Does L-thyroxine treatment downregulate EGF receptor expression in endemic non-toxic nodular goitre?

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Abstract

Introduction: TSH receptor (TSH-R) is mainly expressed in thyroid follicular cells (TFC), regulating thyroid growth. Epidermal growth factor receptor (EGF-R) is often found upregulated in many human tumours. CD44 has been found to be implicated in thyroid tumour formation, carcinogenesis included, and has been selected in our study as a "negative" control for TSH-R and EGF-R, because no evidence exists for its regulatory effects on thyroid growth processes during L-thyroxine treatment.

Material and methods: Fine-needle aspiration biopsies of the thyroid gland were performed in 18 adult patients with endemic non-toxic nodular goitre (NTNG) before and during L-thyroxine treatment (either 50 or 100 µg/daily, depending on goitre volume and patient age) after 6 and/or 12 months, and TSH-R, EGF-R and CD44 expressions were assessed immunocytochemically in TFC.

Results: Before L-thyroxine treatment – positive immunoreactivity for EGF-R [EGF-R (+)], TSH-R [TSH-R (+)] and CD44 [CD44 (+)] was observed in 61, 72 and 82% of NTNG cases, respectively. During L-thyroxine therapy – out of 5 original EGF-R (+) and out of 4 original immunonegative [EGF-R (–)] cases, all were found EGF-R (–); out of the original 7 TSH-R (+) cases, 86% became TSH-R (–), but 2 TSH-R (–) cases became TSH-R (+); out of 7 CD44 (+) cases, 71% remained CD44 (+).

Conclusions: EGF-R expression is completely downregulated in the course of L-thyroxine treatment of endemic nodular goitre, the mechanism which could be considered as being involved in goitre involution.

Key words: EGF receptor, TSH receptor, CD44, thyroid enlargement, goitre treatment.

Introduction

Until the late 1990s, Poland belonged to mild or moderate iodine deficient areas, with rather high prevalence of nodular goitre [1]. In 1997, an obligatory model of iodine prophylaxis was introduced in Poland [2] and, already in 1999, some positive effects were noted, relying on complete elimination of endemic goitre in schoolchildren [2]. However, such prophylaxis could not significantly influence nodular goitre in adults, the goitres having been formed during

a longer time before. During the iodine deficiency period and shortly after the introduction of obligatory iodine prophylaxis, nodular goitre frequently used to be treated with L-thyroxine, with goitre involution observed in most cases [3].

Beside thyroid-stimulating hormone (TSH, thyrotropin), being the main growth factor for the thyroid [4], epidermal growth factor (EGF) has recently gained great significance as a growth factor implicated in thyroid tumorigenesis [5, 6].

The effects of TSH are mediated by a TSH receptor (TSH-R), which belongs to the glycoprotein hormone receptor subfamily of seven-trans-membrane spanning receptors. The TSH receptor is expressed mainly in thyroid follicular cells (TFC) [7] and regulates TFC growth and function [8]. TSH-R, upon TSH binding, couples to G-α(s) protein (Gs), which results in adenylate cyclase activation and in increased cyclic adenosine 3',5' monophosphate (cAMP); *via* the cAMP cascade, thyroid proliferation is activated [9]. Additionally, TSH-R is one of the primary antigens in autoimmune thyroid disease, being the target of antigen-specific T cells and antibodies [10].

The receptor for EGF (EGF-R) is a glycoprotein and a member of receptors with intrinsic tyrosine kinase activity (RPTK) and is widely expressed on many cell types [11]. It should be stressed that EGF-R was frequently found to be upregulated in a wide variety of human tumours [12]. The upregulated EGF-R signalling was correlated with progression to invasion and metastasis [12]. Westermark et al. showed that EGF-R staining was weaker in normal thyroid tissue, compared to the adjacent neoplastic areas [13].

In contrast to TSH and EGF, CD44 is not a growth factor, but, like TSH-R and EGF-R, it is a surface receptor, located, among others, on TFC [14, 15]. CD44 adhesion molecule has been found to be implicated in thyroid tumour formation, carcinogenesis included [16, 17]. CD44 has been selected in our study as a "negative" control for TSH-R and EGF-R, because no evidence exists for its regulatory effects on thyroid growth processes during L-thyroxine treatment.

The goal of our study was to assess immunocytochemically EGF-R, TSH-R and CD44 expression rates in TFC, obtained by fine-needle aspiration biopsy (FNAB) from 18 adult patients with endemic non-toxic nodular goitre (NTNG), before and during L-thyroxine treatment.

It should be stressed that our study is, to our knowledge, the first one assessing the effects of L-thyroxine administration on the aforementioned receptor expressions in TFC of NTNG.

Material and methods

Fine-needle aspiration biopsies of the thyroid gland were performed in 18 patients (mean age: 41.7±8.9; female to male ratio: 2:1) with NTNG before and during L-thyroxine treatment (either 50 or 100 µg/daily, depending on goitre volume and patient age) after 6 and/or 12 months. Written informed consent from all patients was obtained. The duration of the NTNG is unknown due to the fact that the patients were neither diagnosed nor treated with L-thyroxine before the first biopsy. The euthyroid status, before and during L-thyroxine treatment, was confirmed by TSH and FT_4 serum concentrations, remaining within normal ranges (TSH: 0.27-4.20 µIU/ml; FT4: 0.93-1.70 ng/dl). Next, the microscopic specimens, after drying, were submitted to an immunocytochemical analysis, using a DAKO LSAB® Plus Kit/AP system (DakoCytomation, USA). The kit is intended for qualitative demonstration of antigens in cell preparations, paraffin-embedded tissues and cryostat tissues. The LSAB® Plus Kit/AP utilizes a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with alkaline phosphatase-conjugated streptavidin molecules. We used the following components of the DAKO LSAB®2 Rat Kit/AP (DakoCytomation, USA): 1) biotinylated immunoglobulins [in phosphate buffered saline (PBS)], which bind to primary antibodies; and 2) streptavidin, conjugated to alkaline phosphatase in PBS. Next, the Fast Red Substrate System (DakoCytomation, USA), using Fast Red as a chromogenic substrate for AP, was employed. The Fast Red Substrate System is especially useful on cell smears. Monoclonal primary murine antibodies (Novocastra, UK) against EGF-R (clone EGFR.113), TSH-R (clone 3B12), CD44 (clone VFF-11) and PanCK (clone 5D3) were employed. For all the studied proteins, only clear positive staining was classified as a positive reaction. To ensure confidence in the interpretation of experimental results, one control set was always included in each staining procedure: cells stained without primary antibodies.

Pancytokeratin (PanCK) is a marker for tissue of epithelial origin. Cytokeratin AE1/AE3 has been referred to as "pancytokeratin" because of its broad reactivity. Cytokeratin AE1/AE3 is a mixture of two different clones of anti-cytokeratin monoclonal antibodies, AE1 and AE3. PanCK expression, when observed in fine-needle aspiration biopsy samples, confirms the presence of TFC in the samples.

Receptor expression was recognized either as immunopositive (+) or as immunonegative (–).

Statistical analysis

The frequency of events was statistically analyzed using the Ratio Comparison Test. Statistical significance was determined at the level of $P<0.05$.

Results

In all the cytological samples, obtained before and during L-thyroxine treatment, the cytological picture contained several elements to be regarded adequate and representative for nodular goitre. Those elements included: follicular epithelial cells (dispersed and, in various aggregates, mostly monomorphic, but, in single cells, moderate anisocytosis was also observed), macrophages with or without hemosiderin (deriving from the foci of degeneration and haemorrhage), and colloid (a characteristic component of samples from colloid nodules and cystic lesions).

Positive immunoreactivity for TSH receptor [TSH-R (+)] was observed before L-thyroxine treatment in 72% (13/18) and negative reaction for TSH-R [TSH-R (–)] was observed before treatment in 28% (5/18) of NTNG cases (Table I); the difference was statistically significant (P<0.01).

In the course of L-thyroxine therapy, out of 7 analysed TSH-R (+) cases, 86% (6/7) became TSH-R immunonegative [TSH-R (–)], while the remaining 14% (1/7) were further TSH-R immunopositive [TSH-R (+)] (Table I) (the difference was statistically significant; P<0.01). Two (2) cases from the original TSH-R (–) NTNG were analyzed and both were found to be positive for TSH-R protein in the course of L-thyroxine treatment (Table I).

Positive immunoreactivity for EGF-R [EGF-R (+)] was observed before L-thyroxine treatment in 61% (11/18), and a negative reaction was observed for EGF-R [EGF-R (–)], before the treatment, in 39% (7/18) of NTNG cases (Table II).

During L-thyroxine treatment, 5 cases from the original EGF-R (+) NTNG were analyzed and 100% (5/5) were found to become immunonegative for EGF-R protein (Table II). Also, in the course of L-thyroxine administration, 4 cases from the original EGF-R (–) NTNG were analyzed and 100% (4/4) remained immunonegative for EGF-R protein (Table II).

Positive immunoreactivity for CD44 [CD44 (+)] was observed, before treatment, in 82% (14/17) of NTNG cases [in one case the result was questionable (+/-)], and negative reaction for CD44 [CD44 (–)] was observed, before the therapy, in the remaining 18% (3/17) of NTNG cases (the difference was statistically significant, P<0.001) (Table III).

During L-thyroxine treatment, 7 cases from the original CD44 (+) NTNG were analyzed; 71% (5/7) and 29% (2/7) were found to be immunopositive and immunonegative for CD44 protein, respectively (Table III).

Discussion

In our study, positive immunoreactivity for TSH receptor [TSH-R (+)] was observed before L-thyroxine treatment much more frequently than negative immunoreactivity for this receptor. It is worth recalling once again that TSH is one of the most important proliferative and functional stimuli for TFC and TSH receptors are present on normal TFC. Positive staining for TSH-R was observed along the basal cell surface of TFC in normal human thyroid tissues by Mizukami et al. [18]. It is also known that TSH-R expression is

Table I. TSH receptor (TSH-R) expression in thyroid follicular cells (TFC) before and during L-thyroxine (LT4) treatment

Before LT_4	TSH-R (+)			TSH-R (–)		P
	13			5		<0.01
During LT_4	TSH-R (+)	TSH-R (–)	P	TSH-R (+)	TSH-R (–)	P
	1	6	<0.01	2	0	Not available

Table II. EGF receptor (EGF-R) expression in thyroid follicular cells (TFC) before and during L-thyroxine (LT4) treatment

Before LT_4	EGF-R (+)			EGF-R (–)		P
	11			7		0.0978
During LT_4	EGF-R (+)	EGF-R (–)	P	EGF-R (+)	EGF-R (–)	P
	0	5	Not available	0	4	Not available

EGF-R – epidermal growth factor receptor

Table III. CD44 expression in thyroid follicular cells (TFC), before and during L-thyroxine (LT4) treatment

Before LT_4	CD44 (+)			CD44 (–)		P
	14			3		<0.001
During LT_4	CD44 (+)	CD44 (–)	P	CD44 (+)	CD44 (–)	P
	5	2	0.071	2	0	Not available

markedly decreased in pathological TFC (cancer cells included); for example the TSH-R mRNA levels were significantly lower in carcinomas, in comparison to benign thyroid tumours [19]. Moreover, decreased TSH receptor expression is associated with thyroid cell de-differentiation and worse disease prognosis [19]. Thus, the results, as shown above, are not in any way astonishing.

On the other hand, the negative immunocytochemical reaction for TSH-R [TSH-R (–)], which was observed before treatment in almost 30% of NTNG cases, is consistent with the general conviction that TSH is not the only stimulating factor for TFC [4].

It turned out that, in the course of L-thyroxine therapy, 86% of TSH-R (+) cases became TSH-R (–), while the remaining 14% (1 case) continued to be TSH-R (+). Such a considerable disappearance of TSH-R expression may confirm the known beneficial effects of L-thyroxine treatment in cases of NTNG, relying on thyroid growth control. In brief, downregulation of TSH-R expression, which was observed during L-thyroxine treatment in the present study, may have contributed to the gradual decrease of goitre volume. On the other hand, two cases from the original TSH-R (–) NTNG were analyzed and both were found to be positive for TSH-R protein in the course of L-thyroxine treatment. Obviously, such a result seems rather unexpected and difficult to explain. Presumably, in such cases of original TSH-R (–) NTNG, which became TSH-R (+) in the course of L-thyroxine treatment, a kind of insensibility to such therapy should be considered.

Summing up, it does not seem that TSH-R plays any leading role in the control of TFC proliferation in NTNG during L-thyroxine treatment.

Epidermal growth factor (EGF) is one of the most important growth factors for TFC and may have a relevant role in the regulation of normal and neoplastic thyroid cell growth [4-6, 20]. Positive immunoreactivity for EGF-R [EGF-R (+)] was observed in our study before L-thyroxine treatment in 61% of NTNG cases. Obviously, the presence of EGF-R is expected in the majority of examined NTNG cases, while remembering the stimulating role of EGF in TFC proliferation. In earlier studies, the authors demonstrated an increased EGF-R level in tissues of papillary and anaplastic thyroid carcinomas, as compared to adjacent normal tissue [21]. In another study, it was observed that EGF-R levels were increased not only in malignant thyroid tumours but also in well-differentiated benign thyroid nodules [22]. Moreover, Westermark et al. have shown that EGF-R location varies in different thyroid tissues [13]. Namely, in normal thyroid tissue (and in toxic diffuse goitre), the EGF-R expression was observed mainly on the basal or basolateral surface of TFC [13]. In turn, in nodular goitres, the EGF-R staining was basal, lateral and apical and, in neoplastic tissues, pericellular and, sometimes, cytoplasmic EGF-R staining was observed [13]. Later on, Marti et al. showed, for the first time, nuclear localization of EGF-R and EGF in the thyroid, especially in follicular adenomas and carcinomas [23].

A negative immunocytochemical reaction was observed for EGF-R [EGF-R (–)], before the treatment, in almost 40% of NTNG cases; it is consistent with the knowledge confirming the presence of other, apart from EGF, growth factors for TFC.

During L-thyroxine treatment, 100% of the original EGF-R (+) cases were found to become immunonegative for EGF-R protein. Also, in the course of L-thyroxine administration, all cases of the original EGF-R (–) remained immunonegative for EGF-R protein. Thus, it should be stressed that no EGF-R were found in NTNG in the course of L-thyroxine treatment. Those fairly unambiguous results suggest some beneficial effect of L-thyroxine treatment in non-toxic nodular goitre – at least to some extent – *via* downregulating EGF-R expression.

On the basis of the present study, it could be concluded that downregulation of EGF-R, due to L-thyroxine treatment, plays a more important role in goitre involution than downregulation of TSH-R. Although decreased secretion of pituitary TSH is accepted to constitute the most important mechanism in favourable effects of pharmacological treatment of NTNG, downregulation of EGF-R should be considered as another significant cellular mechanism.

As pointed out above, CD44 is a cell-surface glycoprotein, involved in thyroid tumour formation, carcinogenesis included [16, 17]. We selected CD44 in our study as a "negative" control for TSH-R and EGF-R because no evidence exists for its regulatory effects on thyroid growth processes during L-thyroxine treatment. In our study, positive immunoreactivity for CD44 [CD44 (+)] was observed, before treatment, in over 80% of NTNG cases – more frequently than the negative reaction. The considerable CD44 (+) expression may confirm the role of the protein in TFC proliferation (tumorigenesis included). During L-thyroxine treatment, only CD44 (+) NTNG cases were analyzed, from which over 70% still remained immunopositive. Thus, as was expected, CD44 is not greatly involved in goitre involution during L-thyroxine treatment.

In conclusion, downregulation of EGF-R expression may be involved in favourable effects of L-thyroxine administration in patients with endemic nodular goitre.

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