play an important role in this synaptic plasticity process.

Hormonal control of glycosidases

Regular part of glycosylation modification is also programmed removal of mono- or oligosaccharide moieties in the process of building the specific glycomoiety on the protein. Trimming step is performed by variety of glycosidases the action of which is required many times before some glycosyltransferases can add new sugars, and thus it is anticipated that synthesis of complex glycomoieties on hormonally-controlled proteins may involve also hormonal regulation of glycosidase enzymes. For example, glucosidase I, a key trimming enzyme in *N*-glycosylation, was found to be under positive control of hydrocortisone and two peptides, insulin and prolactin, in the mammary gland where they all act as lactogenic hormones (SHAILUBHAI et al. 1990a,b), although it has not been established whether this control takes place at transcriptional, translational or other level, or whether upregulation of enzyme activity is achieved by another mechanism. On the other side, there is good reason to expect that hormonal control of glucosidase I reflects coordinate action of mentioned three hormones as they also stimulate synthesis of α -lactalbumin, a major mammary protein product displaying high degree of glycosylation. Nevertheless, in one of the earliest papers on TSH glycosylation RONIN et al. (1984) suggested that thyroid hormone regu-

lation of this process can involve not only galactosyltransfering activity, but also mannosidase activity, and ratio between these activities determine degree of TSH glycosylation and hormone half-life. In above chapter mentioned work of THOTAKURA et al. (1994) on hormonal control of 2,6-sialyltransferase activity related to TSH glycosylation shows that simultaneous glycosidase activity is necessary to ensure proper sialylation of galactose residues. Similar case, when co-deglycosylation is required for programmed glycosyl transfer, was described by HELTON and MAGNER (1994b) for thyroid hormone regulated production of TSH in hypothyroid mice. Along with β -1,4-galactosyltransferase mRNA expression, drop in thyroid hormone levels upregulates also increase in pituitary α -mannosidase-II mRNA, though not to the same extend and with different kinetics which might reflect temporal-dependent needs for degree or type of TSH glycosylation. Cell-specific and developmentally-dependent distribution of Golgi mannosidases I and II in various rat tissues strongly indicates that glycosidases might be under steroid hormone control, including glucocorticoids and sex steroids (DONG et al. 2000). And indeed, testosterone was recently found to inhibit activity of β -glucuronidase and β -*N*-acetylglucosaminidase in catfish seminal vesicles in concentration-dependent manner (CHOWDHURY and JOY 2001). Very special case of the hormonal control of monoglycosylation process has been analyzed by SLAWSON et al. (2002) by studying the activity of *N*-acetylglucosaminidase (GlcNAcase), the streptozotocin-inhibitable neutral hexosaminidase, involved in removing GlcNAc monoglycosyls from proteins during *Xenopus* oocyte maturation. Progesterone is capable of stimulating oocyte maturation by gradual downregulation of GlcNAcase activity in early stages of oocytes which are characterised by higher levels of GlcNAc monoglycosyls than older and mature stages.

Conclusions

Altogether above discussed results clearly show that steroid hormones and some other members of small lipophilic ligand family are involved in the control of protein glycosylation which in most cases is related to the process of secretion. Based on the number of reports in the literature it seems that there was no specific selection pressure during evolution to favor or eliminate hormonal control of *N*-glycosylation versus *O*-glycosylation, and both types of modifications are more-less equally occurring among hormonally-controlled modifications. However, there is very little evidence that there

was any co-evolution in the systems utilizing both types of glycosylation at the same time and during the same response. This is somewhat surprising if one consider the fact that numerous proteins which are glycosylated and the process of particular glycomodification is under hormonal control, contains multiple and different glycosylation sites. This view is acceptable if hormonally-controlled step in glycosylation machinery may function as rate-limiting or check-point, and therefore its regulation is sufficient to maintain control over the whole process. However, this might be true only for very simple systems in which ligand produces the same response every time and single glycosylated product is used *e.g.* for secretion. Such a simple system if exemplified by a number of tissues or cell types in which hormone controls either sialylation or fucosylation, the last glycosylation step, via upregulation or downregulation of sialyltransferase or fucosyltransferase activity/expression. On the other hand, in more complex systems in which hormone fluctuations can provoke series of different responses reflected in vast panoply of glycosylations of very different proteins with diverse functions, we can hypothesize or even expect that one ligand will regulate given process in various steps and at multiple levels. In spite of this logical assumption the most of the information we have today, in fact, will appear fragmentary. For example, there is striking contrast between the high number of systems in which steroid hormones control glycosyltransferase activity, and low number of systems where steroid hormones control glycosi-

dases, although both types of enzymes are known to be equally required for glycosylation. Nevertheless looking at this problem from opposite point of view, these fragmentary data originating from various systems when combined can suggest that effects of small lipophilic hormones on protein glycosylation are rather complicated. Addressing this problem by means of more complex methodological approach and in well-defined model systems potentially with known genomes, can complement present and extend our knowledge in future concerning coordinate action of hormones on processes associated with or tightly linked to protein glycosylation. It is our firm belief that by adhering to such a complex methodology which in addition can utilize set of mutations or transgenes, we can obtain substantial information on the mechanism how hormonal control of glycosyltransferases and glycosidases is interconnected to the synthesis of substrates, posttranslational maturation, and final destination/function of target proteins which may not necessarily be granule formation and secretion.

Acknowledgements

This work was supported, in part, by the Research Grant 2/999533 from GAV and by Grants No. 2/7194/20 and 2/3025/23 from VEGA Slovakia, and US-Slovak collaborative grant 029/2001 from APVT to R.F. International collaborative grants from the NATO (CRG-972173 and LST.CLG-977559) are also acknowledged.

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ENDOCRINE AND METABOLIC ACTIVITIES OF A RECENTLY ISOLATED PEPTIDE HORMONE GHRELIN, AN ENDOGENOUS LIGAND OF THE GROWTH HORMONE SECRETAGOGUE RECEPTOR

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Ghrelin is a member of the group of growth hormone secretagogues (GHSs). It is a peptide hormone, recently isolated from stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R). Ghrelin is mostly produced by the stomach, although its production has been proved in various tissues. It is a potent releaser of growth hormone (GH) from anterior pituitary cells, but it also stimulates the release of other hypophyseal hormones. Ghrelin stimulates food intake and induces metabolic changes leading to an increase in body weight and body fat mass. This effect seems to be independent of GH action and needs an intact NPY/AGRP (neuropeptide Y/agouti-related protein) system. Plasma ghrelin levels are decreased in obesity, elevated in cachexia and show a diurnal rhythm. Its preprandial elevation suggests, that it might be a signal for meal initiation. Ghrelin further stimulates the release of gastric acid and gastric motility and affects pancreatic functions. It has vasodilatory, cardioprotective and antiproliferative effects. This article is focused on ghrelin's endocrine and metabolic functions.

Key words: Ghrelin - Des-acyl ghrelin - Growth hormone GHRH Metabolism - Nutrition

The history of GHSs dates back to 1977, when Bowers developed a group of small peptides which stimulated the release of GH from anterior pituitary cells in vitro (BOWERS et al. 1977). Further studies led to the development of several more potent peptides, with a strong GH stimulatory effect in vitro and in vivo (BOWERS et al. 1984). However in 1982 growth hormone-releasing hormone (GHRH) was characterised. It was postulated, that GH secretion by the anterior pituitary gland was regulated by an interaction of two hypothalamic hormones – GHRH and somatostatin. This approach however could not explain the effects of GHSs, whose mechanism of action on GH release is different. The effects of GHRH and GHSs on GH release are complementary, their simultaneous administration is the most potent GH releaser to date. This hypothesis was supported by the identification of different receptors and intracellular signalling (ARVAT et al. 2001; BALDEL-LI et al. 2001).

Isolation of the growth hormone secretagogue receptor. Later a GHS-R DNA was isolated (HOWARD et al. 1996). GHS-R is a classical G-protein coupled receptor. Two types of GHS-R DNAs (cDNAs) have so far been identified and designated receptor 1a and 1b. Unlike the GHS-R 1a, the GHS-R 1b fails to bind GHSs and its function remains to be elucidated (MCKEE et al. 1997).

There is strong evidence suggesting, that there are several additional subtypes of the GHS-R, which modulate a variety of endocrine and non-endocrine activities of GHSs. Binding sites, different from the GHS-R 1a and 1b have been found in a wide range of tissues (PAPOTTI et al. 2000; GHIGO et al. 2000). The GHS-R 1a is widely present in various tissues. It was found in the anterior pituitary gland, hypothalamus, stomach, intestine, pancreas, myocardium, aorta, adrenal, lung, liver, skeletal muscle, kidney, thyroid, adipose tissue, lymphatic gland, parathyroid, placenta, mammary gland,

prostatic gland, and spleen (DATE et al. 2000; NAGAYA et al. 2001; PAPOTTI et al. 2000; SHUTO et al. 2001).

Endogenous ligand. Despite intensive research, the isolation of an endogenous ligand of the GHS-R remained elusive until recently. In 1999 Kojima isolated the endogenous ligand, which was later named ghrelin, from a rat stomach (KOJIMA et al. 1999). Ghrelin is predominantly produced by the stomach, with substantially lower amounts derived from the bowel, pancreas, kidney, placenta, pituitary and hypothalamus (DATE et al. 2000a; KORBONITS et al. 2001; KRŠEK et al. 2002; VOLANTE et al. 2002; WIERUP et al. 2002). In stomach it is present in endocrine cells, which account for about 20% of the endocrine cell population in oxyntic glands. Ghrelin cells have no continuity with the lumen, but they are closely associated with the capillary network running through the lamina propria. Ghrelin cells seem not to operate under gastrin control (DORNONVILLE DE LA COUR et al. 2001). Removal of the acid-producing part of the stomach in rats reduces serum ghrelin concentration by cca 80%, which supports the view, that the main source of ghrelin is the stomach (DATE et al. 2000a).

Structure of ghrelin. Ghrelin is a peptide containing 28 amino acids, in which the serine 3 residue is n-octanoylated. This posttranslational peptide modification had not been previously observed and it is of a critical importance for its binding to GHS-R 1a, its GH-releasing and other endocrine activities, as well as for its transport over the blood-brain barrier. Human and rat ghrelin differ in 2 amino acids, in positions 11 and 12 (BEDNAREK et al. 2000; KOJIMA et al. 1999; MATSUMOTO et al. 2001; MUCCIOLI et al. 2001).

Molecular variants of ghrelin. Later studies led to the isolations of another natural endogenous ligands of the GHS-R. One of them is des-Gln¹⁴-ghrelin, a 27-amino acid peptide, where there is glutamin missing in the 14th position. Des-Gln¹⁴-ghrelin possesses the same biologic activities as ghrelin (HOSODA et al. 2000a).

Des-acyl ghrelin, which lacks a hydrofobic chain substitution on position 3, is another molecular form of ghrelin. The ratio of these two forms in a rat's stomach is 2:1 in favour of the desacylated form, which also predominates in systemic circulation in rats (HOSODA et al. 2000b) and humans (YOSHIMOTO et al. 2002). This form of ghrelin lacks ghrelin's endocrine activities, however it is able to exert some non-endocrine actions (BALDANZI et al. 2001).

Secretion of ghrelin. Ghrelin plasma level decreases after food intake and increases during food depriva-

tion in rats and humans (ASAKAWA et al. 2001; TOSHINAI et al. 2001; TSCHÖP et al. 2001). In fasting rats an increase in ghrelin mRNA together with a decrease in its content in the stomach and an increase in plasma ghrelin was observed. These changes were abolished after realimentation (TOSHINAI et al. 2001). In humans plasma ghrelin level is negatively correlated with BMI and percentage body fat content. Plasma ghrelin is decreased in obese patients and increases after weight reduction (TSCHÖP et al. 2001), its level is elevated in patients with anorexia nervosa and decreases after weight normalization (BECKER et al. 1999, OTTO et al. 2001).

Intravenous or peroral administration of glucose leads to the suppression of plasma ghrelin in rats as well as in humans (ARIYASU et al. 2002, NAKAGAWA et al. 2002; SHIYA et al. 2002). In hyperinsulinemic conditions, either causing hypoglycemia, or in euglycaemic clamp, plasma ghrelin concentrations are suppressed (LUCIDI et al. 2002; McCOWEN et al. 2002; SAAD et al. 2002). Based on these observations, insulin might directly or indirectly mediate the effect of nutrition or energy state on plasma ghrelin. The decline in plasma insulin with fasting would lead to an increase in plasma ghrelin, while post-prandial insulin release would suppress plasma ghrelin concentration. Similarly the hyperinsulinemia of obesity would suppress plasma ghrelin, while the lower insulin level in lean subjects would lead to increased ghrelin concentrations. Some studies report a reduction in insulin secretion in humans after ghrelin administration (BROGLIO et al. 2001). It could be a hormonal response to fasting, which maintains glucose level during fasting by inhibition of insulin secretion.

In contrast, another observation was made in rodents. The administration of insulin led to the stimulation of ghrelin secretion in the stomach (TOSHINAI et al. 2001). Whether interspecific or other mechanisms are involved needs to be further clarified.

Acromegalic patients show lower ghrelin levels than healthy controls, which are unaffected by oral glucose tolerance test. Low ghrelin levels might be caused by the putative negative feedback mechanism of GH and IGF-I, or the ghrelin levels might be suppressed by hyperinsulinemia, as the lowest ghrelin levels were found in patients with the most severe insulin resistance (CAPPIELO et al. 2002).

Subjects with growth hormone deficiency (GHD) have lower ghrelin levels in comparison with healthy control subjects, which are not modified after 1 year of

GH replacement therapy, as observed by JANSSEN et al. (2001). This study included patients with idiopathic GHD, patients after hypophysectomy or radiotherapy. The explanation of this remains to be cleared. GHD patients had higher BMI and percentage body fat than the control group, which decrease after one year of GH therapy. So the supposed decrease in ghrelin plasma level due to a negative feedback of GH therapy, could be blunted by the increase in ghrelin level due to a reduction of adiposity. Also the effect of other pituitary hormone deficiency or chronic pituitary hormone replacement could play a role.

An infusion of somatostatin and its analogues causes a significant decrease in plasma ghrelin concentrations (BROGLIO et al. 2002b; NORRELLUND et al. 2002). Glucose and lipid-heparin infusion, which trigger hypothalamic somatostatin release, reduces, but does not eliminate the GH response to ghrelin (BROGLIO et al. 2002), whereas GH response to GHRH is almost completely abolished by somatostatin (BROGLIO et al. 2002; DI VITO et al. 2002).

The infusion of GHRH in rats results in a significant increase in pituitary gene expression of ghrelin and its receptor system, suggesting that this system in the pituitary gland could modulate the regulation of growth hormone secretion by GHRH (KAMEGAI et al. 2001; YOSHIHARA et al. 2002).

Endocrine activities of ghrelin. Ghrelin is a dose-dependent stimulator of GH secretion in rats, *in vivo* and *in vitro*. Its stimulatory effect *in vivo* is much stronger than that of GHRH (KOJIMA et al. 1999; SEOANE et al. 2000). Concentrations of prolactin, ACTH, LH, TSH and leptin are not affected by its peripheral administration in rodents, however its intracerebroventricular (ICV) administration leads to stimulation of secretion of ACTH, and inhibition of secretion of TSH, with no change of LH or prolactin levels (DATE et al. 2000b; WREN et al. 2000). After intravenous administration in rats, ghrelin blocks the stimulated secretion of somatostatin, whereas its basal secretion remained unaffected (TOLLE et al. 2001).

In humans, an intravenous administration of ghrelin causes a dose-dependent increase in serum GH levels. In a comparison to GHRH, ghrelin is a stronger stimulator of GH release (ARVAT et al. 2000; GHIGO et al. 2001; PEINO et al. 2000; TAKAYA et al. 2000). Ghrelin administration leads to an increase in prolactin, ACTH and cortisol levels (ARVAT et al. 2001b). Serum levels of FSH, LH, TSH remain unaffected (TAKAYA et al. 2000). A stimulatory effect on aldosterone secretion in

humans was observed (ARVAT et al. 2001a). Also in patients with isolated GHD ghrelin causes a significant increase in GH level. Its stimulatory effect is stronger than that of GHRH + arginine or insulin induced hypoglycemia, but GH level is lower than in healthy controls. Ghrelin in these patients increases levels of ACTH, cortisol and PRL, which indicates, that this endocrine activity is fully independent of mechanisms underlying the GH-releasing effect (AIMARETTI et al. 2002).

Although the presence of ghrelin-producing cells in hypothalamus has been reported, their number is small. It is questionable, if GH release is controlled by the ghrelin from the hypothalamus or from the stomach, which is the main source of circulating ghrelin. A blood-to-brain and brain-to-blood transport of acylated ghrelin has been observed, whereas non-acylated ghrelin entered the brain by diffusion (BANKS et al. 2002). After a repeated administration of ghrelin in rats, the GH level did not substantially increase, this occurred only after a break of 3-4 hours. Spontaneous episodes of GH secretion were not observed during the 3-hour interval (TOLLE et al. 2001). Also no change in GH mRNA expression in anterior pituitary was observed. As GHRH stimulates GH synthesis and secretion at the same time and GHS-R is expressed in hypothalamic cells secreting GHRH, this is another supporting fact, that ghrelin needs an intact GHRH system for its stimulatory effect. After administration of GHRH antiserum in rats, or hypothalamo-pituitary disconnection, the GH response to GHSs is strongly inhibited (TANNENBAUM et al. 2001). Studies in persons with an inactivating defect of the GHRH receptor gene suggest, that in humans the amplitude of GH pulses is driven by GHRH and the timing of GH pulses is primarily supervised by oscillations in somatostatin and/or ghrelin concentrations (ROELFSEMA et al. 2001).

Central metabolic effects of ghrelin. Ghrelin after administration in rats leads to an increase in food intake together with a dose-dependent weight gain due to a significant increase in fat tissue with no change in lean body mass, the amount of bone tissue or stimulation of growth (NAKAZATO et al. 2001; WREN et al. 2000). Respiratory quotient increases, which means an increase in sacharide metabolism and a decrease in fat utilisation. The lipogenetic effect of ghrelin seems to be independent of GH action, as GH increases energy expenditure and causes a decrease in body fat mass with no change in respiratory quotient. This effect was confirmed in

GH-deficient mice, in which ghrelin also leads to weight gain (Tschöp et al. 2000).

Expression of GHS-R was found in neurons of nucleus arcuatus secreting NPY and AGRP. NPY is one of the most effective stimulators of food intake and weight gain. During ghrelin administration, the expression of markers of neuronal activation (Fos and Egr-1 protein) as well as the expression of NPY mRNA and NPY secretion increased in these neurons (NAKAZATO et al. 2001; SHINTANI et al. 2001). During administration of antibodies against NPY or antagonists for NPY-receptor Y1 and Y5, ghrelin-induced hyperphagia was inhibited. During ICV administration of antibodies against ghrelin no inhibitory effect on NPY induced hyperphagia was observed (NAKAZATO et al. 2001). Neurons producing NPY and AGRP colocalize in nucleus arcuatus, and AGRP antagonists abolish ghrelin induced feeding. This data suggest, that ghrelin uses the NPY/AGRP system for its effects (HORVATH et al. 2001).

Ghrelin blocks leptin-induced feeding reduction and similarly leptin suppresses ghrelin-induced feeding. These results indicate, that ghrelin might antagonize leptin action in the regulation of the NPY system (NAKAZATO et al. 2001).

Ghrelin also inhibits serotonin release from rat hypothalamic neuronal terminals in vitro, the same as orexin A and orexin B. This could also account for the feeding stimulatory effect of this peptide (BRUNETTI et al. 2002).

Eating disorders. Plasma ghrelin levels are negatively correlated with body mass index (BMI), body fat mass, adipocyte size, plasma insulin levels, plasma glucose levels and plasma leptin levels (Tschöp et al. 2001). Circulating ghrelin levels in normal-weight subjects show a diurnal profile similar to GH diurnal rhythm (SHIYA et al. 2002). Plasma ghrelin concentrations rise progressively for one to two hours before each meal and fall to through levels within one to two hours after beginning of eating. In healthy volunteers administered ghrelin caused hunger sensations, so its increase could have a role in meal initiation. Between-meal ghrelin

values rise gradually throughout the day in a diurnal pattern, with a nadir between 9 a.m. and 10 a.m. and a peak between midnight and 2 a.m. (CUMMINGS et al. 2002).

In obese individuals ghrelin levels are decreased, with no change after food intake (ENGLISH et al. 2002). This is a reversible condition, after weight loss the mean plasma ghrelin levels increase. There is a positive correlation between the percentage decrease in body weight and BMI and the percentage increase in the area under the curve of ghrelin secretion (CUMMINGS et al. 2002). Obese patients also show marked impairment in spontaneous secretion of GH as well as in the somatotroph responsiveness to all provocative stimuli. GH insufficiency in obese patients has been reported reversible after long-term diet and marked weight loss. It is likely that alterations in the influence of ghrelin together with the alteration of the NPY/leptin interplay could have a role. Among metabolic alterations, the chronic elevation of free fatty acid levels and hyperinsulinism probably have a key role in causing GH insufficiency in obesity, associated with low ghrelin levels (MACCARIO et al. 2002).

In patients with anorexia nervosa plasma ghrelin levels are high (BECKER et al. 1999). After therapeutic intervention causing an increase in body weight, a significant decrease in circulating ghrelin levels was observed (OTTO et al. 2001). In bulimia nervosa, mean plasma ghrelin levels are significantly higher than those in the control group, which indicates a possible role of habitual binge eating and purging behavior on circulating ghrelin levels and a possible role of ghrelin inducing hyperphagia through the appetite control system in these patients (TANAKA et al. 2002).

The orexigenic effect of ghrelin might also play a crucial role in hyperphagia seen in Prader-Willi syndrome (PWS), which is characterized by excessive appetite and progressive massive obesity. Plasma ghrelin is high in PWS and a positive correlation was found between plasma ghrelin and subjective ratings of hunger in these subjects (DELPARIGI et al. 2002).

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