The thyrotropin receptor in thyroid carcinoma

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The Thyrotropin Receptor In Thyroid Carcinoma

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Ignoranti quem portum petat nullus suus ventus est
If one does not know to which port one is sailing, no wind is favorable.
Lucius Annaeus Seneca (4BC - AD65)

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Chapter 1

General Introduction

INTRODUCTION

Human thyroid tumors originate from epithelial follicular cells or from parafollicular C-cells. Follicular cell-derived tumors range from benign adenomas to differentiated (follicular and papillary) and undifferentiated (anaplastic) carcinomas. Differentiated thyroid carcinoma (DTC) has an overall favorable prognosis, with a 10-year survival of 90-95% (1). However, subgroups of patients are at risk for recurrent disease or death (2). The prognosis is much lower, when distant metastases occur. Distant metastases, usually in the lungs and bones, occur in 10 to 15 % of patients with DTC. With the exception of surgery in solitary metastases, therapy with radioiodine (RaI) is the only curative therapeutic option. The response of metastases to RaI however, is moderate, due to diminished, or lost, ability to accumulate effective dosages of RaI. Alternative conventional treatment options (external radiotherapy or chemotherapy) have limited success (3). The numerous advances that have been made in recent years in the development of anti-cancer drugs have not lead to breakthroughs in the treatment of metastases of DTC. This may be explained by the fact that not many studies with these compounds have yet been conducted in DTC patients. Another explanation is that DTC has unique features that distinguish this endocrine tumor from other non-endocrine tumors. As a consequence, the search for new treatment options in DTC requires the appreciation of the specific features of DTC. In this thesis, we describe the role of one of the unique features of thyroid tissue, the receptor for thyroid stimulating hormone (TSHR), in the treatment and follow-up of DTC. We propose that the TSHR ultimately may be an attractive target for novel therapies for metastatic DTC.
In this introductory chapter a general overview of DTC and the TSHR will be provided and the questions addressed in this thesis will be introduced.

CHARACTERIZATION OF THYROID CARCINOMA

DTC has a low incidence, varying from 2-10/100.000 (4-7) with a female to male preponderance of 2:1. In general, 80% of newly diagnosed thyroid carcinomas are differentiated tumors with a median age at diagnosis of 45 to 50 years (2). DTC has a relatively favourable prognosis with a 10-yr survival of 90-95%. This high survival rate is the result of the biological behavior of most of these tumors and the efficacy of primary therapy, consisting of surgery and RaI therapy. However, when distant metastases occur, the prognosis is worse because the results of RaI therapy, which is virtually the only curative treatment option, are moderate. Depending on the localization and size these metastases may affect quality of life for years.
The tumor-node-metastases (TNM) classification system is based primarily on pathologic findings and separates patients into four stages, with progressively poorer survival with increasing stage (8). Recently, the 6th edition of the TNM system has become available (9). The most important difference with he 5th edition is the fact hat the dimension of T1 has been extended to 1.5 cm and that tumors with limited extrathyroidal extension is designated T3 instead of T4, which has implications for the prognosis of DTC (10). Therefore,

some experts propagate to continue the use of the 5th edition. In the studies in his thesis the 5th edition of he TNM staging system is used (11).

PATHOGENESIS

Human thyroid tumors originate from epithelial follicular cells or from parafollicular C-cells. Follicular cell-derived tumors represent a wide spectrum of lesions, ranging from benign adenomas to differentiated (follicular and papillary) and undifferentiated (anaplastic) carcinomas, thus providing a good model for finding a correlation between specific genetic lesions and histological phenotype.
Recent developments have provided a detailed map of the role of the genetic alterations involved in the pathogenesis of thyroid neoplasms and DTC. The dissection of these genetic alterations has important implications not only for the diagnosis, but also for the understanding of the molecular (patho)physiology of thyroid disorders (12-14). Follicular adenomas and carcinomas frequently have mutations in one of the three RAS genes (figure 1). For instance, mutations of the GSP and thyroid-stimulating hormone (TSH) receptor genes are associated with benign hyperfunctioning thyroid nodules and adenomas. The understanding of the molecular pathogenesis of papillary carcinoma (PTC) has improved considerably by the recent identification of mutations in B-RAF, which are present in 40-60% of the carcinomas. B-RAF is a component of the RET, RAS, RAF cascade that activate MAP kinase. Indeed, mutations and rearrangements of B-RAF, RAS, RAF and TRK (neurotrophic tyrosine kinase receptor) account for almost all cases of PTC. Translocations of RET observed in DTC result in a chimeric protein consisting of an activated RET tyrosine kinase domain. (13;15-30). MET (receptor-tyrosine kinase) overexpression in DTC is thought to be regulated by transcriptional or post-transcriptional mechanisms as a secondary effect (31). The genetic mechanisms underlying follicular thyroid carcinoma (FTC) are less clear (32), but a very interesting observation has been the rearrangement of the PAX-8 and PPAR-gamma genes (33), a unique combination of genes that traditionally are associated with thyroid development (the transcription factor PAX-8) and cell differentiation and metabolism (PPAR gamma). The chimeric protein acts as a dominant negative competitor for PPARgamma. Indeed, in experimental models of DTC, downregulation of the PPARgamma signaling route has been observed (34). Anaplastic carcinomas are frequently associated with mutations of the p53 tumor suppressor gene (35). This is in contrast with many other tumors in which p53 mutations play a role early in the process of tumorgenesis.

In the pathogenesis of thyroid carcinoma, it is believed that the genetic alterations lead to both proliferation via multiple pathways, and the loss of thyroid specific proteins. The disappearance of the functional expression of thyroid specific proteins is a complex chain of events, of which the mechanisms are incompletely understood. From many observations, it is believed hat there is a sequential disappearance of thyroid specific proteins. The disappearance of thyroid peroxidase (TPO) is believed to be an early event, followed by the disappearance of NIS. TSH receptor (TSHR) expression and thyroglobulin (Tg) expression are usually still present in advanced stages (36;37;38). The mechanisms involved in this

decreased expression of thyroid specific proteins may be genetic, involving the absence of thyroid transcription factors, epigenetic changes (observed for NIS and TSHR), mutations (not frequently observed) or post-translational mechanisms (NIS)(39).

DIAGNOSIS

Despite the increasing standards of imaging techniques like ultrasound, fine needle aspiration (FNA) remains the procedure of choice in patients presenting with thyroid enlargement. The sensitivity of FNA for DTC in most series is 90-95%. The specificity of FNA is lower, 60-80% when all patients with a non-benign FNA are referred for surgery (40). As a consequence, the frequency of FTC in hemi-thyroidectomies performed after suspicious results from FNA is only 20-30%. The problem is that the distinction by FNA between benign and malignant follicular neoplasms remains difficult, as the crucial criterion for FTC vs. adenoma (FA) is capsular invasion, which cannot be determined by cytology. In addition, the distinction between FA and Follicular variant of PTC (FVPTC) is also difficult, because the crucial criterion here is the aspect of the nuclei. The implication is that 70-80% of the patients with suspicious results from FNA, who undergo thyroid surgery have a benign tumor (41). Therefore, approaches to improve the accuracy of FNA are warranted (41).

INITIAL THERAPY

The guidelines for the initial therapy of DTC have been extensively reviewed in the guideline papers mentioned above. In all patients with DTC, except unifocal T1 (5th edition TNM (11)) PTC, initial therapy consists of near-total thyroidectomy followed by RaI ablative therapy of thyroid remnants. Although there is still some controversy about the extent of thyroid surgery, there are strong arguments in favor of total or near-total thyroidectomy (leaving only as limited thyroid tissue as is necessary to keep vital structures intact) in all patients (42). Total or near-total thyroidectomy results in a lower recurrence rate than more limited thyroidectomy, because many papillary carcinomas are multifocal and bilateral. Furthermore, total thyroidectomy facilitates total ablation with iodine-131 and reveals a higher specificity of thyroglobulin (Tg) as a tumor marker. (43-47).
Although controversy exists with respect to the routine application of iodine-131 ablation of thyroid remnants, many clinics still follow this procedure. Postoperatively, iodine-131 therapy is given for three reasons. First, it destroys any remaining normal thyroid tissue, thereby increasing the specificity of detectable serum Tg and positive whole-body scintigraphy as markers for persistent or recurrent tumor (2;43;48). Second, iodine-131 therapy may destroy occult microscopic carcinomas, thereby decreasing the long-term risk of recurrent disease (43;49-51). Third, the use of a large amount of iodine-131 for therapy permits post ablative scanning, a test for detecting persistent carcinoma (52;53).
However, in a meta-analysis (54) this presumed beneficial effect of RaI ablation to prevent recurrence or death was doubtful. A beneficial effect was only shown in patients with a

high risk or irradical surgery (45;49;55;56). In addition, doubts have arisen on the safety of routine RaI ablation, and a recent paper suggested a relation between excess non-thyroidal malignancies and RaI treatment (57). This has led to a more careful positioning of RaI ablation in recent papers (58;59). In conclusion, there is consensus about the efficacy of iodine-131 ablation therapy in patients with: (i) tumor stages T2-4; (ii) evidence for remaining thyroid tumor remnants and (iii) metastases (60;60;61).

FOLLOW-UP

The purpose of follow-up protocols in DTC is to detect, and prevent, persistent or recurrent DTC. Recurrences are usually detected during the early years of follow-up, but may be detected later, even after more than 15 years after initial treatment. Most patients during follow up have been cured definitely, and, as a consequence; have a low pre-test probability for recurrent disease. Therefore, the sensitivity of the diagnostic test must be adequate to detect the few patients with evident thyroid carcinoma, whereas specificity must also be high to avoid unnecessary treatments in patients without recurrent disease. In addition, the burden of diagnostic tests for the patient should be kept at a minimum. The most important tools in follow up protocols are serum measurements of Tg, diagnostic whole body RaI scintigraphies and neck-ultrasound.

DETECTION OF RECURRENT DISEASE

Thyroglobulin

Numerous studies have been performed on the diagnostic value of serum thyroglobulin (Tg) measurements. The consensus is that TSH stimulated Tg measurements have superior diagnostic value in DTC (62). The interpretation of many studies, and consequently of the guidelines on Tg, performed so far is difficult, because the analytical aspects of Tg measurements are complicated. The type of analysis (RIA or immunometric assay) affects the interpretation of serum Tg values (63). Currently, the clinical interpretation of serum Tg levels is hampered by pre-analytical (the presence of Tg antibodies), analytical and statistical problems (63;63;64-68). Statistical problems are the use of fixed Tg cut-off levels without using receiver operator curve (ROC) analyses. Therefore, in a recent European consensus paper, it was recommended to define institutional Tg cut-off levels (69). In addition to diagnostic purposes, Tg could also be used as a prognostic factor in DTC.

New serological markers

Because of the limitations of Tg, novel serological markers have been searched for. Of interest is the demonstration of Tg mRNA in peripheral blood, which indicates the presence of circulating Tg producing cells (e.g. thyroid cancer cells). However, in a number of studies, Tg mRNA alone did not have sufficient diagnostic power to discriminate between patients with active tumor and thyroid remnants (70) or thyroid carcinoma and healthy volunteers (71). In contrast, the combination of Tg and Tg mRNA allowed the identification of all patients with active disease in another study (34). Interestingly, RT-PCR can also be applied

to detect cells that produce other thyroid specific proteins. In a study on TPO (72), RT-PCR correlated significantly with metastatic disease.

Diagnostic RaI scans

The results of iodine-131 whole body scanning depend on the presence and the ability of thyroid-cancer tissue to accumulate iodine-131 in the presence of high serum TSH concentrations. The sensitivity of diagnostic RaI scintigraphies is much lower than that of ultrasound and Tg measurements and consequently, the routine use of RaI scintigraphy in the diagnostic follow-up of DTC patients is no longer recommended (58;73).

Ultrasound

In recent publications, ultrasound combined with FNA had the highest sensitivity (even higher than Tg) for local recurrent DTC and lymph node metastases (74-76). This has led to an important place for ultrasound in he follow up of DTC.

18-F Fluorodeoxyglucose-positron emission tomography (FDG-PET)

The diagnostic accuracy of FDG-PET in patients suspected of recurrent DTC is not well defined. Many studies are biased by selection of patients or have other methodological problems (77). The general idea is that FDG-PET may be useful in patients with elevated serum Tg levels, in whom no RaI uptake is observed after diagnostic or post-therapeutic scintigraphy. The sensitivity of FDG-PET is better when serum Tg levels are higher (78). FDG-PET during TSH stimulation may be more sensitive than during suppressive therapy (79).

Somatostatin Receptor Scintigraphy (SRS)

The expression of somatostatin receptors (SSTR3 and SSTR5) by DTC is the rationale for SRS imaging and therapy. Interestingly, in a considerable number of DTC, SRS imaging shows pathological lesions, which has diagnostic and therapeutic consequences (80;81).

TSH-SUPPRESSIVE L-THYROXINE THERAPY

Patients treated for differentiated thyroid carcinoma (DTC) receive thyroxin replacement therapy. The purpose of this therapy is not only to replace endogenous thyroid hormone, but also to suppress serum thyrotropin (TSH) levels in order to prevent relapse or progression of thyroid cancer. The rationale for TSH suppressive thyroxin replacement therapy is based on multiple clinical and experimental observations, reviewed in (82). Only four observational clinical studies have been published on the effects of thyroxin induced TSH suppression on the prevention of DTC recurrence or thyroid carcinoma related death (49;83-85). In the first study, Mazzaferri et al (49) found fewer recurrences and thyroid carcinoma related deaths in patients treated with TSH suppressive thyroxin dosages. In the second study, Cooper et al (84) showed that TSH suppression was an independent predictor in non-radioiodine treated high-risk papillary cancer patients. However, in these 2 studies initial therapy was not uniform with respect to the extend of surgery and radioiodine ablation therapy (49;84). In a recent publication, Jonklaas et al demonstrated in a multicenter study, that the degree of TSH suppression is a predictor of thyroid carcinoma

specific survival in high risk patients, independently of radioiodine ablation therapy and the extent of thyroid surgery. As initial therapy in their cohort was not distributed uniformly, it was not studied whether TSH suppression after uniform initial therapy consisting of both near total thyroidectomy and radioiodine ablation has additional value. In addition, they did not study the value of TSH suppression in patients who were cured after initial therapy. In the fourth study, Pujol et al (83) studied 121 DTC patients who where all treated by total thyroidectomy and thyroid remnant ablation. They showed that a percentage of undetectable TSH values of less than 10% significantly predicted a lower relapse free survival. In this study, only the comparison of extreme TSH values showed a significant difference in relapse fee survival. The low number of thyroid carcinoma related deaths, did not allow to assess the prognostic value of TSH with respect to mortality. This lack of compelling evidence that prolonged suppression of serum TSH levels is associated with a better prognosis in low risk DTC together with the adverse effects of hyperthyroidism on bone mineral density (86) and cardiac function (87) was also reflected in recent guidelines to aim at normal TSH levels in low-risk DTC patients (58). To assess the relation between the degree of TSH suppression and prognosis in more detail, we studied in Chapter 2 the association between the degree of TSH suppression and long-term prognosis in a group of 366 consecutive DTC patients.

THERAPY FOR RELAPSING OR METASTATIC DISEASE

CONVENTIONAL THERAPIES

RaI Therapy

Distant metastases, usually in the lungs and bones, occur in 10 to 15 % of patients with DTC. Lung metastases are most frequent in young patients with papillary carcinomas. In general, bone metastases are more common in older patients and in those with FTC.
In case of residual disease or metastases, surgery can be attempted when the lesion is accessible. In other cases, RaI therapy will be given in patients with metastases that accumulate RaI. The remission rate in pulmonary metastases treated with iodine -131 is 50%, varying from 90% in patients with microscopic metastases to only 10% in macronodular disease (61;88;89). The remission rates of bone metastases in the same studies are worse, varying between 7-20 %. A major problem in this category of patients is the diminished or lost ability of thyroid cancer cells to accumulate RaI, indicated by negative post-therapeutic whole body scintigraphy. In these cases the prognosis is poor, as alternative treatment options (external radiotherapy or chemotherapy) have limited success (90).

Chemotherapy

Although differentiated thyroid carcinoma is a low prevalent malignancy, many chemotherapeutic protocols that have been developed over the last decades for more common malignancies have been tried in progressive thyroid carcinoma. Overall, these approaches have been disappointing. Of the classical chemotherapeutic agents, adriamycin, alone or combined with cisplatin and bleomycin may induce temporary remissions or stationary

disease in about 30-50% of the patients (90;91). The same has been reported for paclitaxel (92). Most remissions however, last only a few months and at the cost of a considerable reduction in quality of life, thus leading to the recommendation hat there is no place in principle for chemotherapy (58;73).

NEW THERAPEUTIC APPROACHES FOR THYROID CARCINOMA:
1 REDIFFERENTIATION

Epigenetic therapies

One of the mechanisms by which cells can block the expression of certain genes is by enzymes that methylate these genes or de-acetylate the histones that envelope a particular gene. These mechanisms also play a role in the silencing of genes in cancer. Therefore, compounds that can reverse methylation or inhibit histone deacetylation may lead to the re-expression of genes that are silenced in cancer. Demethylation therapy has been proven successful in leukemia. In an in-vitro study in thyroid carcinoma, the demethylating agent 5-azacytidine led to re-induction of NIS expression, accompanied by RaI uptake in thyroid cancer cell lines (93). In parallel, the histone deacetylase inhibitor Depsipetide has been reported to reinduce NIS mRNA expression and RaI uptake in DTC (94;95), although toxicity may be a serious problem (96).

Retinoids

Retinoids are derivatives of vitamin A (i.e. retinol). Beneficial effects of retinoids have been reported in promyelocytic leukaemia and several types of carcinoma (97-99). In vitro studies have reported that retinoids have beneficial effects in DTC (100-103) including increased NIS mRNA expression and iodide uptake in some thyroid cancer cell lines (100). Interestingly, the promoter of the NIS gene has a retinoic acid response element (104).
A limited number of human studies have been performed on the effects of retinoids on I-131 uptake with mixed results (105-109), all using the RAR agonist 13-cis retinoic acid. However, recent studies indicated a differential expression of both RAR and the retinoid receptor RXR in thyroid carcinoma cell-lines and tissues (110;111), which corresponded to the responsiveness to ligands for these receptors. The importance of RXR expression with respect to responsiveness to retinoid treatment was demonstrated in the latter study (111).
Bexarotene (Targretin, Ligand Pharmaceuticals, San Diego) a RXR agonist, which also induces RAR by transcriptional activation. (112) has been tested in a prospective controlled clinical trial in 12 patients with metastases of DTC and decreased or absent I-131 uptake. Bexarotene treatment was able to induce I-131 uptake in metastases of 8/11 patients (113). Thus, Bexarotene partially restores I-131 uptake in metastases of DTC. A subsequent clinical trial was performed to study the effectiveness of high-dose I-131 together with Bexarotene in thyroid carcinoma patients. Unfortunately, this therapy was not successful.

Statins and PPAR-gamma agonists

An interesting new class of drugs is the class of PPARgamma agonists. These drugs have been introduced as anti-diabetic agents. Their proposed mechanism is the differentia-

tion of pre-adipocytes into adipocytes, thereby increasing the fatty-acid storing capacity of adipose tissue. The involvement of PPAR-gamma in differentiation processes extents beyond the area of adipose tissue. Indeed, altered expression of PPAR-gamma and in vitro beneficial effects of PPAR-gamma agonists have been described in a number of malignancies. In DTC, these compounds influence differentiation (114), induce apoptosis in thyroid tumors and prevent their growth in nude mice (115). In a recently published clinical study, rosiglitazone induced RaI uptake in DTC (114).
Statins (e.g. lovastatin) have been shown to be potent inhibitors of the HMG-CoA reductase. They are able to bind HMG-CoA reductase, the rate-limiting enzyme of the mevalonate (MVA) pathway, approximately 1000-fold more effective than the natural substrate (116;117). They are regarded as safe and effective drugs in the treatment of hypercholesterolemia. In addition to their primary use, the anticancer activity of statins was intensively studied and in vitro studies show an effect on growth and invasion of tumor cells (118;119). Several phase I-II clinical trials have been conducted. However, the overall antitumor response rates in these trials were disappointing.
Until recently statins and thiazolidinediones were only tested separately for anticancer effects. Yao et al. tested this combination and found that combined use of troglitazone and lovastatin resulted in a dramatic synergistic effect against human glioblastoma and CL1-0 human lung cancer cells lines in vitro at low concentrations (120). There is hope that this combination can induce this effect in vivo, because the effects were found at clinically achievable concentrations of lovastatin and troglitazone. Both lovastatin and troglitazone have been shown to have re-differentiating properties, in addition to reduction of growth and invasion of tumors (118;121). Indeed, in addition to a beneficial effect on tumor growth, glitazones have also been reported to reinduce the expression of NIS. Within the group of thiazolidinediones, troglitazone displayed the highest potential to re-establish NIS expression and Iodine uptake in thyrocytes in vitro. We decided to further explore the potential beneficial effects of statins and glitazones in the follicular thyroid carcinoma cell-line FTC-133. In Chapter 3, we tested the combinational effect of low concentrations of troglitazone, lovastatin and the combination on growth and explored the mechanism. In addition, we also studied the effects of this combination on the re-expression of thyroid specific proteins, e.g. NIS and the TSH receptor.

NEW THERAPEUTIC APPROACHES FOR THYROID CARCINOMA: 2 OTHER TARGETS

Neovascularization

Molecular pathways involved in neovascularization have been demonstrated in thyroid carcinoma (122). The cascade of approaches to target tumor-induced neovascularization has led to a number of promising compounds that are now being tested in clinical trials in prevalent tumors. Reports have been published on beneficial effects of anti-VEGF antibodies in thyroid carcinoma cell-lines (123) and endostatin in animal experiments (124). A recently published clinical trial, including thyroid carcinoma patients was also successful (125).

Tyrosine kinase inhibitors

Another intriguing development is the advent of tyrosine kinase inhibitors. The development of imatinib mesylate (Gleevec) is prototypical for the innovative design of modern drugs with the molecular pathogenic defect as a starting point. Following imatinib, other small molecules have been developed, aimed at other tyrosine kinase activated pathways such as the epithelial growth factor receptor (EGFR) activated pathway (13;126). Activation of tyrosine kinase pathways is relevant for thyroid carcinoma. Several studies have been published reporting successful treatment with the tyrosine kinase inhibitors aimed at RET, VEGF or the EGFR (127-129).

NEW THERAPEUTIC APPROACHES FOR THYROID CARCINOMA: 3 MEMBRANE RECEPTOR TARGETED THERAPIES

Somatostatin receptors

The expression of somatostatin receptors by DTC make these tumors candidates for SRS based therapy. Recent studies have reported moderate effects of indium labeled octreotide (130) and promising effects of lutetium octreotate (131).

TSH receptor targeted therapy

An interesting and potentially promising approach would be to make use of specific proteins expressed by DTC as a target for therapies. One of the most obvious thyroid specific proteins is the TSHR.

TSHR TARGETED THERAPY

TSH

The main role of Thyroid Stimulating Hormone (TSH) or thyrotropin is the regulation of hormone production by the thyroid gland by binding to the TSH-receptor and achieving homeostasis in target organs by the classical feedback loop. Within this feedback loop TSH production in the pituitary is positively regulated by TSH releasing hormone (TRH) and, directly or indirectly, inhibited by T3 and T4. TSH also regulates its own secretion by an ultra short negative feedback loop (132-136).

TSH structure

Thyroid Stimulating Hormone belongs to the family of glycoprotein hormones (GPH), which are non-covalently linked heterodimers consisting of an alpha and beta chain. The α- chain is identical for all the members of the glycoprotein-hormone family, which also includes CG, LH and FSH and consists of 92 amino acids whereas the 118 amino acid beta chain is unique to TSH and determines specificity (132;137;138). Although being specific to their receptors, the beta chains of the glycoprotein hormones still display a high homology as they originate from a common ancestral beta chain (139). In vivo TSH is heavily glycosylated and the carbohydrate groups constitute 15-25% of the total weight of TSH adding up to a total weight of 28- to 30-kDa.

TSH RECEPTOR

TSH-receptor expression

The human TSHr gene is located on chromosome 14q31and is encoded by 10 exons of which the last exon encodes the entire transmembrane and intracellular region (132). Expression of the TSHr is regulated by thyroid specific and non-specific transcription regulatory elements. So far, binding sites for thyroid hormone receptor (TR)-α1/ retinoid-X receptor (RXR) heterodimer, GA-binding protein (GABP), cAMP responsive-element and TTF-1 have been identified (140-144).

Structure and activation of the TSH-receptor

The TSHr is a member of the family of the leucine-rich repeat containing G-protein-coupled receptors and specifically binds the glycoprotein TSH. It is similar to other glycoprotein hormone receptors as luteinising hormone receptor and follicle stimulating hormone receptor but has unique insertions. In its unglycosilated form TSH receptor has a molecular weight of 84kDa but the glycosylated form is 95-100 kDa (139). It consists of an extracellular domain containing of a leucine rich repeat (LRR) and a membrane associated part consisting of 7 transmembrane domains connected with 3 external(E 1-3) and 3 internal loops (I 1-3).

Two insertions are unique to the TSHr and make it the largest of the glycoprotein hormone receptors, a small 8aa insertion and a 50aa insertion. Within the 8aa fragment the Cys41 seems to be of particular importance as substitution of this amino-acid results in loss of TSH binding to its receptor whereas substitutions of the other aminoacids in this fragment have no effect on TSH binding (145).

The 50aa insertion forms a loop stabilized by 3 disulphide bridges formed between the cysteins 283-408, 284-398 and 301-390. The loop itself is susceptible to proteolytic cleavage at the sites 302-317 and 366-378 (146). Cleavage of the two cleavage sites results in a separate A- and B-TSHR subunit (or α and β) and a small C-peptide. After proteolytic cleavage the A and B subunit are connected by disulfide bonds which can be destroyed resulting in the release of the A subunit in the medium, a process known as shedding. This appears to be happening in an in vivo situation as an excess of B-subunit was found in thyroid tissue (147).

In the normal situation TSH can bind to the TSHr resulting in an activation of both Gs and Gq protein in human cells (132). An exception are patients with the autoimmune disease Graves hyperthyroidism, whom posses TSHr-stimulating auto-antibodies. Furthermore, in the absence of a ligand, TSHr is known to have a relatively high basal activity when compared to LH (148).

Once the TSHR is activated, it induces phopholipase C(PLC) and the protein kinase A(PKA) signal transduction system, each inducing different effects. Phopholipase C (PLC) regulates iodine efflux, H_2O_2 production and thyroglobulin iodination, whereas adenylate cyclase regulates iodine uptake and transcription of Tg, TPO and NIS via PKA (144). The degree of activation by TSH can be measured by determining intracellular cAmp levels or by using other downstream effectors (see Read out systems).

The TSH receptor is constitutively internalized via clathrin coated pits and partly recycled

to the cell surface, a process increased 3-fold after incubation with TSH (149). Furthermore, TSHr signalling is regulated by several posttranslational modifications. Glycosylation, phosphorylation, sialyation and dimerisation influence cell-surface expression or signalling of the receptor (132;137;139;146;149;150)

Autoimmunity to the TSHR

One of the mayor diseases associated with the TSHr is the autoimmune disease Graves' hyperthyroidism. This disease is characterized by thyroid enlargement, goiter and high thyroid hormone levels. Graves hyperthyroidism is one of the autoimmune diseases known as autoimmune thyroid disease (AITD) which include Graves' hyperthyroidism, Hashimoto's thyroiditis and idiopathic thyroid failure. These diseases are closely related and partly display the same symptoms. Hyperthyroidism in Graves' disease is caused by specific TSHr binding antibodies. TSHr binding antibodies called TRAb (TSH receptor antibodies) can be distinguished into 3 different types: stimulating(TSAb), blocking(TBAb) and binding with no apparent effect on stimulation. TBII (TSH binding inhibitory immunoglobulins) are a generic term for both thyroid stimulating antibodies (TSAb) and thyroid blocking antibodies (TBAb) and inhibit binding of TSH to its receptor. Hyperthyroidism in GD is caused solely by TSAb, which bind to, and activate, the TSHR, thus stimulating thyroid hormone production (151-154).

The cause of the autoantibodies in Graves' hyperthyroidism is unknown and there is no evidence that thyroid antigens in Graves' hyperthyroidism are abnormal. It is likely that the cause of GD is associated with a combination of genetic, environmental, and endogenous factors, which are responsible for the emergence of auto reactivity of T and B cells to the thyrotropin receptor (TSHR).

TSH receptor expression in thyroid carcinoma

TSHR expression is persistent in thyroid carcinoma. Although TSHR expression is lost in poorly differentiated thyroid carcinoma, TSHR is expressed more persistently than other thyroid specific proteins. This is the base of clinical practice in which the TSH dependant tumor marker thyroglobulin is increased after stimulation with TSH. In addition, TSHR expression is found immunohistochemically in a large panel of thyroid carcinomas (155;156).

TSH receptor expression in other tissues

Expression of the TSH-receptor has been reported in other tissues such as lymphocytes, thymus, pituitary, testis, kidney, heart and orbital tissues (157-159). Thus although TSHR appears to reside in non-thyroid tissues, the TSHR in those tissues is only found at very low levels. Moreover, it is likely that these small quantities of TSHR are due to 'leaky' transcription which presumably occurs incidentally rather than intentionally implicating a lack of function of the TSHR in the extra thyroidal expression (144;157). However, recently some papers reported a more active role of the TSHR in non-thyroid tissues like bone. An important development has been the discovery of the TSH receptor (TSHR) in bone (160-163). TSHR knockout and haploinsufficient mice with normal thyroid hormone levels have decreased bone mass suggesting that TSH might directly influence bone remodeling (161;164). This is intriguing, because effects on bone metabolism that were previously

ascribed to high thyroid hormone levels could also be attributed to suppressed TSH levels (144;164;165). Furthermore, in animal studies, low doses of TSH increased bone volume and improved microarchitecture in ovariectomized rats (166), without increasing serum thyroid hormone levels. However, the concept has been challenged recently by a report concluding that bone loss in thyrotoxicosis is mediated predominantly by thyroid hormone receptor (TR) alpha (167). Osteoblasts like cells posses TSH receptors and display increased levels of cAMP when exposed to TSH, although these effects are small and it is unlikely that TSH plays a physical role in bone remodelling It is still debated whether functional TSHR exists outside the thyroid and pituitary (161;164;167).

APPROACHES FOR TSH TARGETED THERAPIES

Ligands

In the development of therapies against cancer, the ideal therapy would be to target only tumor cells. Unfortunately, most therapies lack this specificity and also affect healthy cells. After the discovery of potent bacterial and plant toxins, the idea emerged to use specific surface markers to guide these toxins to tumor cells. One of the most versatile binding agents are part of our own immune system namely antibodies. The combination of the binding domains of antibodies and a toxic compound resulted in the field of immunotoxins. In the case of thyroid cancers, one of the promising specific targets is the TSH receptor. Its natural binding agent TSH, or a derivate of TSH, may provide the specificity to guide toxins to thyroid tumor cells.

TSH

Within the TSH structure several regions are particularly important for binding and biological activities. The unique seat belt region of the β-chain, which includes the highly conserved “determinant loop” (β88-95), wraps around the alpha chain, thus stabilizing the linking of the α-and β chain. Within the alpha chain several regions are highly conserved: (33-38), the α-Helix (40-46), α-Lys and the glycosilation site α-Asn(51,52) and the α-carboxyterminus (88-92) (138). The exploration of the functions of different regions within TSH provides a platform to introduce calculated modifications to TSH. In the past the group of Weintraub and Szkudlinski have done extensive research in this field and succeeded in bioengineering superactive analogs using homology studies between species and other members of the glycoprotein hormone family (168;169).
Further modifications can be made by fusing the separate alpha and beta chain, which bypasses the rate limiting assembly step essential for secretion and hormone specific glycosilation of TSH (170;171). Using this knowledge, modified TSH analogues may be able to guide components directly to TSHr expression cells in the future (168;172;173).

Antibodies

Antibodies offer a wide range of specific binding properties and are often the first choice when toxins need to be guided to tumor cells. In the past whole antibodies were fused chemically to toxins, but nowadays recombinant immunotoxins offer the opportunity to further optimize the antibody derived binding domains. A drastic reduction in size,

while maintaining binding properties, can be achieved by removing a part of the constant regions the Fc, which has no antigen binding affinity but interacts with Fc receptors and complement. The resulting Fab's contain the antigen-binding site. A further reduction in size can be achieved by reduction to the binding site only. A major disadvantage is the loss of the disulphide bond, which lies in the removed portion of the Fab. In order to stabilize these variable regions a short amino acid linker can be used. However, this is not always sufficient and aggregates may form due to dissociation. This problem can be overcome by the introduction of a disulphide bridge within the Fv framework or by mutagenesis (174;175). An interesting approach are highly potent monoclonal anti-TSHR antibodies that exhibit potent TSHR stimulating activity. For instance, nanogram concentrations of the IgG mAbs KSAb1 and KSAb2 and their Fab induce full stimulation of the TSH receptor that is matched by the ligand TSH and, thus, act as full agonists for the receptor (176). In addition to antibodies, other cell binding proteins can be used such as growth factors or cytokines.

LYTIC COMPOUNDS

Bacterial Toxins

Typically toxins used in immunotoxins consist of several domains. A binding domain concentrates the toxins on the cell surface of the target cells, and subsequently the translocation domain facilitates translocation across the membrane to the cytosol. Once the cytosol is reached, the death activity domain inactivates cellular processes and kills the cell (174). A wide range of toxins from various organisms have been used in immunotoxins, e.g. ricin, diphteria toxin, pseudomonas exotoxinA, abrin, anthrax, Shiga, cholera, Clostridial neurotoxins and pertussis. Initially, the plant toxin ricin was often used to construct immunotoxins, but this resulted in vascular leak syndrome (VLS) a process where ricin damages vascular endothelial cells causing fluid to enter the bloodstream. However, genetic engineering of the toxin has led to a more favourable modified ricin (177;178). At present, the two toxins most commonly used in immunotoxins are of bacterial origin; Diphteria Toxin (DT) and Pseudomonas exotoxinA(PE). (174;179;179-183).

Pseudomonas exotoxinA(PE)

The toxin Pseudomonas exotoxinA originates from the bacterium Pseudomonas aeruginosa and consists of 3 domains. Domain Ia which is located at the N-terminus facilitates binding to the target cells via the a2-macroglobulin receptor (also known as LRP1) which is expressed in many cell types. Once bound the toxin is transported into the cell via Clathrin coated pits into endosomes. In the acidic environment of the endosome PE is proteolytically cleaved by furin between amino acids 279 and 280 and the disulphide bond between residues 265 and 287 is reduced. The c-terminal halve of the cleaved toxin is then transported to the endoplasmatic reticulum (ER) via the trans golgi network by exploiting an ER retrieval system (184). This transport is presumably regulated by the C-terminal REDLK sequence that functions as a KDEL sequence after removal of the terminal lysine residue. REDL binds to the native KDEL receptor which is normally involved in the reverse translocation of misfolded proteins from the ER thus guiding PE to the cytosol (185-187). In the final step domainIII (transferaseIII) is translocated into the cytosol, where it inactivates EF-2)

thereby crippling protein production and sending the cell into apoptosis (174;179;179-183).

Diphteria Toxin (DT)

The diphteria toxin originates from Corynebacterium diphteriae and inactivates the elongation factor 2(EF-2) in a similar way as PE (188). Apart from the similar EF-2 inactivation step, there are a few differences. It has a different orientation and the ADP-ribosylating activity occurs at the N-terminus whereas the binding domain is present at the C-terminus. The binding domain binds to the heparin binding epidermal growth factor(EGF)-like precursor (189) followed by transport into the cell via Clathrin coated pits. Once the toxin has reached the acidic endosome, DT is processed in a similar way to PE, DT is proteolytically cleaved by furin and the disulphide bond between the A and B fragment is reduced resulting in an enzymatically active fragment A. In contrast to PE, DT is structurally changed by the acidic environment in such a way that it can cross the endocytic membrane directly into the cytosol via insertion of the T domain in the membrane. When DT-related proteins were produced in the E.coli periplasm DT containing the B fragment were lethal to E.coli at low PH by insertion into the membrane whereas cells were unharmed at PH7 (190). Once the N-terminal domain (fragment A) has reached the cytosol it inactivates the EF-2 in a PE-like fashion.

Modified toxins

The idea of combing the high toxicity of DT and PE to a cancer specific binding domain led to the development of the first generation of immunotoxins decades ago. These first generation immunotoxins consisted of a chemical conjugated whole toxin and specific antibodies which was also toxic to normal cells. Although removal of the natural binding domain has overcome some of the problems, chemical linking is still very costly to produce, gives heterogeneous products and results in large products that have slow penetration rates. Modern immunotoxins are made by recombinant approaches, which are beneficial in both production and optimization of the products. Large scale production of a homogeneous product is now possible in an organism of choice. However, it must be kept in mind, that these toxins are toxic to normal animal cells. Nowadays, most immunotoxins are produced in Escherichia coli which produce large amounts of immunotoxins economically.
The toxins used in these recombinant immunotoxins can, and have been optimized, by removing unnecessary elements such as the binding domain and domain Ib. A further increase in cytotoxic activity of PE can be achieved by replacing the C-terminal REDL sequence into the characteristic endoplasmic reticulum retention sequence KDEL. In addition to optimization of the toxins, the newly attached specific binding domains (often an antibody) have been optimized to achieve optimal binding with minimal size.
The immunotoxins with the highest potency in the clinic will result from the combination of the potency of the toxin and the specificity of the replaced binding domain (174;179;179-183;191).
In Chapter 4, we conducted a series of experiments to generate modified recombinant TSH. We also studied the feasibility of fusing proteins with modified TSH as a model for TSH toxins and still maintain biological activity.

READ OUT SYSTEMS

An essential part of the development of TSHR targeted compounds the validation. Validation of compounds involves different subsequent stages, including biochemical, in-vitro and in vivo experiments. For the in-vitro testing, a sensitive and reliable assay should be available that not only is capable of testing TSHR binding, but also activation, as a successful TSHR targeted toxin needs to be internalized. Obvious candidates for the in vitro testing of TSHR binding are commercially available assays for TBII, as used in Graves' disease.

Assays for detection of TBII

Most commercially available assays for TBII are based on immunoglobulin-mediated inhibition of the binding of radio labelled or luminescent TSH to the TSHR. The sensitivity of these assays ranges from 80 to 99 percent (192). A number of studies have been published on bioassays for TBII. Initially, radioimmunoassays were used to measure cAMP activity in FRTL-5 cells or cell-lines stably transfected with the TSHR (193-197). However, this method is relatively cumbersome and expensive. More recently, bioassays have been developed based on the incorporation of a luciferase construct in TSHR transfected cell-lines. In these assays, cAMP that is generated by TSH-receptor activation induces luciferase expression. With these methods, the presence of TSAb (198;199) as well as TBAb (200;201) in sera from patients with a history of GD have been demonstrated convincingly. However, the threshold of the luciferase based assays published is relatively low, ranging from 1 mU/l bovine TSH (198) to 100 mU/l (199).
In Chapter 5, we aimed to develop a superior luciferase-based bioassay for TSHR binding and activation. We validated this assay in sera of de novo patients with GD. As a byproduct of our project we found in Chapter 6 that this bioassay has attractive properties for diagnosing de novo GD, but also for the determination of the induction of TSHR antibodies after RaI therapy for benign thyroid disease.

OUTLINE OF THE PRESENT THESIS

In the present thesis, questions regarding the role of the TSHR in the therapy and follow-up of DTC will be addressed. These questions arise from the need for a DTC specific approach in the search for novel therapeutic approaches in metastatic DTC as well as uncertainties with respect to the role of TSHR suppressive thyroxine replacement therapy in DTC.

In **Chapter 2** we describe a study aimed at the optimal degree of TSHR suppressive thyroxine replacement therapy in patients with DTC. We studied the relationship between degree of TSH suppression and risk of recurrence and death in 366 patients DTC patients with varying degrees of TSH suppression.

In **Chapter 3** we describe an in vitro investigation aimed at the additional effects of combined treatment with troglitazone and lovastatin on growth and redifferentiation of the follicular thyroid carcinoma cell line FTC133, and the underlying molecular mechanism.

In **Chapter 4** we describe an extensive project in which we cloned TSH alpha and beta chains from a human pituitary tumor, generated recombinant human single chain TSH (scTSH), introduced mutations leading to superior biological activity and also introduced extensions to the scTSH while preserving its biological activity.

In **Chapter 5** we describe the generation of a novel luciferase based TSHR binding and activation assay and its validation in patients with de novo Graves disease.

In **Chapter 6** we describe the application of the luciferase based TSHR binding assay in the detection of TSHR activating antibodies, induced by RaI therapy in patients with benign thyroid disease.

Finally, in **Chapter 7** the results of the present thesis are summarized and put into perspective, followed by a dutch translation in **Chapter 8**.

Chapter 2

Associations of Serum Thyrotropin Concentrations with Recurrence and Death in Differentiated Thyroid Cancer

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ABSTRACT

Objective: The relation between serum TSH levels and risk for recurrence or thyroid carcinoma related death in patients with differentiated thyroid carcinoma (DTC) has only been studied to a limited extent.

Design: Single-centre observational study in 366 consecutive patients with DTC, who had all been treated according to the same protocol for initial therapy and follow-up. Median duration of follow-up was 8.85 years.

Methods: The relation between summarizing variables of unstimulated serum TSH concentrations (25th, 50th 75th percentiles, the percentage of suppressed and unsuppressed TSH values) and risk for recurrence or thyroid carcinoma related death was analyzed by Cox survival analyses in patients with at least 4 TSH measurements.

Results: In Cox-regression analysis, we found a positive association between serum TSH concentrations and risk for thyroid carcinoma related death and relapse, even in initially cured patients. The median of the individual TSH concentrations was the best indicator for thyroid carcinoma related death (hazard ratio (HR): 2.03 (confidence interval (CI): 1.22 – 3.37) and relapse 1.41 (CI 1.03 – 1.95). A threshold of 2 mU/l differentiated best between relapse free survival and thyroid carcinoma related death or relapse.

Conclusion: Our study supports current guidelines, which advise to aim at TSH levels in the low normal range in cured low risk patients, whereas TSH levels should be suppressed in non-cured or high-risk patients.

INTRODUCTION

Patients treated for differentiated thyroid carcinoma (DTC) receive thyroxin replacement therapy. The purpose of this therapy is not only to replace endogenous thyroid hormone, but also to suppress serum thyrotropin (TSH) levels in order to prevent relapse or progression of thyroid cancer. The rationale for TSH suppressive thyroxin replacement therapy is based on multiple clinical and experimental observations, reviewed in (82). It has been observed in case reports that high TSH levels may promote tumor growth, which is reversed by thyroxin therapy (202-204). Experimental studies have confirmed the expression and functional activity of TSH receptors in DTC (205-207) and the proliferative effects of TSH on thyroid carcinoma cells in vitro (208;209). TSH also stimulates protein synthesis and metabolism in DTC, which is the rationale for clinical diagnostic procedures, like TSH stimulated serum thyroglobulin (Tg) measurements (3;210) and TSH stimulated FDG-PET scintigraphy (79). Despite this notion, only four observational clinical studies have been published on the effects of thyroxin induced TSH suppression on the prevention of DTC recurrence or thyroid carcinoma related death (49;83-85). In the first study, Mazzaferri et al (49) found fewer recurrences and thyroid carcinoma related deaths in patients treated with TSH suppressive thyroxin dosages. In the second study, Cooper et al (84) showed that TSH suppression was an independent predictor in non-radioiodine treated high-risk papillary cancer patients. However, in these 2 studies initial therapy was not uniform with respect to the extend of surgery and radioiodine ablation therapy (49;84). In a recent publication, Jonklaas et al demonstrated in a multicenter study, that the degree of TSH suppression is a predictor of thyroid carcinoma specific survival in high risk patients, independently of radioiodine ablation therapy and the extent of thyroid surgery. As initial therapy in their cohort was not distributed uniformly, it was not studied whether TSH suppression after uniform initial therapy consisting of both near total thyroidectomy and radioiodine ablation has additional value. In addition, they did not study the value of TSH suppression in patients who were cured after initial therapy. In the fourth study, Pujol et al (83) studied 121 DTC patients who where all treated by total thyroidectomy and thyroid remnant ablation. They showed that a percentage of undetectable TSH values of less than 10% significantly predicted a lower relapse free survival. In this study, only the comparison of extreme TSH values showed a significant difference in relapse fee survival. The low number of thyroid carcinoma related deaths, did not allow to assess the prognostic value of TSH with respect to mortality. This lack of compelling evidence that prolonged suppression of serum TSH levels is associated with a better prognosis in low risk DTC together with the adverse effects of hyperthyroidism on bone mineral density (86) and cardiac function (87) was also reflected in recent guidelines to aim at normal TSH levels in low-risk DTC patients (211).

To assess the relation between the degree of TSH suppression and prognosis in more detail, we studied the association between the degree of TSH suppression and long-term prognosis in a group of 366 consecutive DTC patients who were all treated by total thyroidectomy and radioiodine ablation therapy. Because the median duration of follow-up was 8.9 years, the number of thyroid carcinoma related deaths allowed us to study both relapse free survival and mortality, both in the total group and in a subgroup of patients

who were cured 1 year after initial therapy.

MATERIAL AND METHODS

PATIENTS AND METHODS

Three-hundred-and-sixty-six consecutive patients were included in the study. These patients had received initial therapy for DTC between January 1986 and January 2000. January 1986 was chosen as a starting date, because from that date forward, all relevant patient data were registered in a computerized database. Initial surgery and radioiodine ablation therapy were performed at the Leiden University Medical Centre or at one of the connected general hospitals. All hospitals are affiliated in the Regional Comprehensive Cancer Centre, using the same standardized protocol for the treatment and follow-up of DTC.

All patients were treated by near-total thyroidectomy, followed by routine radioiodine ablative therapy with 2800 MBq I-131. In case of incomplete tumor resection or when metastases were present, 6000 MBq was administered after thyroidectomy. Lymph node surgery was performed as follows: when lymph node metastases were the presenting symptom, a modified radical neck dissection (removal of lateral lymph nodes with preservation of sternocleidomastoid muscle, internal jugular vein and accessory nerve) was performed at the time of total thyroidectomy. When lymph node metastases were not the presenting symptom, neck inspection was performed during thyroidectomy and suspected lymph nodes removed. When lymph node metastases became apparent during follow up, a modified radical neck dissection was performed.

Follow-up was performed according to a standard protocol, consisting of unstimulated and at least one TSH stimulated serum Tg measurement, diagnostic 185 MBq I-131 scintigraphy and ultrasound. After initial therapy, levothyroxine therapy was started in a dose to suppress TSH levels (below 0.1 mU/l).

Cure, 1 year after therapy was defined as the absence of I-131 accumulation at diagnostic 185 MBq scintigraphy, Tg serum concentrations below 2 ug/l after TSH stimulation and no other indication for disease (212). When Tg antibodies were present at the time of evaluation of initial therapy, only those patients were considered cured in whom after prolonged follow-up no tumor became discernable.

Tumor presence during follow-up was defined as histologically or radiologically (X-ray, CT-scan, MRI-scan, FDG-PET scan or I-131 scintigraphy proven DTC and stimulated Tg levels above 2 ug/l (213). In case of recurrent disease or metastases, surgery was attempted if the lesion was solitary and accessible, followed by additional radioiodine therapy (6000 MBq). If the tumor could not be removed surgically, radioiodine therapy was given and repeated if necessary. All radioiodine therapies were followed after 7 days by whole body scintigraphy.

The following data were registered: age at diagnosis, sex, date of diagnosis, histology, TNM stage, serum Tg, Tg antibodies and TSH levels at regular intervals, date of cure, disease state one year after initial therapy, date of recurrence after cure, date of death, cause of

death and date of last follow up. TNM stage was registered according to the 5th edition (11). This was done because most patients were analyzed before the latest edition of the TNM classification. We used the following end-points of follow-up: date of death (82 patients), date of emigration (12 patients) and date of most recent contact (272 patients). Death causes were analyzed in all 82 patients who had died during follow-up. Death cause was investigated using medical records, death certificates, enquiries with other physicians involved in the treatment of each patient, enquiries in other hospitals, enquiries with general practitioners and autopsy findings. Death causes were divided into thyroid cancer related death and other causes.

TSH ANALYSES

Data from 6245 TSH measurements in 366 patients were retrieved from the Department of Clinical Chemistry. Of these, stimulated TSH levels (verifying all TSH levels > 10 mU/l) were discarded, which left 5680 measurements. Only patients were analyzed in whom at least 4 TSH measurements were available, leaving 4805 measurements in 310 patients.
After verifying that there was no time-dependency of TSH in our patient group (the average slope of TSH in all patients during the observation period being –0.001 mU/l*year (CI –0.002 – 0.000 mU/l*year), and because from a biological point of view there is no indication that the relation between TSH and thyroid carcinoma cells does change over time, we chose express TSH exposure using the following TSH summary parameters: for each patient: the 25th , 50th and 75th percentiles of all TSH measurements, the percentage of all TSH levels below 0.1 mU/l, 0.4 mU/l and 4.5 mU/l which is in line with the methods used in earlier papers (49;83-85).
The prognostic significance of TSH for thyroid carcinoma related death was analyzed in all patients as well as in patients who were cured 1 year after initial therapy. The prognostic significance of TSH for tumor relapse was analyzed in patients who were cured 1 year after initial therapy.

LABORATORY MEASUREMENTS

Serum TSH was determined throughout the study period with Elecsys E-170 on a Modular Analytics E-170 system (Roche Diagnostic Systems, Basel, Switzerland), reference range 0.4 – 4.5 mU/l, detection limit: 0.005 mU/l, intra-assay variability: 0.88-10.66%, inter-assay variability: 0.91-12.05%). Serum Tg was determined with IRMA (Tg kit, Brahms, Berlin Germany) on a Wallac (Wallac, Turku, Finland), intra-assay variability: 0.14-13.9%, inter-assay variability: 12.3-17.4 %). Serum Tg antibodies were determined with IRMA (Sorin Biomedica, Amsterdam, The Netherlands) on a Wallac (Wallac, Turku, Finland) intra-assay variability: 3.6-4.1%, inter-assay variability: 11.6%).
Until January 1997 serum Tg was measured using an immunoradiometric assay (IRMA), the Dynotest TG (Brahms Diagnostica GmbH, Germany) with a sensitivity of 0.3 µg/l. From January 1997, the Dynotest TG-s (Brahms Diagnostica GmbH, Germany) was used, with a sensitivity of 0.05 µg/l and an inter-assay variability of 0.3 µg/l. The comparability of the 2 methods is excellent: R2: 0.99, slope 0.99, intercept 0.09 (214). Serum Tg-antibodies were

also measured at these specific time points by the Ab-HTGK-3 IRMA (DiaSorin Biomedics, Italy).

STATISTICAL ANALYSES

Normally distributed data are presented as mean ± SD. Data that are not distributed normally are expressed as median and 25 and 75th percentiles. Categorical data are expressed as percentages. All statistical analyses were performed using SPSS for windows version 12.0 (SPSS Inc., Chicago, IL). Prognostic indicators for recurrence or thyroid carcinoma related death were identified using Cox-regression analyses. Indicators that were identified as significant for survival in were entered into a stepwise model. A p-value of < 0.05 was considered significant.

RESULTS

Characteristics of the patients are shown in Table 1. Mean age at time of surgery was 48 ± 18 years. Median duration of follow-up was 8.85 years (0.75 – 16.98) years. Three-hundred-and-ten patients (75 males, 235 females) were available, in whom at least 4 unstimulated TSH measurements were available. Thirty-nine (13%) of these patients died of thyroid carcinoma. Two-hundred-and-fifty patients (81%) were cured 1 year after initial therapy. Of the patients who were cured 1 year after initial therapy, 39 (16%) developed a relapse, whereas 10 (4%) died because of thyroid carcinoma.

ALL PATIENTS

TSH related parameters of all 310 patients are given in Table 2. The median of the individual percentages of TSH values below the lower limit of normal (0.4 mU/l) was 73% and the median of the percentages below 0.1 mU/l was 50%. No differences in these percentages were observed between the different TNM stages. By univariate Cox regression analysis, significant indicators for thyroid carcinoma related death were: extrathyroidal tumor extension (T4), the presence of distant metastases and older age (Table 1). Significant TSH related predictors for thyroid carcinoma related death were the 25th and the 50th percentiles (median) of the serum TSH concentrations for each patient: the hazard ratio (HR) for the 25th percentile was 1.35 (a HR > 1 meaning a higher risk for the endpoint), the HR for the median TSH was 1.22.
The 25th percentile for a patient indicates that 25% of the measurements are below (or equal) to this value and 75% of the measurements are above (or equal) to this value. A higher 25th percentile value in a patient indicates that up to 75% of the TSH values are higher in this patient than in a patient with a lower 25th percentile.
When the significant variables assessed by univariate Cox regression analysis were introduced into a stepwise multivariate model, only T4, M1 and older age remained significant predictors (Tables 1,3).

TABLE 1. Patient characteristics

	N	N with 4 or more TSH Measurements	Thyroid Carcinoma Related Deaths N (%)	Cured Patients after 1 Year N (%)	Patients with Relapse after Cure N (%)	Thyroid Carcinoma Related Deaths after N (%)
Total	366	310	39 (13)	250 (81)	38 (16)	10 (4)
Gender	91 / 275	75 (24) /	13 (14) /	57 (76) /	11 (19) /	1 (2) /
(Male/ Female)		235 (76)	26 (14)	193 (82)	28 (15)	9 (5)
Stages						
T1	22	19	0 (0)	19 (100)	1 (5)	0 (0)
T2	188	164	8 (5)	153 (93)	17 (11)	4 (3)
T3	56	52	8 (15)	45 (87)	9 (20)	4 (9)
T4	96	75	23 (31) * #	33 (44) * #	12 (36)* #	2 (6) * #
N1	107	85	15 (18)	55 (65)	12 (22)	2 (4)
M1	52	42	19 (45) * #	10 (24) * #	10 (100)* #	2 (20) *
Histology						
Papillary	203	171	18 (11)	141 (82)	25 (20)	4 (3)
Follicular	72	63	13 (21) *	50 (79)	10 (7)	5 (10)
Follicular variant papillary carcinoma	68	56	5 (9)	45 (80)	3 (7)	1 (2)
Hürthle Cell	23	20	3 (15)	14 (70)	1 (7)	0 (0)
Age (continuous)			* #	* #	* #	* #
< 55 yr	210	204	4 (2)	188 (92)	18 (10)	1 (1)
> 55 yr	156	106	35 (33)	62 (58)	21 (34)	9 (15)

* Significant at univariate analysis
# Significant at multivariate analysis

TABLE 2.. Summarizing data of serum tsh concentrations in 310 patients after initial therapy for differentiated thyroid carcinoma . Data are expressed as median (25^{th} - 75^{th} percentiles). TSH values are expressed with 1 digit.

	All patients	**Patients Cured After 1 Year**
Individual TSH related parameters:	Median (25^{th} - 75^{th} percentiles)	Median (25^{th} - 75^{th} percentiles)
Percentiles of TSH values per patient:	(n=310)	(n=250)
25^{th} Percentile (mU/L)	0.0 (0.0 – 0.1)	0.0 (0.0 – 0.1)
50^{th} Percentile (mU/L)	0.1 (0.0 – 0.4)	0.1 (0.0 – 0.4)
75^{th} Percentile (mU/L)	0.4 (0.1 – 1.4)	0.4 (0.1 – 1.4)
Percentage of TSH values below the indicated values per patient:		
<0.1 mU/L	50 (30 – 73)	50 (29 – 73)
< 0.4 mU/L	73 (50 – 89)	72 (50 – 89)
< 4.5 mU/L	100 (90 – 100)	100 (90 – 100)

TABLE 3. Hazard ratios of serum tsh levels for thyroid carcinoma related death (a) and relapse (b) as calculated with cox-survival analysis. Only patients with a tsh value equal to or exceeding 4 were included.

		Univariate Analysis – single covariate			Stepwise analyses with significant co-variates	
A. Thyroid Carcinoma Related Death			**p**	**Hazard Ratio (CI)**	**p**	**Hazard Ratio (CI)**
Total Group	All stages combined	25^{th} percentile	0.007	1.35 (1.08 – 1.69)	n.s.	
		Median TSH	0.028	1.22 (1.02 – 1.46)		
	T1-3 M0	Median TSH	0.044	1.63 (1.01 – 2.64)	n.s.	
	T4 M1	n.s.				
Patients cured 1 year after initial therapy	All stages combined	n.s.				
	T1-3 M0	Median TSH	0.006	2.03 (1.22 – 3.37)	0.013	2.14 (1.18 – 3.89)
		Percentage TSH values > 4.5 mU/L	0.009	1.06 (1.01 – 1.11)	0.021	1.07 (1.01 – 1.13)
	T4 M1	n.s.				
B. Relapse			**p**	**Hazard Ratio (CI)**	**p**	**Hazard Ratio (CI)**
	All stages combined	n.s.				
	T1-3 M0	Median TSH	0.020	1.46 (1.06 – 2.01)	0.033	1.41 (1.03 – 1.95)
	T4 M1	n.s.				

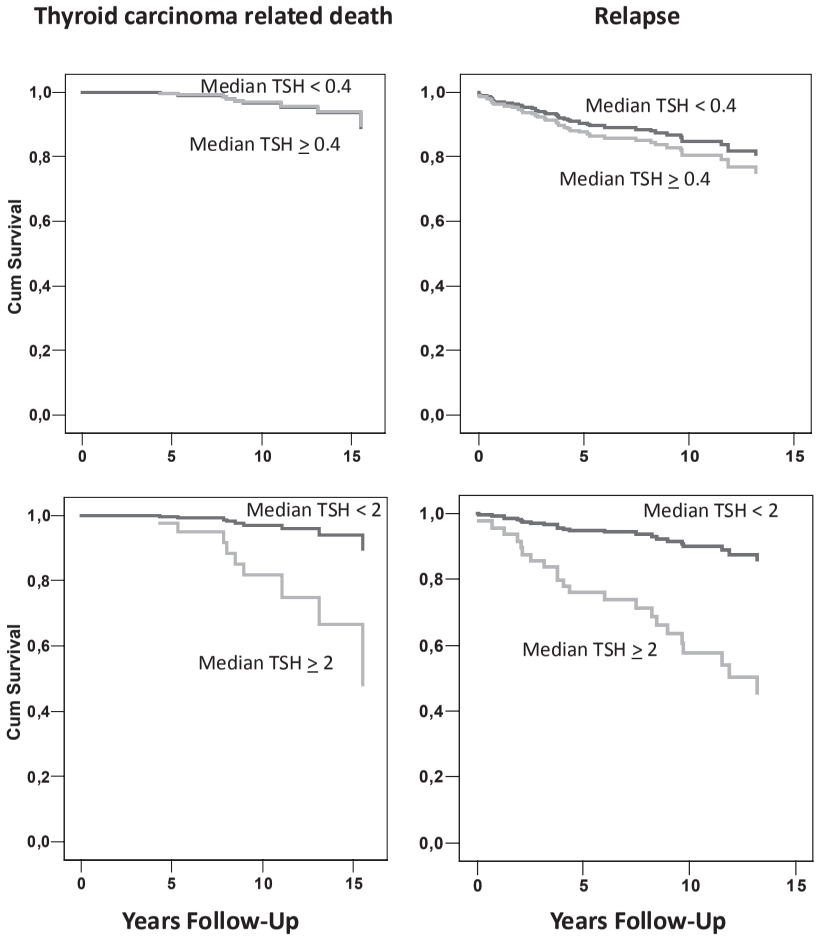

FIGURE 1. Relation between the median (50th percentile) of serum TSH concentrations and thyroid carcinoma related death and relapse in 250 patients who were cured 1 year after initial therapy for differentiated thyroid carcinoma.

a. Relation between a median serum TSH concentration cut-off value of 0.4 mU/L and thyroid carcinoma related death.
b. Relation between a median serum TSH concentration cut-off value of 2 mU/L and thyroid carcinoma related death.
c. Relation between a median serum TSH concentration cut-off value of 0.4 mU/L and thyroid carcinoma relapse.
d. Relation between a median serum TSH concentration cut-off value of 2 mU/L and thyroid carcinoma related relapse.

PATIENTS WHO WERE CURED ONE YEAR AFTER INITIAL THERAPY

Two-hundred-and-fifty patients were cured one year after initial therapy. When all 250 patients were analyzed, the median of the proportion of TSH values below the lower limit of normal (0.4 mU/l) was 72% and the median of the proportion below 0.1 mU/l was 50% (Table 2). No differences in these percentages were observed between the different TNM stages.

Thyroid Cancer Related Death

By univariate Cox regression analysis, significant indicators for thyroid carcinoma related death were: extrathyroidal tumor extension (T4), the presence of distant metastases and older age (Table 1). Significant TSH related predictors for thyroid carcinoma related death were the 50th percentile (HR: 2.03) and the percentage of TSH values above 4.5 mU/l (HR: 1.06).

When the significant variables detected by univariate Cox regression analysis were introduced into a stepwise multivariate model, T4, M1 and older age remained significant predictors for thyroid carcinoma related death (Table 1). For all cured patients, the 50th percentiles (HR: 2.14) of TSH values and the percentage of TSH values above 4.5 mU/l (HR: 1.07) were significant independent predictors for thyroid carcinoma related death (Table 3).

The effect of median TSH on thyroid carcinoma related death became only discernible at a cut-off level of 2 mU/l (Figure 1). At cut-off levels of 0.1 and 0.4, no significant difference in thyroid carcinoma related death was observed (Figure 1).

To investigate the relation of TSH in patients with recurrent and/or metastatic whose tumor did- or did not accumulate radioiodine we studied the 38 patients with recurrence after initial cure. Of these patients, 8 had metastases with radioiodine uptake. None of these patients died, whereas the 12 of the 30 patients who had no uptake of radioiodine died. In an additional analysis in which we built an interaction term comprising TSH and radioiodine uptake, we found a hazard ratio for thyroid carcinoma related death of 2.24 (CI 1.53 – 3.29) in patients without radioiodine uptake vs. patients in whom radioiodine uptake was present.

Relapse Free Survival

At univariate Cox regression analysis, significant indicators for relapse in patients who were cured 1 year after initial therapy were: extrathyroidal tumor extension (T4), the presence of distant metastases and older age (Table 1). A significant TSH related predictor was the 50th percentile (HR: 1.46)

When the significant variables obtained by univariate Cox regression analysis, were introduced into a stepwise multivariate model, T4, M1 and older age remained significant predictors for relapse (Table 1). For all cured patients, the 50th percentile of TSH (HR: 1.41) was a significant independent predictor for thyroid carcinoma related death (Table 3). The effect of median TSH on relapse became only discernible at a cut-off level of 2 mU/l (Figure 1). At cut-off levels of 0.1 and 0.4 mU/l, no significant differences in relapse were observed (Figure 1)

DISCUSSION

In the present study we investigated the association between serum TSH concentrations in patients during follow-up for DTC, thyroid carcinoma specific mortality and risk for recurrence. The study differed from earlier studies in the homogeneity of the patient group with respect to initial therapy (49;84;85), the study size and the duration of follow-up (83).
We found positive associations between serum TSH concentrations and risk for thyroid carcinoma related death and relapse. In a multivariate Cox-regression analysis model, in which tumor stage and age were also included, this association remained significant in patients who have been cured 1 year after initial therapy. The median of the TSH concentrations in each patient appeared to be the best predictor for thyroid carcinoma related death and relapse. However, subsequent analyses revealed that this effect became apparent at higher median TSH values (cut-off level of 2 mU/l). No differences in risks for thyroid carcinoma related death and relapse were observed between suppressed TSH levels (both TSH < 0.4 mU/l and <0.1 mU/l) and unsuppressed TSH levels (TSH levels within the reference range). Interestingly, this association between TSH levels and risk for relapse or thyroid carcinoma related death was present both in patients with initial stages T1-3 and M0 and with stages T4 or M1. Even for initial tumor stage T1-3 and M0, median TSH was an independent predictor for thyroid carcinoma related death. These results differ from the studies of Mazzaferri et al (49) and Cooper et al (84), who did not find an independent relation between TSH and prognosis. Our patient group is comparable with the study of Pujol et al. (83). Pujol et al found a difference in relapse between the extremes of TSH suppression (continuously undetectable vs. continuously unsuppressed). Pujol et al, however, did not report the relation between TSH levels and thyroid carcinoma related death. Our study results are in line with the recent report of Jonklaas et al. (85) who demonstrated that the degree of TSH suppression is a predictor of thyroid carcinoma specific survival in high risk patients, independently of radioiodine ablation therapy and the extent of thyroid surgery. Our analysis extends their findings in the respect that in patients who received total thyroidectomy and radioiodine ablation, and who were cured 1 year after initial therapy, TSH remains an independent predictor for disease specific survival. Our study confirms the findings of Jonklaas et al. that this relation is only present at TSH levels in the higher normal range, so that sustained TSH suppression is not recommended in low risk patients.
Because our study is based on retrospective data, the analyses might have been susceptible to bias. However, we could not find differences in summarizing parameters of serum TSH levels between high-risk and low-risk patients. In addition, differences in follow-up intensity between patients with higher and lower TSH levels could result in bias. However, the amount of TSH measurement per year did not differ significantly between patients who died of thyroid carcinoma and who did not. Moreover, even if such a difference would have been present, lower, rather than higher, TSH levels would be expected in high risk patients. Therefore, we believe that the results of our study are valid.
The percentage of patients reaching the target TSH range was lower than favorable (~73% below < 0.4 mU/l). We found no differences in TSH levels between high- and low-risk patients so that physician-related differences in target TSH levels between high- and low risk patients is an unlikely explanation. Another explanation could be that over time, the

physicians would have been less focussed on keeping TSH at the target levels. However, we did not find any time dependency of TSH.

The results of our study, e.g. that the deleterious effects of TSH on thyroid carcinoma recurrence or thyroid carcinoma related death become apparent above a median TSH of 2 mU/l, provide a rationale for the advice in the recently published European and United States guidelines for the follow-up of thyroid carcinoma to aim at TSH levels in the lower normal range (0.4 – 1 mU/l) in low-risk DTC patients (215;216) as unnecessary TSH suppression is associated with lower bone mineral density (86) and cardiac dysfunction (87;217).

Although relation between TSH levels and risk for thyroid carcinoma related death or recurrence was also present in non-cured patients and patients with an initially high risk, subgroup analysis did not reveal a safe TSH threshold in these patients. Because we found indications that the hazard of elevated TSH levels for thyroid carcinoma related death is especially important in non-iodine accumulating metastases, and given the findings of Jonklaas et al (85) we advice to maintain suppressed TSH levels (<0.1 mU/l) in patient categories with initial high risk and/or recurrent tumor.

Chapter 3

Treatment of the follicular thyroid carcinoma cell-line FTC-133 with Troglitazone and Lovastatin synergistically increases expression of G1 transition inhibitors and induces re-differentiation

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Submitted

ABSTRACT

Studies have shown that thiazolidinediones e.g. troglitazone and statins e.g. lovastatin are, in addition to their primary indication, also effective inhibitors of growth and invasion of tumor cells of various origins. Recently it was demonstrated that a combination of clinically achievable concentrations of lovastatin and troglitazone can produce a dramatic synergistic effect on growth in human glioblastoma and CL1-0 human lung cancer cells lines in vitro. The exact mechanism is still unclear but it was demonstrated that p27kip1 protein was significantly elevated and the phosphorylation status of Rb was reduced. A possible mechanism for this cell cycle arrest and apoptosis by both thiazolidinediones and statins may result from PTEN upregulation.

In addition to anti-cancer effects, thiazolidinediones and statins have been shown to have redifferentiating effects in DTC. This re-differentiating effect may be highly beneficial in patients with differentiated thyroid cancer as iodine uptake can be lost in DTC metastases due to de-differentiation. Once NIS is lost, treatment becomes problematic as I-uptake via NIS is vital for successful treatment with radioactive iodine.

We decided to further explore the beneficial in vitro effects of a combination of lovastatin and troglitazone in the follicular thyroid carcinoma cell-line FTC-133 on growth and apoptosis. After exposing the cells to Troglitazone and/or Lovastatin treatments for up to 4 days we tested for cell-growth and induction of apoptosis by MTS-assay and FACS analysis. To further elucidate the mechanisms leading to cell-cycle arrest we tested the expression levels of inhibitors of CDK4/6 cyclin complex assembly (p15, p16 and p27) by RT-PCR. In addition, we also evaluated the beneficial in vitro effects of a combination of lovastatin and troglitazone on the expression of the TSH receptor and NIS genes via RT-PCR.

In our study we found that in the human thyroid follicular cell-line FTC-133, the combination of lovastatin and troglitazone resulted in a remarkable synergistic effect on morphology and cell density. These effects coincide with redifferentiation as was demonstrated by an increase in TSH-receptor and NIS expression.

INTRODUCTION

The primary clinical indication of statins is in hypercholesterolemia and the prevention of myocardial infarction, whereas that of the thiazolidinediones is in improving insulin sensitivity in type 2 diabetes mellitus patients.
Studies have shown that both classes of drugs are, in addition to their primary indication, also effective inhibitors of growth and invasion of tumor cells of various origins. The anticancer activity of statins was intensively studied and in vitro studies show an effect on growth and invasion of tumor cells e.g. anaplastic thyroid cancer, melanoma, prostate cancer and pancreatic cancer (118;119;218-220). In vitro beneficial effects of thiazolidinediones have been described in a number of malignancies e.g. breast cancer, hepatocellular carcinoma, pancreatic cancer, ovarian carcinoma, melanoma, lung carcinoma, and lymphoma cells (221-226). On the molecular level statins and thiazolidinediones have different cellular targets. Statins (e.g. lovastatin) are potent inhibitors of HMG-CoA reductase by binding to HMG-CoA reductase, the rate-limiting enzyme of the mevalonate (MVA) pathway, approximately 1000-fold more effective than the natural substrate (Wong,2002;Demierre,2005) whereas thiazolidinediones (e.g. troglitazone) are peroxisome proliferator-activated receptor (PPAR) agonists. Until recently statins and thiazolidinediones were only tested separately for anticancer effects. Recently, Yao et al. found that a combination of clinically achievable concentrations of lovastatin and troglitazone can produce a dramatic synergistic effect against human glioblastoma and CL1-0 human lung cancer cells lines in vitro at low concentrations. They found a significant elevation of p27kip1 protein and a reduced phosphorylation status of Rb (120). The exact mechanism is still unclear but it has been suggested that PTEN upregulation is a possible mechanism for cell cycle arrest and apoptosis by both thiazolidinediones and statins (227;228). In addition to growth related anti-cancer effects, thiazolidinediones and statins have been shown to have redifferentiating effects in DTC. Frohlich et al. investigated the effects of troglitazone, rosiglitazone and pioglitazone on differentiation in normal porcine thyrocytes and in follicular carcinoma cell-lines FTC-133 and FTC-238. Troglitazone was most effective of the tested thiazolidinediones in re-differentiating the carcinoma cell-lines as demonstrated by significantly increased radio-iodine uptake and subsequent apoptosis (229). In a clinical study it was demonstrated that rosiglitazone was able to induce uptake of radioiodine in DTC in vivo (114). In the thyroid derived cell-lines FTC-133, FTC-238 and ARO an increase in differentiation has previously been shown. Wang et al. also found a significant effect of lovastatin on differentiation of the anaplastic thyroid cancer ARO cell-line. At a dose of 25 μM, lovastatin was able to significantly increase iodine uptake (118). This re-differentiating effect, which was observed at clinically achievable concentrations of lovastatin and troglitazone, may be highly beneficial in patients with differentiated thyroid cancer as iodine uptake can be lost in DTC metastases due to de-differentiation. Once NIS is lost treatment becomes problematic as I-uptake via NIS is vital for successful treatment with radioactive iodine.
We decided to further explore the beneficial in vitro effects of a combination of lovastatin and troglitazone in the follicular thyroid carcinoma cell-line FTC-133 on growth and apoptosis and to further elucidate the mechanisms leading to cell-cycle arrest by inhibitors

of CDK4/6 cyclin complex assembly (p15, p16 and p27). In addition, we also evaluated the beneficial in vitro effects of a combination of lovastatin and troglitazone on the expression of the TSH receptor and NIS genes

MATERIALS & METHODS

CELL CULTURE

FTC-133 cells were cultured routinely in Ham's F12 medium (Gibco BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 100 IU/ml penicillin (Life Technologies, Rockville, USA) and 100 µg/ml streptomycin (Life Technologies, Rockville, USA) in a humidified atmosphere of 5% CO2 and 95% O2 at 37ºC. Cells were trypsinased and transferred (1:3) to new medium every 3-4 days. For experiments, the cells were seeded at a density of 1x104/cm2 and the various treatments started after 24 h.

TREATMENTS

Troglitazone was purchased from the Cayman Chemical Company (Tallinn, Estonia), Lovastatin was purchased from Ag Scientific (San Diego, USA) and Geranylgeranyl transferase I (GGTI) purchased from Sigma (St.Louis, USA). Troglitazone and lovastatin were added to FTC-133 cells at a concentration of respectively 10µM and 1µM and the cells were exposed to this treatment for 2 days unless stated otherwise. GGTI (25µM) was used to selectively block the geranylgeranylation of proteins whereas lovastatin blocks both geranylgeranylation and farnesylation.

CELLTITER 96® AQUEOUS ONE SOLUTION CELL PROLIFERATION ASSAY

The effect of troglitazone, lovastatin or the combinational treatment for up to 4 days on growth (n=6) was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison,USA) which is based on the conversion of a MTS tetrazolium compound (Owen's reagent) by cells into a colored formazan product. In brief, 20µl of CellTiter 96® AQueous One Solution Reagent was added into each well of a 96-well assay plate containing the samples in 100µl of culture medium. The plate was incubated for 2 hours at 37°C in a humidified, 5% CO2 atmosphere. Absorbance was recorded at 490nm using the SPECTRAmax GEMINI Microplate Spectrofluorometer plate reader (molecular devices, Sunnyvale, CA, USA).

FACS EXPERIMENTS

Annexin V-FITC (Bender MedSystems, Vienna, Austria) was used to detect phosphatidylserine on the outer leaflet of the cell membrane thus measuring initiation of apoptosis. In brief, after treatment with 10µM troglitazone and/or 1µM Lovastatin for 1 or 2 days, cells

were harvested by centrifugation, washed 1 time with ice-cold PBS and resuspended in binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2,5 mM CaCl2) at a concentration of 1 × 106 cells/ml. A total of 5 µl of Annexin V-FITC and 5 µl of 20 µg/ml Propidium iodide (PI) were added to 100 µl of cell suspension and incubated for 15 min in the dark before addition of 400 µl of binding buffer. Quantitative analysis of the apoptotic percentage of 10,000 cells was performed using the FACScan Analyzer (Becton Dickinson , Franklin Lakes, USA). Cell debri and PI positive cells were excluded by gating the cells and the shift in the percentage of Annexin positive cells was taken as a measure of apoptosis.

WESTERNBLOT

Whole cell extracts were subjected to SDS-polyacrylamidegel electrophoresis and transferred to a nitrocellulose membrane (0.45 µm) (Pierce, Rockford, IL,USA) all subsequent steps were performed in the presence of a commercial protease inhibitor cocktail in the recommended dose (Roche, Basel Switzerland). After 20 minutes of blocking at room temperature the membrane was incubated with the primary antibody GAPDH (sc-25778) (Santa Cruz Biotechnology,inc, Santa Cruz, USA) at 4C O/N followed by a 1h incubation with horseradish peroxidase conjugated secondary antibody. The immune complexes were visualized using the Molecular Imager ChemiDoc XRS System (Biorad, Hercules, CA, USA). Following equalization of the amounts of protein by comparing using GAPDH expression, GAPDH, Rb and p-Rb expression was visualized using the primary antibodies Rb(4H1) mAb and phosho-Rb(Ser807/811) (cell signaling Technology, Danvers, USA) as described before.

cDNA

Total RNA was extracted by using Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) and RNA concentrations were determined by measuring the absorbance at 260 nm. Genomic DNA was eliminated and RNA was reversed transcribed using the RT2 first strand kit (Superarray bioscience corporation, Frederick, USA)

RT-PCR

Gene expression was examined using the RT2 system from superarray according to the manufacturers guidelines (Superarray bioscience corporation, Frederick, USA). In short, a 25µl mix was made by mixing 1µl primerset (NIS (SLC5A5), TSHr (TSHR) or P27 (CDKN1B)) with 11,5 µl H2O containing 25ng cDNA and 12,5µl RT2 Real-time sybr green/Fluorescein PCR Master MIX (Superarray bioscience corporation, Frederick, USA). All RT-PCR's were performed in triplicate using a two-step cycling program (1 cycle 10'95C followed by 40 cycles 15"95C; 1'60C) on the BioRad iCycler (Biorad, Hercules, CA, USA)
Results were expressed as fold induction compared to untreated cells:

fold induction = 2^((control-beta)-(gene-beta))

Gene = Ct gene of interest in treated group

Beta = Ct beta-actin
Control = Ct gene of interest in untreated group

STATISTICAL ANALYSES

Results are expressed as the mean plus or minus the standard error of mean. Student's t-tests were used for all hypotheses testing. All statistical analysis was performed using SPSS version 14.0 (SPSS, Inc., Chicago, IL). A p value of <0.05 was considered significant.

RESULTS

GROWTH

The follicular thyroid carcinoma cell-line, FTC-133 was exposed to Troglitazone and/or Lovastatin treatments for 2 days resulting in a remarkable synergistic effect on morphology and cell density when treatments were combined (Figure1). Cells which received only one of the treatments appear normal whereas the troglitazone/lovastatin combination resulted in decreased growth and rounding up of cells. Similar to the troglitazone/lovastatin combination the geranylgeranylation blocker GGTI in combination with 10M troglitazone also resulted in decreased growth and rounding up of the cells whereas GGTI alone did not (Figure 1).
Initially only the combination of troglitazone and lovastatin inhibited cell growth(89%, P=0,0019) whereas longer exposure times also resulted in impaired cell growth for the individual treatments (Figure 2). At day 2 the troglitazone and lovastatin treatment alone had no effect on growth while the combination resulted in a lower amount of viable cells (89% of the untreated cells). A 4 day exposure resulted in impaired growth of the cells which received treatment of troglitazone or lovastatin (90% troglitazone, P=0,0432; 68% lovastatin,P=0,0009) while the combination gave an additional effect (46%, P<0.0001) (Figure 2). After transfer to normal medium without lovastatin and troglitazone most cells were still viable and resumed normal growth and morphology (data not shown).

FACS ANALYSES

In order to test if impaired cell growth and detachment of the cells was due to apoptosis we performed a FACS analysis using ANNEXIN V which binds to the apoptotic marker phosphatidylserine (PS). None of the treatments resulted in increased binding of Annexin V to the cell surface of PI negative cells which received treatment for 1 or 2 days (Figure 3).

WESTERN-BLOT

As the phosphorylation state of the Rb protein plays a pivotal role in the negative regulation of the cell cycle we performed a western blot with antibodies specific for the state of phosphorylation (Figure 4). These data confirmed that the combination of troglitazone and

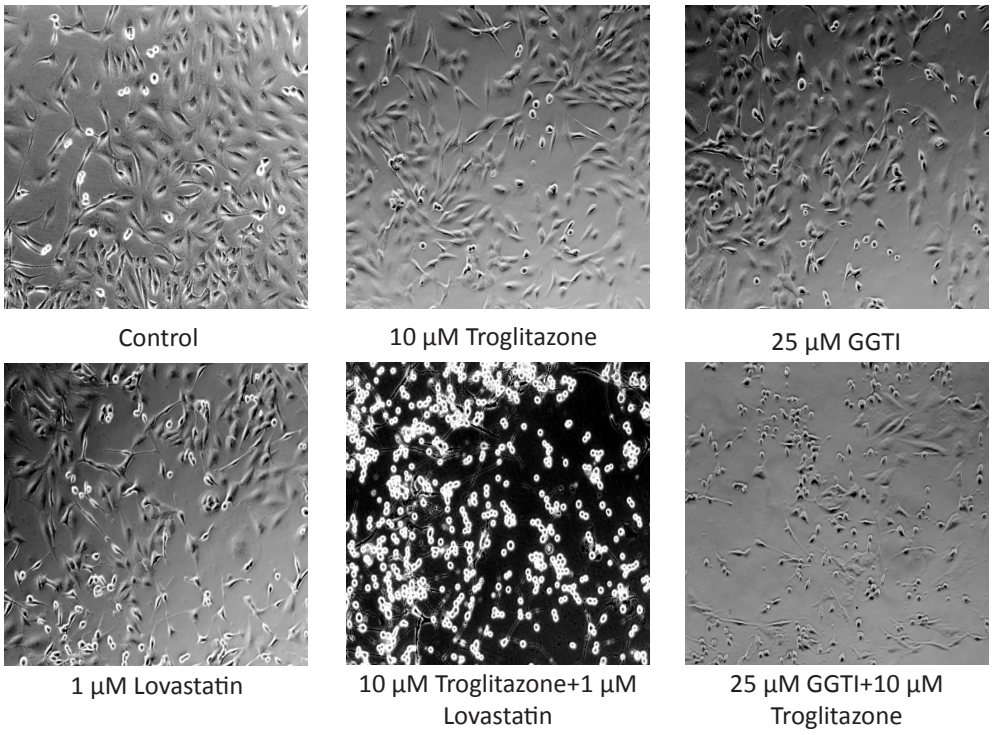

FIGURE 1. Effect of Troglitazone, Lovastatin and GGTI voluit treatments on FTC-133 cells. Cells were exposed to 10 µM Troglitazone, 1µM lovastatin, 25 µM GGTI or a combination for 2 days. The combination of troglitazone and lovastatin resulted in a remarkable effect on morphology and cell density, the cells appear to be less dense and rounded up (50x).

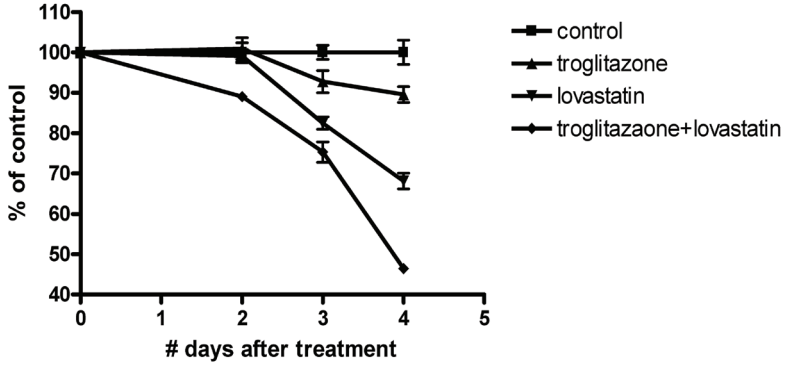

FIGURE 2. The effect of troglitazone, lovastatin or the combinational treatment for up to 4 days on growth was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay(Promega, Madison,USA) as described in materials and methods. Viable FTC-133 cells are depicted as percentage of untreated cells. After 2 days of treatment the troglitazone-lovastatin combination resulted in reduced growth vs. control of 89% (P=0,0019) Individual treatments with either troglitazone or lovastatin also resulted in impaired cell growth from day 3. Measurements were performed at least in triplicate. (troglitazone, 90%, P=0,0432; lovastatin, 68%, P=0,0009; T+L, 46%, P<0.0001)

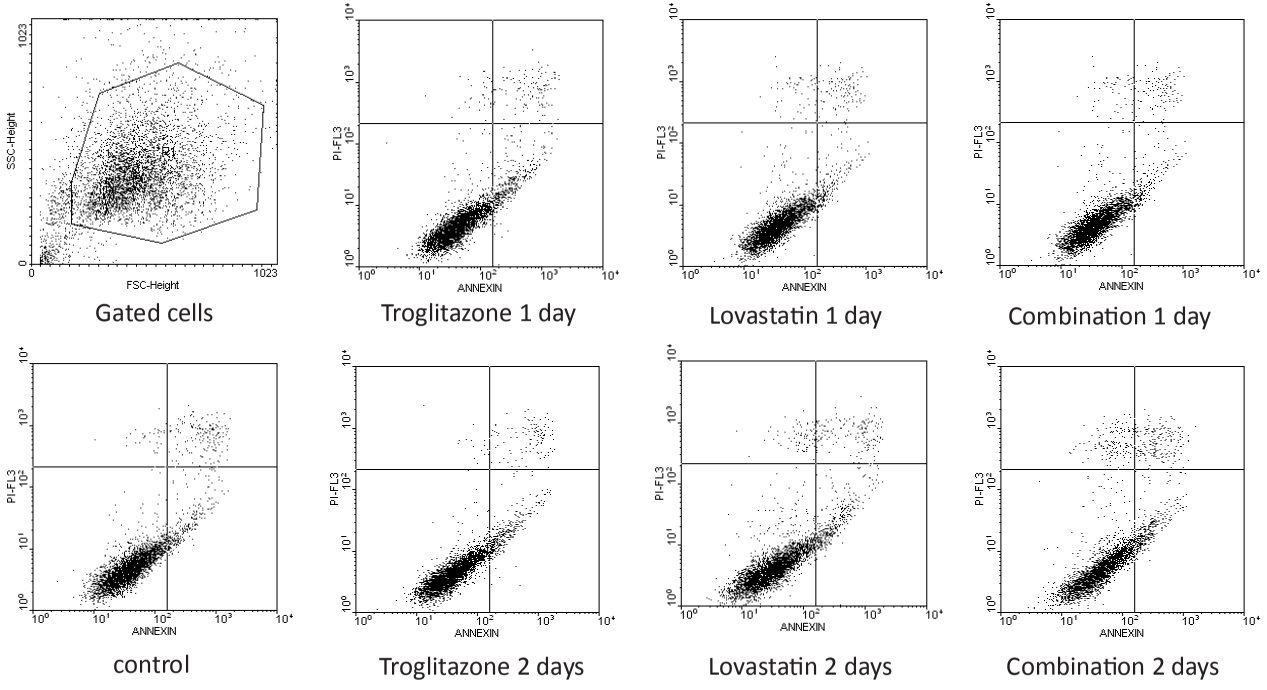

FIGURE 3. FACS analyses for detection of apoptosis using the apoptosis marker phosphatidylserine as described in materials and methods. ANNEXIN V-FITC binding is depicted on the horizontal axis and PI on the vertical axis. None of the treatments resulted in an increased binding of Annexin V to the cell surface. There was a tendency for increased binding of annexin V after the combination of treatments for two days but this was not significant(P=0,06). Evaluation of the cell populations which were positive for AnnexinV/ and negative for PI staining are depicted below the histograms.

Percentage lower right of total gated cells

	Control	Troglitazone	Lovastatin	Combination
1 day treatment	6,81	7,6	6,09	8,705
		(p=0,63)	(p=0,60)	(p=0,53)
2 days treatment	6,81	7,125	7,525	9,085
		(p=0,85)	(p=0,38)	(p=0,056)

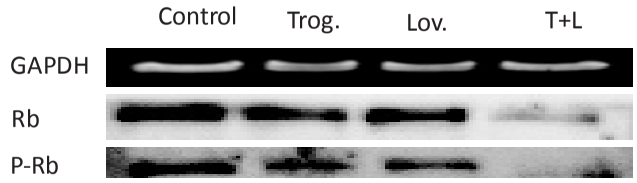

FIGURE 4. Synergistic effects of lovastatin and troglitazone on the phosphorylation status of Rb. Cells were exposed to 10 µM Troglitazone, 1µM lovastatin or the combination for 2 days and cell lysates were analysed by westernblot as described in materials and methods.

TABLE 1. FTC-133 cells were treated with troglitazone(10 µM) and /or lovastatin(1µM) for 2 days. Fold in- or decrease of P15, P16,P27, NIS and TSHr mRNA expression was compared to untreated cells (control) using RT-PCR as described in materials and methods. Fold in- or decrease was calculated using the formula: fold induction= 2^((control-beta)-(gene-beta)).

Treatments	**P15**		**P16**		**P27**		**TSHR**		**NIS**	
	fold difference	*p*	*fold difference*	*p*	*fold difference*	*p*	*fold difference*	*p*	*fold difference*	*p*
Troglitazone vs control	1,32	0,837	8,83	0,089	2,35	0,038	0,91	0,235	2,81	0,038
Lovastatin vs control	2,29	0,076	7,09	0,002	2,37	0,002	5,38	<0.001	12,26	<0,001
Trog.+Lov. vs control	9,85	0,008	12,94	0,014	3,35	0,061	3,16	0,02	10,1	0,005

lovastatin induced a downregulation of phosphorylated Rb.

RT-PCR

In order to explore the mechanisms behind the negative effect on cell-cycle progression by troglitazone, lovastatin and the combination we tested the expression of P15, P16 and P27 which are known to play a role in cell-cycle progression using RT-PCR (Table 1). In addition, we studied the effects of troglitazone and/or lovastatin on cellular differentiation by a RT-PCR on the thyroid specific genes NIS and the TSHreceptor. Treatment with troglitazone gave no rise in TSHr expression but induced NIS expression almost 3-fold (P=0,038). Lovastatin induced TSHr expression more than 5-fold (P<0,0001) while NIS was induced 12-fold(P=0,0005). When combined troglitazone and lovastatin treatments induced TSHr expression 3-fold (P=0,02) and NIS 10-fold (P=0,005).

DISCUSSION

The combination of a Troglitazone and/or Lovastatin treatment resulted in a remarkable synergistic effect on morphology and cell density in the human follicular thyroid carcinoma cell-line, FTC-133. An effect which was previously reported by Yao et al. in human glioblastoma and CL1-0 human lung cancer cells lines in vitro at similar low concentrations (120). They contributed the effects in part to the inhibition of the mevanolate pathway and tested this by counteracting the effects of the combined therapy with the addition of mevalonolactone (120). Blocking the mevanolate pathway will result in both inhibition of farnesylation and geranylgeranylation. Specific blockers can block either farnesylation (Farnesyl transferase inhibitor, FTI) which activates RAS proteins or geranylgeranylation (GGTI) which activates Rho proteins (230).
We could mimic the effect on cell growth and morphology of the troglitazone/lovastatin combination by the combination of the geranylgeranylation blocker GGTI with 10M troglitazone indicating that inhibition of geranylgeranylation in combination with troglitazone treatment is sufficient to induce the effects observed on growth and morphology. This points to a Rho related mechanism rather then Ras as GGTI inhibits geranylgeranylation of Rho (230).
In order to determine if the impaired cell growth and detachment of the cells was due to apoptosis or only to G1 cell arrest which was demonstrated by the phosphorylation state of Rb we performed a FACS analysis using ANNEXIN V. None of the treatments resulted in increased binding of ANNEXIN V to the cell surface. Additionally, most cells were still viable and resumed normal growth and morphology after transfer to normal medium showing that the cells appear to arrest rather than move into apoptosis after receiving the Troglitazone/Lovastatin combination treatment. Higher doses of lovastatin do appear to cause apoptosis as Wang et al. observed apoptosis in ARO-cells with a lovastatin dose of 50 µM (118).
One possible explanation for the observed growth inhibition may lay in Rho related inhibition via p27 an inhibitor of CDK4/6 cyclinD complex assembly. Geranylgeranylation of Rho

is essential for degradation of this inhibitor and facilitates progression of G1 to S phase (231). To initiate this degradation, Rho needs to be activated by geranylgeranylated during the G1 phase a process blocked by lovastatin and GGTI (232;233). Geranylgeranylation enables RhoA to be positioned at the inner face of the plasma membrane where it serves as a switch in cytoplasmic cascades by switching between an active (GTP) and inactive state (GDP)(232;233). Troglitazone also appears to have an effect on several cell cycle regulators including an increase of p21 and p27 levels and reduction in phospho-Rb in several cell lines such at the mRNA and protein level in rat and human hepatoma cells. Furthermore, forced expression of p27 results in G1 phase cell-cycle arrest in most cell-lines (234).
On the protein level Yao et al. (120) observed this effect on p27 when using the combination treatment. In addition to the known effects on degradation of p27 via Rho we observed 12 fold increase in p16 expression and an almost 10 fold increase of P15 expression when the troglitazone and lovastatin treatment were combined.
P15INK4b and P16INK4a are members of INK4b-ARF-INK4 a tumor suppressor locus. An excess of these inhibitors can cause G1 cell-cycle arrest by blocking the assembly of the catalytical active CDK4/6 cyclinD complex which facilitates Rb phosphorylation (235).
P15 and p16 are more primarily associated with growth arrest whereas p21 and p27 are more associated with apoptosis (236). This seems to correspond with our findings that the FTC-133 cells only experience growth arrest and no apoptosis after treatment. So an accumulation of these CDK inhibitors is likely to result in G1 phase cell-cycle arrest rather then the induction of apoptosis. The effects on p15 and p16 give at least a partial explanation for the inhibitory effects of the troglitazone/lovastatin treatment but multiple pathways may be involved.
Upregulation of PTEN expression via ppar-gamma has been suggested as a possible mechanism for cell cycle arrest and apoptosis by both glitazones and statins (227;228). Troglitazone is a well known PPAR-gamma agonist and statins have also been shown to activate PPAR gamma in a dose dependent manner by inhibiting the mevanolate pathway. Using double negative constructs Yano et al. determined that activation of ppar-gamma by statins seems to be regulated via RhoA rather then Ras or Rac (237). The hypothesis that PTEN expression could be involved in the induction of growth arrest via PPAR-gamma is supported by the presence of two PPAR-gamma response elements in the genomic sequence upstream of PTEN (227). Furthermore Teresi et al. showed that both lovastatin and the glitazone, rosiglitazone are able to increase PTEN expression through PPAR-gamma in a dose dependant manner. After 2 days a dose of 1-10µM of lovastatin proved to be most effective in stimulation PTEN expression. In our experiments we used the thyroid cancer cell-line FTC-133 which harbours a splice variant resulting in a PTEN negative phenotype (238). As we saw identical effects on growth compared to the effects described by Yao et al. we conclude that PTEN upregulation is not essential for the induction of growth arrest in cell-lines treated with troglitazone and lovastatin. However, PTEN expression may play a role in the induction of apoptosis after troglitazone and lovastatin treatment in cells which are capable of PTEN expression.
Besides reduced growth and invasion of tumors, both lovastatin and troglitazone have previously been shown to promote cellular differentiation in thyroid derived cell-lines(FTC-133, FTC-238 and ARO), a feature which may be beneficial in improving conventional RAI

therapy which is based on I131-uptake by NIS (118;121).
Using RT-PCR we observed an increase in NIS and TSHr expression when we combine d the lovastatin and troglitazone treatments. This redifferentiating effect can be largely accounted for by the lovastatin treatment although the troglitazone treatment alone was able to upregulate NIS expression.
The effects of the combined troglitazone/lovastatin treatment we observed seems to be universal for cancer cell-lines as Yao et al. discovered similar effects in human glioblastoma, lung-, prostate-, pancreatic- and cervical cancer cells lines (120). The effects on the CDK inhibitors give at least a partial explanation for the inhibitory effects of the troglitazone/lovastatin treatment but multiple pathways may be involved. Although the synergism of troglitazone and lovastatin is dramatic in vitro its usefulness will still have to be proven in vivo. However, there is hope that the combination of troglitazone and lovastatin can induce the effects on growth and differentiation status in vivo, because the synergistic effects were found at clinically achievable concentrations in the human follicular thyroid carcinoma cell-line, FTC-133 (116;239;240). Therefore we think that a combined Troglitazone/Lovastatin treatment may proof to be beneficial in patients with DTC as remarkable reduction of growth coincides with increased NIS expression.

Chapter 4

Superior Thyrotropin Receptor Binding and Activation of a Novel, Modified, Single Chain Thyroid Stimulating Hormone

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Submitted

ABSTRACT

We describe the design of a modified single chain TSH molecule which is capable of potently stimulating the TSH-receptor. Furthermore, the enhanced stimulating effect is maintained after the fusion of small proteins to the N-terminus indicating that our modified single chain TSH may be able to function as a carrier of proteins.

TSH subunits α and β were cloned from a human pituitary tumor, amplified with overlapping primers and fused in a second PCR resulting in a β-α orientated TSH with an intact β-chain secretion signal. In order to create a superactive scTSH, we introduced several mutations in the scTSH. We tested the properties of scTSH for binding to and activation of the TSH receptor using a TSHR expressing CRE-Luc modified CHO cell-line and the relevant biological endpoint of iodine uptake by FRTL-5 cells. We subsequently fused a 6xhistidine tag with flexible linker, alone and in combination with a six amino-acid sequence to the N terminus of scTSH. We found that the modified scTSH has superior TSHR binding and activation properties in comparison with wtTSH which lead to an increased uptake of radioiodine by FRTL-5 cells. Addition of a 6xhistidine tag with a flexible linker with and without a small protein extension did not compromise these properties.

A possible application of modified TSH may lie in the specific targeting of metastases derived from differentiated thyroid carcinoma (DTC). The success of conventional radioiodine therapy in metastases of differentiated thyroid carcinoma (DTC) is limited by insufficient uptake of radioiodine. Therefore, new therapeutic strategies are needed. The retained expression of the receptor for thyroid stimulating hormone (TSHR) in most advanced DTC tumors offers a perspective for a targeted approach. The development of this modified TSH may be the first step in the development of a carrier-protein for cytotoxic drugs capable of specifically targeting thyroid tumor cells through the TSHR.

INTRODUCTION

Thyroid stimulating hormone (TSH) is a 28- to 30-kDa member of the glycoprotein hormone family, which also includes chorionic gonadotrophin (CG), luteinising hormone (LH) and follicle stimulating hormone (FSH). These hormones consist of a common α- and a hormone-specific β-chain, which are non-covalently linked (132;137;241). TSH which is synthesized and secreted by the pituitary regulates thyroid hormone production in the thyroid gland and is essential for thyroid hormone homeostasis in target organs by the classical pituitary-thyroid feedback loop. In addition, TSH inhibits its own secretion by an ultra short negative feedback loop within the pituitary gland (132;242). Both the α- and β-chain of TSH are important for binding to, and activation of the TSH receptor (TSHR). The α- chain is identical for all the members of the glycoprotein-hormone family and consists of 92 amino acids whereas the 118 amino acid beta chain is unique to TSH and determines specificity (132;137;138). The TSHR, as well as other proteins involved in iodine metabolism and thyroid hormone production, are largely specific for the thyroid and thyroid derived tumors. For decades, one of these unique features, i.e. the expression of the sodium iodine symporter (NIS), has been successfully used in the treatment of thyroid tumors by using radioactive iodine therapy, since through the presence of NIS, iodine selectively accumulates in thyroid tissue. In part because of the success of radioiodine therapy, thyroid cancer has an excellent prognosis. However, in a subset of patients, undifferentiated thyroid tumors, and more particularly distant metastases, have lost this unique characteristic. In these patients, radioactive iodine therapy is ineffective and new treatment modalities are needed. The specificity of TSH for the TSHR may be used for delivering of drugs specifically to the tumor cells, for example by using TSH as a carrier of fusion proteins or cytotoxic drugs. Although TSHR expression is lost in poorly differentiated thyroid carcinoma, TSHR expression is more persistent than other thyroid specific proteins. This has been demonstrated by the presence of TSHR expression in a large panel of thyroid carcinomas by immunohistochemistry (155;156). Before TSH can be used as a selective carrier of toxic proteins to thyroid carcinomas, various problems need to be solved. Wild type (wt) TSH is produced as separate alpha and beta chains and assembly of these alpha and beta chains is a rate limiting step in TSH formation. This may be a problem in the pharmaceutical production of recombinant TSH. Furthermore, TSH is posttranslationally modified by glycosylation, which is important for both hormone stability and bioactivity. This posttranslational glycosylation is another problem in the production of recombinant fusion proteins (170;171). At least part of these problems can be circumvented by the production of TSH as a single chain hormone (scTSH), in which the beta-chain is fused to one side of the alpha chain. Furthermore, a scTSH has improved hormone stability and increased serum half-life. This stabilizing effect of scTSH also compensated mutagenesis-induced defects in TSH that impaired dimer formation (172). The possibility to use these favourable characteristics of scTSH over wtTSH in creating a carrier of fusion proteins or cytotoxic drugs to thyroid carcinomas has not been explored in much detail.

Much of the success of TSH as a carrier of drugs depends on its specificity for thyroid derived cells and its affinity for the TSHR. The conversion of TSH to a scTSH alone does not result in a more potent TSH (168;243). However, specific mutations in the hairpin loops βL3 and αL3 improve binding dramatically in recombinant TSH consisting of a non-covalently bound α

and β chain (168;243). Whether the incorporation of these mutations in a scTSH also results in improved TSHR binding characteristics is unknown.

MATERIALS AND METHODS

CELL-LINES

We used TSHR expressing JP26-26 cells (151;194), stably transfected with a cAMP responsive element (CRE)-luciferase construct (TSHR-luc cells) as a read out for TSHR activation (244). B1 and CHO cells were routinely cultured in Ham's F12 medium (Gibco BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 100 IU/ml penicilline (Life Technologies, Rockville, USA) and 100 µg/ml streptomycin (Life Technologies, Rockville, USA). TSHR and CREe-luciferase in TSHR-luc cells were maintained by addition of 2µg/ml blasticidin and 400µg/ml geneticin (Life Technologies, Rockville, USA).

RECOMBINANT HUMAN TSH

Recombinant human TSH used as a comparison to our TSH constructs was obtained from Fitzgerald industries (Fitzgerald Industries, Concord, USA)

PITUITARY cDNA

Total RNA was extracted from a pituitary tumor using Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) and treated with RQ-DNAse (Promega, Madison, USA) to remove genomic contamination. RNA was reverse transcribed into cDNA using the Superscript First-Strand Synthesis System for RT-PCR in the presence of oligo(dT)$_{12\text{-}18}$ according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

CONSTRUCTION MSC-TSH BY PCR

All PCR reactions were done using the proofreading polymerase Pfu-ultra high fidelity (Stratagene, La Jolla, CA). The TSH α- and β-subunits were amplified by PCR from pituitary cDNA and subsequently fused in a second PCR using overlapping primers at the TSH beta terminus and TSH alpha start site removing the TAA stopcodon of the β-chain and the ATG startcodon of the α-chain. After the introduction of HINDIII and EcoRI restriction sites at the flanking regions (Figure 1) the single chain TSH construct was inserted into the expression vector pcDNA3.1 and sequence verified. Subsequently the mutations N66K (N(AAC) > K(AAG)), I58R (I(ATC)>R(CGC)) and E63R (E(GAA)>R(CGG)) were introduced into the L3 loops of the alpha and beta chain by overlapping primers resulting in a modified single chain TSH (Figure 2).
After the creation of the modified single chain TSH (mscTSH) six histidine residues were placed directly behind the β-chain secretion signal followed by 12 base pares coding for

a flexible linker (GAGG) with and without an additional 27 base pares using overlapping primers.

TABLE 1. Primers used for the conversion of wtTSH into a modified single chain TSH

	primer	primer sequence
Conversion to single chain TSH		
Introduction HindIII restriction site	1 fw	atatatat **aagctt** gccaccatgactgctctctttctgatgtc
Elimination stop codon β-chain	2 rev	cacatcaggagcgacagaaaatcctac
Elimination secretion signal+ stop α-chain	3 fw	ggattttctgtcgctcctgatgtgcag
Introduction EcoRI restriction site	4 rev	atatatat **gaattc** ttaagatttgtgataataacaagtact
Modifications		
I(ATC)> R(CGC)	5 rev	tacagtcctgta **gcg** gaagt
E(GAA)> R(CGG)	6 fw	acaggactgta **cgg** atacca
N(AAC)> K(AAG)	7 rev	ccattactgtgaccct**ctt**atatgatt
N(AAC)> K(AAG)	8 fw	**aag** agggtcacagtaatgggggtttc
Extensions		
His tag	rev	accagcaccatggtgatggtgatgatgagacatcgcttgcccacatg
	fw	caccatcaccatggtgctggtggcttttgtattccaactgagtata
His tag+extension	fw	caccatcaccatggtgctggtggctgttgtcctggctgttgcggtggcggc ttttgtattccaactgagtata

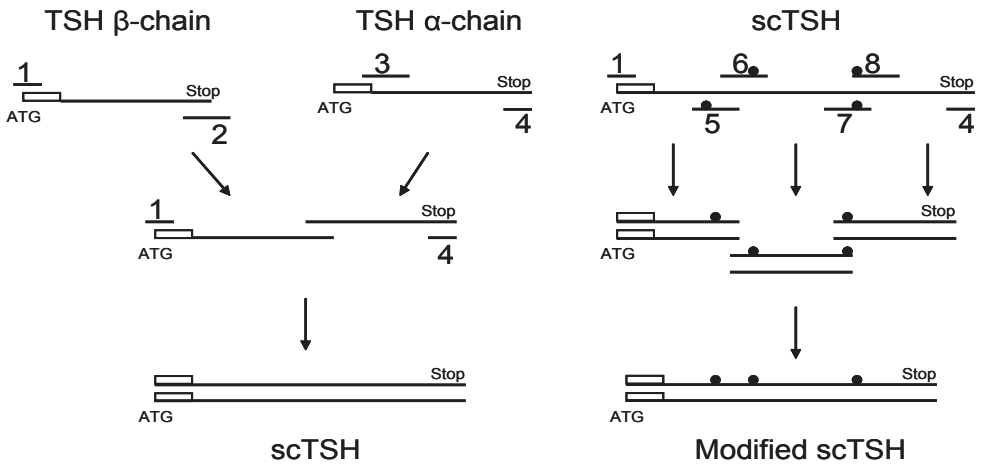

FIGURE 1. PCR scheme of the creation of modified single chain TSH constructs for insertion into the expression vector pcDNA3.1. The primer numbers correspond with primers depicted in Table 1. (A) The β chain with intact secretion signal minus termination signal was directly fused to the α chain removing the secretion signal in a two step PCR. (B) Mutations were introduced by modified primers (Table 1) resulting in three fragments which were subsequently fused in a PCR reaction using overlapping sequences.

STABLE TRANSFECTION OF CHO WITH MSCTSH

CHO cells were seeded at a density of $1,0*10^4$ cells/cm2 in a 6-wells plate and were incubated overnight before transfection. The next day the cells were transfected using Fugene 6 transfection reagent according to the manufacturer's instructions (Roche, Basel, Switzerland). The lipid–DNA complex was prepared by mixing 105 µl α-MEM and 2,5 µl fugene with 1µg pcDNA3.1-mscTSH. The mixture was incubated for 15' at room temperature and the lipid/DNA mix was added to the cells drop by drop. The cells were incubated for 8 h at 37°C/5%CO2 with the lipid–DNA complex, the medium was refreshed and 400µg/ml geneticine (Life Technologies, Rockville, USA) was added. Cells were incubated for 4 days at 37°C/5%CO_2 in an incubator and stably transfected clones were isolated. Conditioned medium which had been in contact with the mscTSH expressing cells for 3 days was tested for TSHR activating properties using the TSHR/CRE-luc transfected cell-line B1. Subsequently TSH levels were measured with the electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, USA) on the Modular Analytics E-170 (Roche Diagnostics, Indianapolis, USA).

CLONE SELECTION BY THE LUC BIOASSAY FOR TSH RECEPTOR ACTIVATION

B1 cells were seeded at a density of $2,5*10^4$ cells per well in 24 well plates in normal medium supplemented with blasticidin and incubated at 37˚C/5%CO_2 for 24 h followed by an interval in minimal medium (Ham's F12 medium supplemented with 0,5 % BSA). After 4h B1-cells were stimulated with conditioned medium from mscTSH producing cells. Luminescence was measured after 20h with the Luciferase Reporter assay system (Promega, Madison, USA) according to the protocol. Ten microliter of cell lysate was assayed for firefly luciferase using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin–Elmer, Boston, MA, USA).

PURIFICATION 6XHIS TAGGED MSCTSH BY NICKEL AFFINITY GEL

Purification of his-tagged proteins was done using HIS-Select™ Nickel Affinity Gel according to the manufacturer's protocol (Sigma-Aldrich Biotechnology, Saint Louis, USA)

IODINE UPTAKE

For I-uptake experiments, cells were grown in 12-well plates. Cells were incubated O/N (15h) after addition of 10mU/l of the TSH variants at 37 °C in a humidified atmosphere. Prior to the uptake studies, the cells were washed three times in Hanks' Balanced Salt Solution (HBSS), buffered with 10 mm Hepes (pH 7·5) Thereafter, HBSS containing 20 µm Na125I with a specific activity of 100 mCi/mmol was added to the cells and incubated for 30 min with the radioactive solutions.
The reaction was terminated by aspirating the radioactive mixture and cells were washed three times with ice-cold HBSS. Accumulated 125-I was determined by permeabilizing the cells with 500 µl ethanol for 20 min at 20 °C and measuring the released radioisotope in

a gamma counter. The DNA content of each well was subsequently determined after trichloroacetic acid precipitation by the diphenylamine method (245). Based on the specific activity of the substrates, the efficiency of the gamma counter and the DNA content of each well, iodide uptake was expressed as picomoles of substrate transported per microgram of DNA or as percentage of control conditions.

STABILITY

Recombinant hTSH and the mscTSH constructs were stored in CHO-II-SFM medium for 0, 1, 2 and 4 days at 37- and 56°C. TSHR stimulating activity at these time points was determined with the Luc-bioassay.

COMPETITION ASSAY

To measure binding competition we used a modified version of the Medizym T.R.A. (Medipan, Berlin, Germany) which is a competitive enzyme immunoassay test for autoantibodies to the TSHR. Here, we abandoned the step where patient serum should be added and instead we added our mscTSH directly to the TSH complex for competition. Subsequently, the protocol was followed according to the manufacturer (Medipan, Berlin, Germany). The optical density was measured at 450 nm versus 690 nm within 20 min after adding the stop solution.

STATISTICAL ANALYSIS

Results are expressed as the mean plus or minus the standard error of mean. Student's t-tests were used for all hypotheses testing. All statistical analysis was performed using graphpad prism (Graphpad software, inc., San Diego,USA)

RESULTS

DESIGN AND CONSTRUCTION OF MSCTSH

Total mRNA was isolated from a human pituitary tumor and converted to cDNA. TSH subunits α and β were amplified with overlapping primers and fused in a second PCR resulting in a β-α orientated TSH with an intact β-chain secretion signal. The sequence of the single chain TSH was verified and subsequently mutations were introduced into the alpha and beta chain resulting in a modified single chain TSH. Furthermore the accessible N-terminus of mscTSH was modified by inserting a 6xHis tag and 4-13 aminoacids in between the cleavage signal and the mature protein (Figure 2). Following construction all constructs were sequence verified.

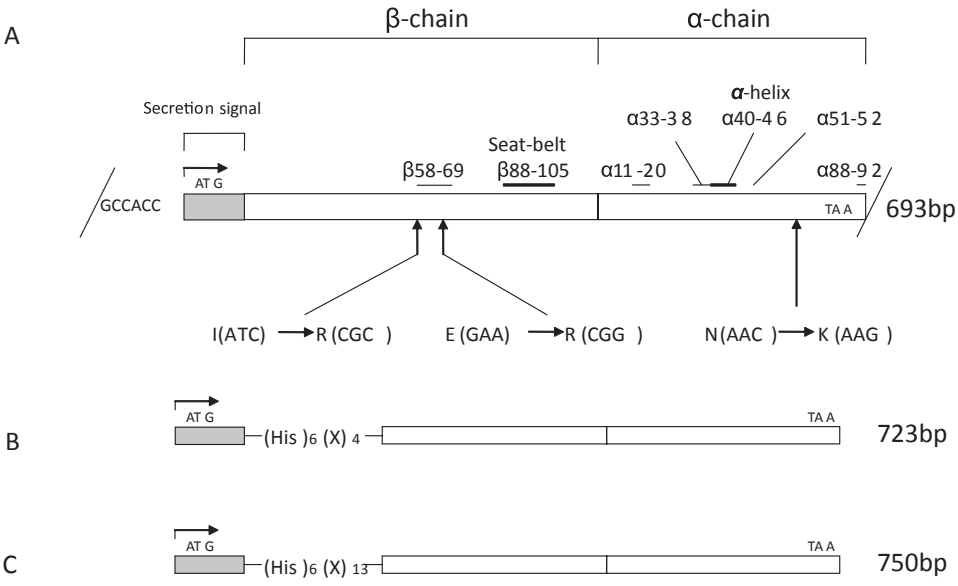

FIGURE 2. Modified single chain TSH constructs for insertion into the expression vector pcDNA3.1. (A) The β chain with intact secretion signal minus termination signal was directly fused to the α chain lacking the secretion signal. Modifications of TSH are depicted by arrows. Furthermore, structural features within TSH which have been shown to be important for binding to and activation of the TSH receptor (168;169) are depicted above the construct. The numbers represent the position of the modified aminoacids. The modified N184 corresponds with N66 in the native α chain. (B) Six histidine residues were placed directly behind the β-chain secretion signal followed by either 4(B) or 13(C) aminoacids coding for a flexible linker (GAGG).

EXPRESSION OF THE CONSTRUCT

scTSH and mscTSH expression constructs were stably transfected in CHO cells. For each construct 8 clones were picked and the presence of TSH was measured in conditioned

medium. In none of the scTSH transfected clones, TSH protein was detected above control levels. In contrast, in conditioned medium of the mTSH transfected clones tested, TSH was detected (Figure 3) suggesting beneficial effects of the point mutations on protein expression and/or secretion into the medium. Subsequent experiments were performed with the mscTSH construct.

Following the creation of a modified scTSH we examined the feasibility of TSH-fusion proteins. Therefore, we generated expression constructs containing extended mscTSH constructs as depicted in Figure 2. mRNA expression levels of the TSH constructs in stably transfected CHO clones were comparable (Figure 4). To optimize production mscTSH transfected cells were grown in CHO-II-SFM resulting in an amount of approximately 4mU/l TSH excreted in the medium.

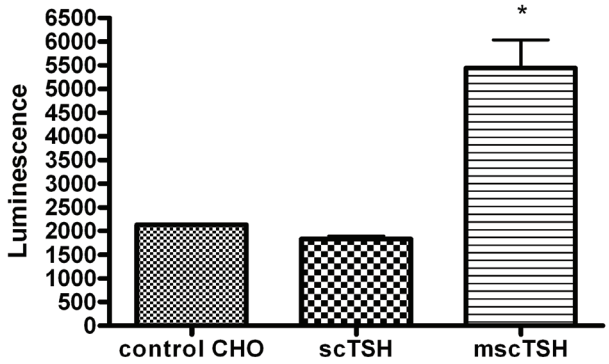

FIGURE 3. TSH-receptor stimulation by conditioned medium of 8 stably transfected CHO cell-lines per construct: 1) non transfected cells, 2) scTSH, 3) mscTSH. TSHReceptor activation increase by mscTSH when compared to control was significant (t-test; p=0,0417)

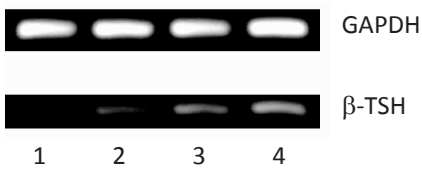

FIGURE 4. mRNA expression levels in stably transfected CHO clones: 1) pcDNA3.1 only, 2) mscTSH, 3) 6xHis-mscTSH and 4) 6xHis-13X-mscTSH.

TSHR ACTIVATION BY THE MODIFIED SINGLE CHAIN TSH CONSTRUCTS

TSHR activation of the modified single chain TSH constructs was compared with commercial recombinant human TSH (Fitzgerald industries, USA) using the TSHR/CRE-luciferase transfected B1 cell-line. All mscTSH constructs had similar activities being 20 fold more potent than commercial recombinant human TSH at 20mU/l (Figure 5).
The Presence and accessibility of the 6xHis tag was confirmed by His-gel purification (Figure 6) showing binding and elution of His tagged TSH constructs whereas mscTSH lacking the 6XHis tag did not bind to the gel.
Binding of mscTSH to the TSHR receptor was tested with a modified version of the Medipan kit and showed a 10fold improved binding to the receptor when compared to rhTSH.
In order to test if modified TSH is still biological active we tested mscTSH for its ability to promote iodine uptake in FRTL-5 cells (Figure 8). mTSH not only was biologically active but showed increased ability to promote I-uptake when compared to rhTSH.

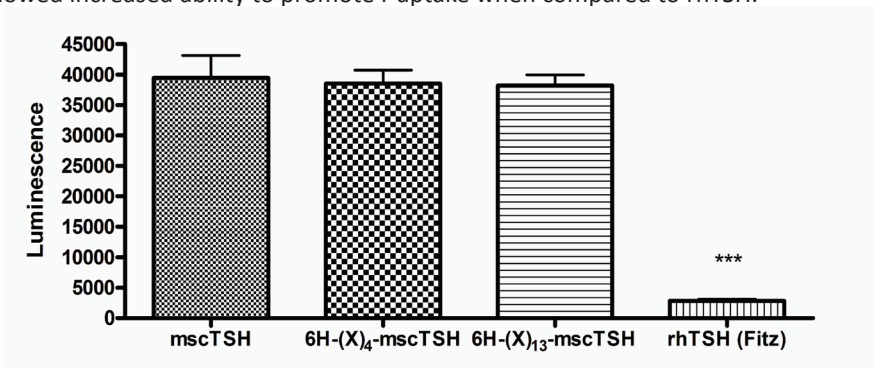

FIGURE 5. TSHR activation by the mscTSH constructs and rhTSH as measured with our TSHR activation assay at a TSH concentration of 20mU/L (Hovens, 2006). T-test (P<0.0001)

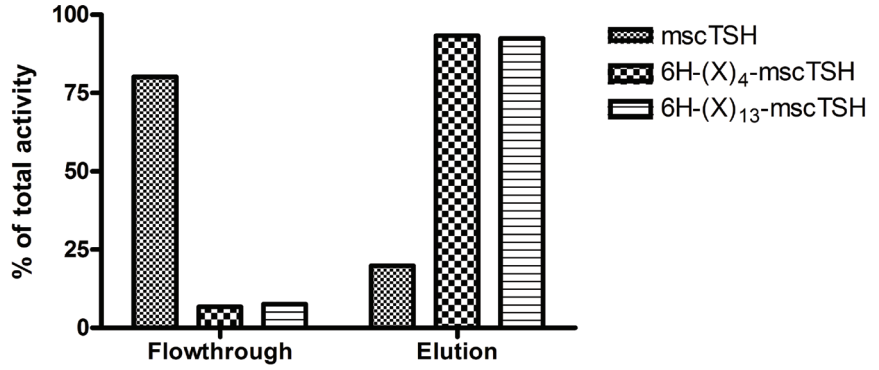

FIGURE 6. TSHR activation by conditioned medium of CHO cells expressing the three TSH constructs as measured with the B1-luc assay 1) mscTSH, 2) 6xHis-mscTSH and 3) 6xHis-13N-mscTSH in non binding-(flowthrough) and binding fractions (after elution) to His tag affinity gel. Results are shown as % of total TSHR activation after addition of medium that had been conditioned for two days.

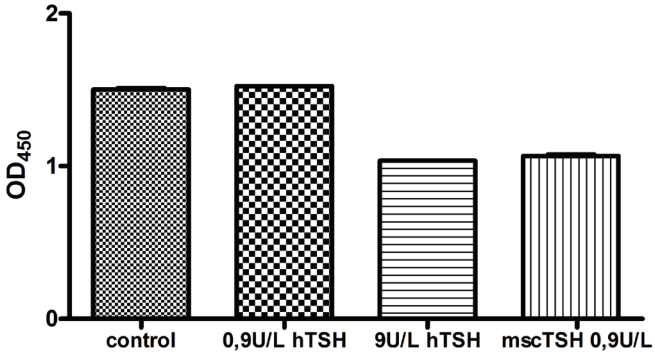

FIGURE 7 Binding of rhTSH and mscTSH to the TSHR as measured with the modified medizym TRA kit. Added TSH competes with TSH conjugate of the kit for binding to the TSHR. Improved binding results in lower levels of bound TSH-conjugate and consequently lower OD_{450}.

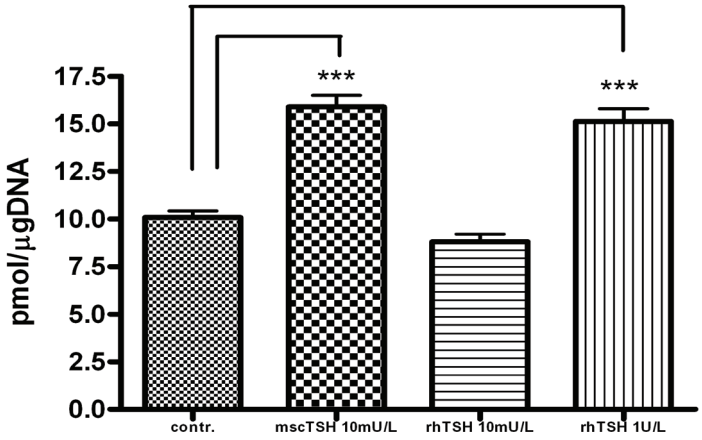

FIGURE 8. Iodine uptake after TSH stimulation was corrected for DNA content and is depicted as pmol/ugDNA. Conditions were as follows: 1) control lacking TSH, 2)mscTSH: modified scTSH at 10mU/L and 3) rhTSH: recombinant human TSH at 10 and 1000mU/L. I-uptake after stimulation with 10mU/L mscTSH significantly increased when compared to control (t-test; p<0,0001) whereas rhTSH did not stimulate I-uptake at 10mU/L. A 100-fold increase of the rhTSH concentration resulted in an I-uptake comparable to mscTSH at 10mU/L (t-test; p=0.0003)

STABILITY OF MSCTSH

Stability of the single chain constructs was tested for up to four days at 37 and 56°C.
Both the rhTSH and single chainTSH variants displayed loss of biological activity at 37ºC (Figure 9A), expressed as luciferase reporter activation in our TSHR activation assay (244). All mscTSH constructs displayed a higher stability than rhTSH as approximately 50% of scTSH and 25% of rhTSH activity remained after 48h. The presence of a His tail with and without a

protein extension did not impair stability of the TSH constructs.
In addition to the effect we found at 37ºC, we saw a sharp decrease of activity at 56ºC with approximately 25% of activity remaining in all TSH constructs after 24h, while activity was almost completely abolished after 48h.

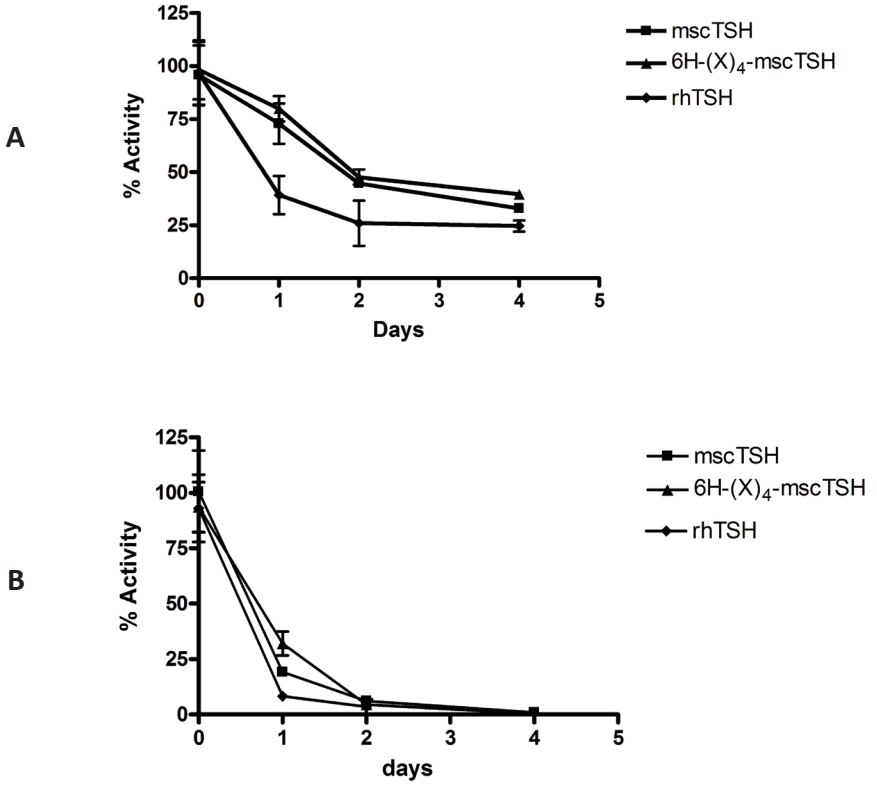

FIGURE 9. Stability of (His)-modified single chain TSH compared to rhTSH at 37°C(A) and 56°C(B). Remaining activity of the TSH variants was measured with the B1Luc-bioassay. Activity is displayed as % of total activity at t=0.

DISCUSSION

In this manuscript we describe the design of a modified single chain TSH molecule, which has powerful TSH stimulating effects. We aimed to develop a protein capable of specifically targeting the thyroid, and, more specifically, thyroid tumor cells. As the TSHR is almost exclusively expressed in thyroid tissue and its expression is maintained in various thyroid tumors (155;156) the TSHR is an interesting target for directing proteins (e.g. toxins) to the

thyroid or thyroid tumor cells. As TSH is the natural ligand of the TSHR, a TSH-fusion product would in theory be able to bind exclusively to TSHR bearing cells. After binding, the TSH fusion protein could be transported into the cell, which is essential for certain applications such as a TSH-toxin fusion protein.

STABILITY

By fusing the beta and alpha chain of TSH we intended to create a more stable protein (168;243). Furthermore, the fusion of the subunits bypasses the rate limiting assembly step, which is essential for secretion, and hormone specific glycosylation of TSH (170;171). Using an immunoassay specific for heterodimeric TSH, Grossmann et al. showed previously that single chain TSH as well as rhTSH were stable at 37C for at least 21 days, while mscTSH was significantly more stable than hTSH at 55C (172). In contrast, we found degradation of both the rhTSH and single chain TSH at 37ºC, when using our TSHR activation assay (244). The mscTSH constructs displayed a higher stability than rhTSH, as approximately 50% of mscTSH and 25% of rhTSH activity remained after 48h. In contrast to the effect we found at 37ºC, there was a sharp decrease of activity at 56ºC with approximately 25% of activity remaining in all TSH constructs after 24h, while activity was almost completely abolished after 48h. These contradictionary effects of temperature on TSH stability between the study of Grossmann et al. and our study may be due to the different methods used for measuring stability. In the study by Grossmann et al. the results were based on an immunoassay specific for heterodimeric TSH rather than on biological activity, whereas our method is based on actual TSHR activating properties. This discrepancy between the two studies suggests that loss of activity may not be directly linked to dissociation of the subunits but may occur prior to this event.

IMPROVING TSH

When using scTSH as a vehicle to guide components to, and into, TSHR bearing cells improved binding to, and activation of, the TSHR is likely to improve specificity and internalization of the scTSH in vivo, as the TSHR internalization rate increases 3-fold after activation (149). In order to create a super-active scTSH we introduced several mutations in our single chain TSH, known to improve rhTSH binding to the TSH-receptor (168;169;172). We tested the properties of our modified scTSH for binding to, and activation of, the receptor and the relevant biological endpoint of iodine uptake. Both binding to, and activating of, the TSH-receptor by mscTSH were improved when compared to commercially available rhTSH, by respectively 10 and 20 fold.
One possible application of super agonistic TSH analogues may lie in improved ^{131}I treatment. Radioiodine ^{131}I is routinely used in the management of thyroid cancer for treatment and diagnostic purposes. As TSH stimulates ^{131}I uptake, patients used to be treated with thyroid withdrawal protocols to increase TSH levels. In recent years recombinant hTSH has become an alternative and phase III trials have demonstrated that rhTSH treatment is nearly or as effective in stimulating ^{131}I uptake as traditional methods (3;246). In FRTL-5 cells our mscTSH was almost twice as effective in inducing ^{131}I uptake compared to rhTSH, making mcTSH a

potential candidate for inducing more efficient ^{131}I uptake also in vivo.
Direct labelling of mscTSH with a radioactive ligand may be another feasible application, especially when distant metastases are involved, which sometimes lose the ability for iodine, and thus ^{131}I, uptake but maintain TSHR expression (155;156).

FUSION PRODUCTS

We wanted to know whether it would be possible to fuse a protein to our hyperactive single chain TSH and still maintain biological activity. As a model for mscTSH fusion proteins, we fused a 6xhistidine tag with flexible linker, alone and in combination with a six amino-acid sequence to the N terminus of mscTSH since the α-carboxy terminus (α 88-92) is unavailable for binding due to its critical role in TSHR binding and activation (138;173). Use of a nickel gel purification step confirmed the presence, and the accessibility, of the 6xHis tag.
We subsequently tested the biologic potential of this 6xHis tagged with and without the small protein extension mscTSH constructs with our bio-luc assay and found that the full TSHR stimulating potential was maintained. Furthermore, the addition of a His tag and the small protein extension to the mscTSH construct did not impair the stability, when compared to the single chain TSH. This suggests, that the conformation of mscTSH was not dramatically influenced by the additional extension on the N-terminus.
The maintained TSHR activating potential of TSH is essential for a formed TSH-TSHR complex to be internalized into the TSHR bearing cell. As the His-mscTSH and His-13X-mscTSH fusion products still possess the full potential of the modified single chain TSH, it is feasible that our mscTSH is able to guide proteins into the thyroid and thyroid tumors in vivo.
After TSHR activation the normal route of TSH leads to the lysosymes. Triggered by the activation of the receptor the TSH-TSHR complex is internalized through clathrin-coated vesicles followed by the recycling of the majority of receptors to the surface and degraded of TSH by lysosomes (149). In theory, this mechanism would enable TSH bound components, e.g. toxins, to enter thyroid (tumor) cells expressing the TSHR, because various toxins of bacterial origin (e.g. pseudomonas exotoxin (PE), Diphteria toxin (DT), Ricin, Shiga toxin use the lysosomal route to kill eukaryotic cells and it is likely that they, when fused to mscTSH, would be able to follow their normal route into the target cell (174;183;247) .
The normal cell binding domain of these toxins can be replaced with a different binding domain and possibly with our mscTSH. Within the group of toxins the ones with a cell binding domain on the carboxy terminal side will be best compatible with our mscTSH as the carboxy terminus (α 88-92) of mscTSH is unavailable due to its crucial role in TSHR binding and activation (241;248).
Other applications of a TSH fusion protein may lie in the field of diagnostics. Our mscTSH may be able to guide markers towards TSHR bearing cells. However, for diagnostic purposes internalization of mscTSH may not be needed, or even be undesired, and a receptor blocking TSH molecule would be more favourable. The introduction of novel mutations, which abolish oligosaccharide chain formation, might be able to achieve this goal (249). In this way it could be possible to attach markers to the surface of TSHR bearing cells without risking degradation.

A limitation of the use of cytotoxic TSH molecules, is the presence of the TSHR in non-thyroid tissues. A number of papers have reported the prevalence of TSHR mRNA and/or protein in non-thyroid tissues such as lymphocytes, thymus, pituitary, testis, kidney, heart and orbital tissues (157;158). Although TSHR appears to reside in non-thyroid tissues, the TSHR in those tissues is only found at very low levels and the relevance of the TSHR in these non-thyroidal tissues remains to be elucidated. Nonetheless, the use of mscTSH-toxin constructs and subsequent destruction of TSHR bearing tissues may cause problems if the TSHR really plays an active role in other tissues. Finally, when using mscTSH constructs for visualization of the thyroid or thyroid derived tumors the presence of the TSHR in other tissues is unlikely to interfere due to the high expression rate of TSHR present in the thyroid when compared to other tissues.

IN SHORT

Compared to rhTSH our mscTSH has higher stability and increased activity, which is potentially very useful for diagnostic purposes and thyroid cancer treatment. Our improved single chain has proven to maintain biologic activity when fused to short extensions. This opens the way to using TSH as a highly specific vehicle to deliver proteins to TSHR bearing cells e.g. toxins to thyroid tumors.

Chapter 5

A Bio-Luminescence Assay for Thyrotropin Receptor Antibodies Predicts Serum Thyroid Hormone Levels in Patients with de novo Graves' Disease

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ABSTRACT

Background: TSH receptor antibodies (TBII) in Graves Disease (GD) can be distinguished in TSH receptor stimulating (TSAb) and blocking (TBAb) antibodies. In commercially available assays however, only total TBII titres can be measured, without discriminating between TSAb and TBAb.

Objective: To design a TBII bioassay for the detection of TSAb and to correlate TSAb activity with severity of hyperthyroidism in de novo GD patients.
Patients: Thirty-five patients with de novo GD and 27 controls.

Methods: The JP-26-26 cell-line, which constitutively expresses the TSH receptor (TSHR), was stably transfected with a cAMP Responsive Element - Luciferase construct. The clone B1 exhibited a near linear increase in luminescence from 0.2 mU/l to 50mU/l bovine TSH and was used as a TBII bioassay.
TBII, free T4 and TSH were measured in the sera of all patients and controls.

Results: In the sera of 35 GD patients, TBII titres did not correlate with serum free T4 concentrations. In contrast, a strong and highly significant correlation was found between TSHR stimulating activity (luminescence) as measured with the TBII bioassay and serum free T4 levels ($R=0.80$, $p<0.0001$).
Interestingly, the luminescence/TBII ratio had a wide range at low TBII titres, whereas high TBII titres were associated with a low degree of TSHR activation. The TBII bioassay also detected TBAb in GD patients who spontaneously developed hypothyroidism.

Conclusions: The B1-TBII-bioassay as developed in our laboratory has a high sensitivity for the detection of TSAb in GD and predicts the severity of hyperthyroidism in untreated GD patients. In addition, we found that high TBII titres are associated with weak TSHR activation.

INTRODUCTION

Graves disease (GD) is the most prevalent cause of hyperthyroidism with a yearly incidence of 5/1000 (250). GD is characterized by the presence of auto-antibodies against the TSH receptor (TSHR) that are referred to as TRAb (TSH receptor antibodies) or TBII (TSH receptor binding inhibiting immunoglobulins). TBII are a generic term for both thyroid stimulating antibodies (TSAb) and thyroid blocking antibodies (TBAb). Hyperthyroidism in GD is caused by TSAb, which bind to and activate the TSHR (251-253).

Although the clinical presentation often presents no difficulties in confirming the diagnosis of GD, the demonstration of TBII may be helpful, especially in selected cases, for instance, when a typical pattern of diffuse accumulation of radioiodine at scintigraphy is absent or in certain clinical conditions such as pregnancy.

Most commercially available assays for TBII are based on immunoglobulin-mediated inhibition of the binding of radio labelled or luminescent TSH to the TSHR. The sensitivity of these assays ranges from 80 to 99 percent (254). However, the obvious disadvantage of these tests is the inability to detect the biological activity of the antibodies. Consequently, it is not possible to correlate the test-result with the degree of TBII. This is particularly important in pregnancy, where the distinction between TSAb and TBAb rather than the demonstration of TBII has clinical consequences (255).

A number of studies have been published on bioassays for TBII. Initially, radioimmunoassays were used to measure cAMP activity in FRTL-5 cells or cell-lines stably transfected with the TSHR (256-260). However, this method is relatively cumbersome and expensive. More recently, bioassays have been developed based on the incorporation of a luciferase construct in TSHR transfected cell-lines. In these assays, cAMP that is generated by TSH-receptor activation induces luciferase expression. With these methods, the presence of TSAb (261;262) as well as TBAb (263;264) in sera from patients with a history of GD have been demonstrated convincingly. However, the threshold of the luciferase based assays published is relatively low, ranging from 1 mU/l bovine TSH (265) to 100 mU/l (266). In addition, in 2 studies, both de novo GD patients and patients who received medical treatment for GD were incorporated (267;268). As a result, it was not possible to study the relationship between TSAb and the actual serum free T4 as a clinical end-point of TSHR activation.

We have developed a luciferase-based bioassay for TSAb and found a lower TSH threshold than in previously published assays. We have validated this assay using sera of de novo patients with GD and found a strong correlation between in vitro TSHR stimulating activity and serum free T4 levels. Interestingly, we found that high TBII titres were associated with a low degree of TSHR activation.

In addition, we tested the biological activity of TBII in a subgroup of patients with a history of GD who spontaneously developed hypothyroidism. The demonstration of TBAb may add to the diagnosis of GD induced hypothyroidism (269-271).

MATERIALS AND METHODS

PATIENTS

The following patient categories were selected from the outpatient clinic of the Department of Endocrinology and Metabolic Diseases of the Leiden University Medical Center. 1) 35 patients with de novo GD as confirmed by elevated serum free T4 and suppressed TSH levels and intense diffuse uptake of Tc-pertechnetate at thyroid scintigraphy. Only patients at first presentation and without medical treatment were included. Their clinical data are presented in Table 1. Eight patients had signs of Graves Ophthalmopathy as assessed by the NOSPECS classification (272). Six patients were smokers. 2) Four patients with de novo toxic multinodular goitre were selected, diagnosed by elevated serum free T4 and

TABLE 1. Clinical Data of Graves Patients

Parameter	
Age	42 ± 11
Men / Women	12/23
Duration of Symptoms of Hyperthyroidism (*Months*)	6 (0.5 –48)
Smokers / Non-smokers	6/29
Goiter Size (*Times Normal*)	2 (1-3)
Ophthalmopathie (nr of Patients)	8
	(5 NOSPECS class III, 3 NOSPECS class IV)
Serum free T4 (*pmol/L*)	40.6 (23.1 – 100)
Serum TSH (*mU/L*)	<0.005 (<0.005 – 0.014)
TBII (*TRAb, measured by the TRAK assay,See Methods, U/L*)	
- Total	21 (<5 – 34)
- Ophthalmopathy	32 (8 – 147)
- No ophthalmopathy	23 (<5 – 99) *
Bio-Luminescence (*assessed by the B1 cell-line, See Methods, Luminescence Units*)	
- Total	4042 (2139 – 7848)
- Ophthalmopathy	4090 (2139 – 7858)
- No ophthalmopathy	4365 (2409 – 7648) **

* p=0.187 vs. No Ophthalmopathy, ** p=0.984 vs. No Ophthalmopathy

suppressed TSH levels and typical focal accumulation of radioactivity at scintigraphy. Only patients without medical treatment were included. 3) Nine patients were selected with positive TBII who had been diagnosed with GD and who spontaneously developed hypothyroidism, as confirmed by elevated serum TSH levels.
4) Twenty-seven subjects were used as controls. These subjects were patients with post-surgical hypopituitarism for pituitary adenoma and stably substituted with hydrocortisone, recombinant human growth hormone (rhGH), thyroid hormone and/or sex steroids when appropriate.

CELL-LINES

JP26-26 cell-line

The JP26-26 cell-line was kindly donated by Dr. G. Vassart, Service de Génétiques, ULB, Campus Erasme, Brussels 1070, Belgium. The JP26-26 cell-line is a sub-line of the initial TSH-receptor expressing JP26 clone (273) that was cultured routinely in Ham's F12 medium (Gibco BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 100 IU/ml penicillin (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.) and 400μg/ml geneticine (Life Technologies, Inc.), to maintain TSHR expression Cells were treated with trypsin and transferred (1:10) to new medium every 3-4 days.

Generating the B1 cell-line

The JP26-26 cell-line was transfected with a cAMP Responsive Element-luciferase construct (kindly provided by Himmler A, Ernst Boehringer Institute, Bender + Co. GmbH, Vienna, Austria) using the Fugene-6 method (Roche, Basel, Switzerland). Ten thousand B1 cells per cm2 well were incubated overnight before transfection. A blasticidin resistance gene expression vector, that was developed in our laboratory, was used to select transfected clones with 2 μg/ml blasticidin.
The selected cell-lines were tested for luminescence after stimulation by bovine TSH (Sigma-Aldrich, Zwijndrecht, The Netherlands) and the cell-line with the highest stimulation/non-stimulated ratio was used for further experiments. This clone was named B1.

Luc bioassay for TSH receptor activation
B1 cells were seeded at a density of 2.5*104 cells per well in 24 well plates in the JP26-26 medium supplemented with blasticidin and incubated at 37˚C/5%CO2 for 24 h followed by an interval in minimal medium (Ham's F12 medium supplemented with 0.5 % BSA). After 4h B1-cells were stimulated with 200μl serum in 200μl minimal medium supplemented with 5%PEG-6000 (MERCK-Schuchardt, Hohenbrunn bei München, Germany) to improve TSHR binding. Luminescence was measured after 20h with the Luciferase Reporter assay system (Promega, Madison, USA) according to the manual. Ten μl of cell lysate was assayed for firefly luciferase using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin–Elmer, Boston, MA, USA).
The relation between luminescence and bovine TSH (bTSH) concentrations and human pituitary TSH (hTSH, Sigma-Aldrich, Zwijndrecht, The Netherlands) was determined by

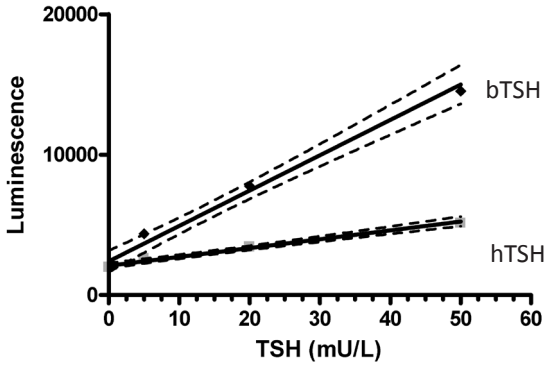

FIGURE 1. Dose response curve of the B1 cell-line with bovine TSH and human TSH. Results are expressed as mean ± SEM. Dotted lines represent the 95% confidence intervals of the regression line.

titrating bTSH or hTSH in TSH/TBII negative serum obtained from 5 selected patients in the follow-up for thyroid carcinoma who received TSH suppressive thyroxin replacement therapy (TSH levels <0.005 mU/l). The results were used to express the stimulation in equivalents TSH based on the standard curve according to the formula:

TSH(mU/l) =(Luminescence – luminescence control sera)/ slope

LABORATORY ASSAYS

Free thyroxin (T4) was measured on a Modular Analytics E-170 (Roche Diagnostic Systems, Basel, Switzerland; intra-assay variability: 2.47-7.57%, inter-assay variability: 5.6-12.4% at different levels). TSH was determined with on a Modular Analytics E-170 (Roche Diagnostic Systems, Basel, Switzerland), intra-assay variability: 0.88-10.66%, inter-assay variability: 0.91-12.05%). TBII were measured with the TRAK assay on the scintigraphy (TRAK RIA Kit, Brahms, Berlin Germany), detection threshold 10 U/l, intra-assay variability: 5.1-6.8%; inter-assay variability 10.2-13.2%).

STATISTICS

Data are expressed as mean ± standard deviation for normally distributed data. Non-normally distributed data were expressed as median and range. Differences between groups (relation between Graves Ophthalmopathy, smoking, serum free T4, TBII and luminescence) were performed by multivariate analysis. Correlation analysis between serum free T4 levels and TBII and luminescence were performed by univariate analysis of variance, with free T4 as dependant variable and TBII and luminescence as covariates. All statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL).

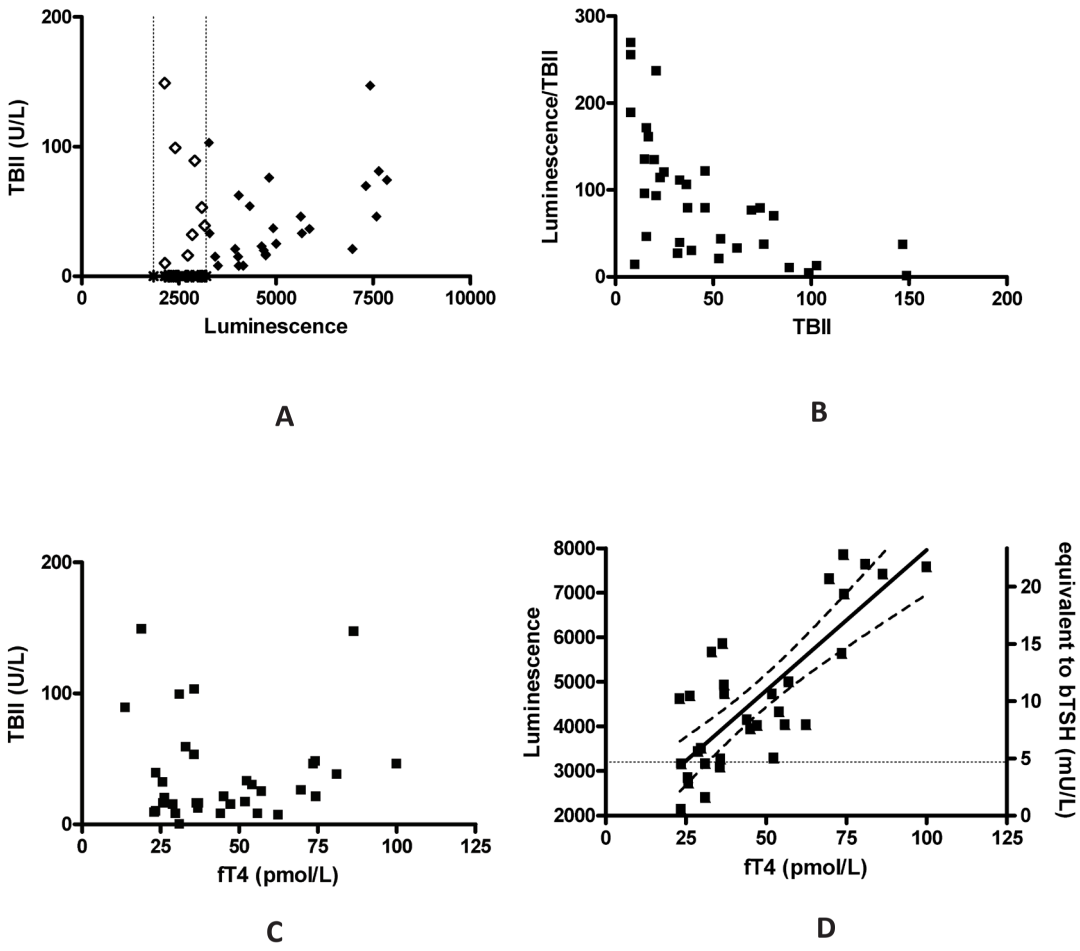

FIGURE 2.

A: Relationship between titres of TBII (as measured with the TRAK assay (see Methods)) and luminescence of sera obtained from 35 patients with GD (◆)and 27 negative controls (✳). There was no significant correlation: R^2= 0.001, p=0,8722. Dotted lines represent the minimal and maximal luminescence of controls. 8(◇) out of 35 GD sera have luminescence levels within the control group range.

B: Relation between luminescence/TBII ratio and total TBII levels in sera obtained from 35 patients with GD. The ratio between luminescence and TBII titres ranged from 1 to 270. At lower TBII levels a wide range in the Luminescence/TBII ratio was observed, whereas high TBII titres were associated with a low level of luminescence.

C: Absence of a relation between TBII as measured with the TRAK assay and serum levels of free T4 of 35 patients with GD. R^2 =0.0623, p=0.2188.

D: A strong correlation was present between luminescence and serum free T4 levels of 35 patients with GD. $R^{2:}$ =0.80, p<0.001. The dotted line represents the maximal luminescence of controls.

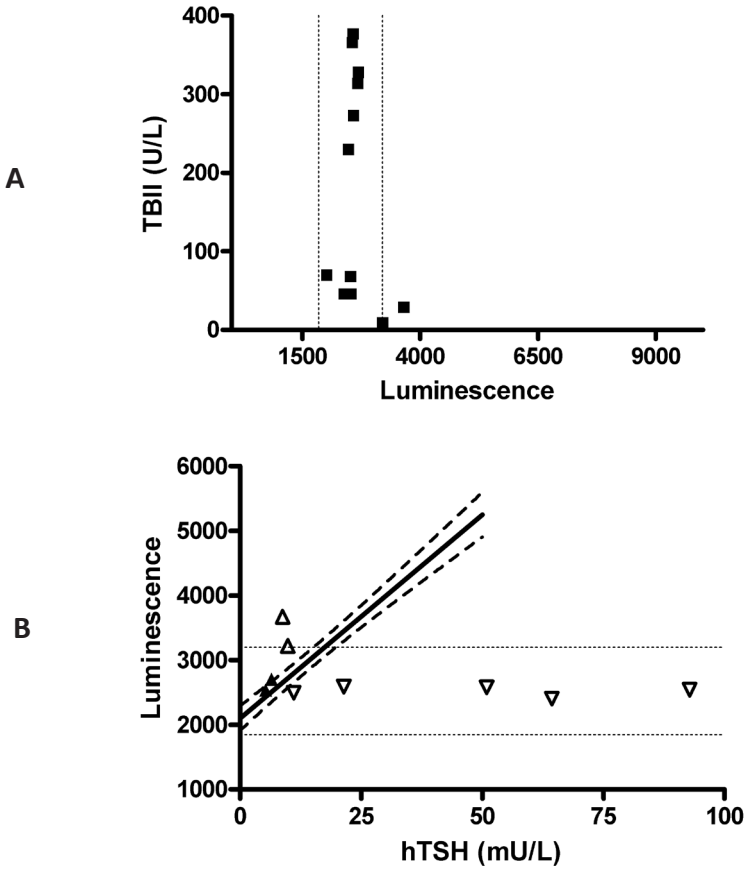

FIGURE 3.

A: Relationship between TBII (measured by the TRAK assay) and luminescence as measured by the B1 cell-line in sera from patients with a history of GD who spontaneously developed hypothyroidism. Despite high titres of TBII, luminescence did not exceed the upper limit of luminescence found in controls (right dotted vertical line).

B: Relation between luminescence and serum TSH levels in patients with a history of GD who spontaneously developed hypothyroidism. The regression line with 95% confidence intervals represents the expected relationship between luminescence and hTSH concentrations. A lower luminescence than expected from the actual TSH concentration was observed in 5 (▽) patient sera, indicating the presence of TBAb. Two patients (△) had a higher luminescence level than was expected, indicating the presence of TSAb. Dotted lines represent the upper and lower values of controls.

RESULTS

EVALUATION OF THE B1- TBII BIOASSAY WITH BOVINE TSH.

After stable transfection of the JP26-26 cell-line with CRE-luc we selected the clone with the highest stimulated/non-stimulated ratio. This clone, named B1, was used for further experiments. Luminescence was detectable at 0.2 mU/l bTSH in minimal medium supplemented with 5% dextran T-70.
In order to test the TSHR stimulation by bTSH in sera we selected sera from patients who received TSH suppressive thyroxin therapy. We added a dose range of bTSH in 50% serum/50% medium to B1, which resulted in a near linear increase in luminescence in the lower range to 50 mU/l bTSH (Figure 1). Baseline luminescence in serum without bTSH was 1999 luminescence units (LU) and a plateau of 26000 LU was reached at 2000 mU/l bTSH, a 13-fold induction compared to untreated cells whereas the slope was 252 $\pm$14 LU * l / mU bTSH. This enabled us to express luminescence in mU/l bTSH (Figure 1). For hTSH, the slope was 63 $\pm$ 4 mU/l hTSH (Figure 1).

EVALUATION OF THE B1- TBII BIOASSAY IN GRAVES PATIENTS AND THE RELATION BETWEEN TBII, LUMINESCENCE AND SERUM FREE T4 LEVELS.

The sera from 35 GD patients were tested for the presence of TBII with the TRAK assay and for TSAb with the B1-TBII-bioassay. As negative controls, 31 sera lacking TRAb were used (27 controls and 4 patients with toxic multinodular goiter). The luminescence in these controls ranged from 1845 to 3210 LU with an average of 2547 LU. The TSH levels in the Graves sera never exceeded 0.014 mU/l, and consequently; the TSHR activation resulted entirely from TSAb (Table 1). The results show a great variability in the relationship between TBII titres as measured with the TRAK assay and luminescence (Figure 2a). Interestingly, the variability in TSHR activation decreased at higher TBII titres: high TBII titres were associated with low levels of TSHR activation. Thus, the variability in TSHR activation per unit TBII is related to the TBII titers (Figure 2b).
The TBII titres measured by the TRAK assay in sera from GD patients showed no significant correlation with the serum free T4 concentrations in these patients (Figure 2c). In contrast, a significant correlation was observed between luminescence and the serum free T4 concentrations in GD patients (Figure 2d, $R2=0.80$, $p<0.0001$). When the bioluminescence found in the sera of GD patients was converted to TSH levels according to the formula given in the Methods section, an equivalent of 0.5 to 22 mU/l bTSH or 2.0- to 88 mU/l hTSH was found.
We did not find a difference in TBII as assessed by the TRAK assay and TSAb as assessed by the bioluminescence assay between patients with and without Graves Ophthalmopathy (Table 1). In addition, we did not find a significant correlation either between smoking and the degree of hyperthyroidism (serum free T4, $p=0.509$), TBII titres (TRAK, $p= 0.509$) or luminescence ($p=0.836$) by multivariate analysis.

B1- LUC ASSAY IN PATIENTS GRAVES HYPOTHYROIDISM

Despite the high titres of TBII in the 12 patients with a history of GD who spontaneously developed hypothyroidism, luminescence did not exceed the upper limit of luminescence found in controls in any of the patients (Figure 3a). In addition, when the relation between luminescence and serum TSH levels of these patients was studied, a lower luminescence than expected from the actual TSH concentration was observed in the sera from 4 of the 12 patients, indicating the presence of TBAb (Figure 3b).

DISCUSSION

The present study was performed to develop a novel luciferase based bio-assay to detect TSAb in sera of patients with GD. We used serum free T4 levels as a clinical in vivo end-point of TSH receptor activation. The results of our assay revealed a strong correlation between TSHR activation and serum free T4 levels in the 35 untreated GD patients. In contrast, TBII titres did not correlate with serum free T4 levels. In addition, we found that high TBII titres were associated with weak TSHR activation. Although the diagnosis of GD presents no difficulties in the majority of patients, advantages of determining the presence of TSAb in patients are obvious: A reliable biochemical test to establish GD alleviates the diagnostic use of thyroid scintigraphy. Most commercially available tests only measure TBII, without discriminating between TSAb and TBAb. Establishing TSAb rather than TBII points directly to the cause of hyperthyroidism. In addition, the existence of a relation between in vitro TSHR stimulation and degree of hyperthyroidism could have clinical implications, not only in de novo GD, but especially in selected categories of patients, notably patients with a history of GD with positive TBII titres.

Bio-luminescence assays published so far have demonstrated the feasibility of this approach. These studies gave a good indication of the spectrum of TSHR activation in these patients (274-277). The purpose of our study was to develop a test with a higher in vitro sensitivity for TSH than those previously published and to study the direct correlation between in vitro TSHR stimulation and serum free T4 levels as a clinical end-point of TSHR stimulation. This correlation could not be studied in earlier studies due to the fact that de novo, untreated GD patients as well as treated patients were studied (278;279).

As a platform for our assay, we used TSHR transfected CHO cells from the JP series. JP09, JP26 and JP26/26 have been used earlier in RIA based bioassays (280-282). Of these clones, the JP09 clone was used for a luminescent bioassay named "lulu" that was able to detect TSAb (283) and TBAb (284). We decided to use the JP26/26 cell-line that expresses the highest density of TSHR, resulting in the highest specificity when used in a RIA assay (285). Compared to the JP09 based lulu cell-line, our JP26/26 based B1-luc assay had a lower detection limit. In addition, we found a linear correlation between bTSH and luminescence, which enabled us to convert luminescence to bTSH and hTSH equivalents. At higher TSH levels the linear correlation is lost probably due to saturation or downregulation of the TSH receptor (286-288).

The activity of pituitary human TSH in our assays was ~25% of bTSH. The TSHR activation

ratio between bTSH and hTSH differs between various assays (from 2 in an FRTL-5 cell-line (289) , 5 when porcine thyroid cells were used (290) to 29 when I-125 uptake was used as a readout in FRTL-5 cells (291).

We found a strong and highly significant correlation between the in vitro TSHR stimulating activity of GD patients sera and their serum free T4 levels, in contrast to the absence of a relationship between TBII levels as assessed by TRAK and serum free T4 levels. To our knowledge, only one animal study has been published demonstrating a relation between the TSHR stimulating hamster antibody MS-1 and free T4 levels in mice (292). In our study, we found a strong correlation between activating properties of TSAb in patients with GD and serum free T4 levels, irrespective of TBII titres.

A relation was found between TBII titres and the luminescence (TSHR activation) per TBII unit. At lower TBII levels, a wide range in luminescence/TBII was observed, whereas at high TBII titres, predominantly weak TSHR activation was observed. An explanation for this observation may be the coexistence of TSAb and TBAb in GD. Previous reports show that the percentage TBAb of TBII in sera of untreated GD may be up to 30% (293). TBAb have a higher TSHR binding capacity than TSAb (294). At lower TBII titres, the availability of sufficient free TSHR may lead to optimal TSHR stimulation by the TSAb present. At higher TBII titres, competition between TBAb and TSAb may lead to suboptimal TSHR stimulation by TSAb. An alternative explanation could be that at high TBII titres, the distribution between TBAb and TSAb is more in favour of TBAb.

It is well known that TSAb are related to the activity of Graves Ophthalmopathy (295;296). In our study, we did not find a significant difference in TBII or luminescence values between patients with or without GO (Table 1). An explanation can be that the number of patients with GO in our series was too low to detect these differences. An important difference with the studies of Gerding et al (297) is that our patients were untreated, whereas the relation between GO and TRAb has been established in euthyroid (treated) patients (298).

Another category of patients in which the determination of TSAb or TBAb could be helpful is patients with a history of GD who developed hypothyroidism either spontaneously or after radioiodine treatment. In our analysis we found that most patients had TBAb, which is in line with earlier studies (299;300). Another application of the bioassay may be the prediction of recurrence of hyperthyroidism. A longitudinal study should be performed to this purpose.

We conclude that the newly developed B1-TBII bioassay has several advantages: The use of the bioassay enables an insight into the degree of TSHR activation in contrast to the standard TRAK assay, which only determines antibody binding to the TSH receptor. This is illustrated by the strong correlation between the free T4 levels and the luminescence. In addition, the bioassay may be useful to establish the nature and thus the clinical consequences of TBII in other categories of patients with a history of GD, especially in pregnancy.

Chapter 6

Induction of Stimulating Thyrotropin Receptor Antibodies after Radioiodine Therapy for Toxic Multinodular Goiter and Graves Disease Measured with a Novel Bio-assay.

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ABSTRACT

Background: Radioactive iodine therapy (RaI) in toxic multinodular goiter (TMNG) has been associated with the occurrence of Graves like hyperthyroidism. It has been postulated that pre-existing autoimmunity may contribute to this phenomenon.

Objective: To study whether RaI induces thyrotropin receptor stimulating antibodies (TSAb) on the short term in TMNG and whether pre-existing autoimmunity is relevant.
Patients: Thirty-one patients with relapsing Graves disease and 17 patients with TMNG, all eligible for RaI.

Methods: Before and 6 weeks after RaI, sera were collected and analyzed for the presence of thyroglobulin (Tg), Tg-antibodies (TgAb) thyroid peroxidase antibodies (TPO-Ab) and thyrotropin receptor binding antibodies (TBII). TSAb were analyzed with a novel high-sensitive luciferase based bioassay based on the JP-26-26 cell-line, which constitutively expresses the TSH receptor.

Results: In Graves disease, RaI did not induce or increase the levels and proportion of patients with measurable levels of any of the antibodies measured, despite a significant increase in Tg. In contrast, in TMNG, RaI induced TBII in 3 TMNG patients, which was accompanied by measurable TSAb in one occasion.

Conclusions: We conclude from the present study that induction of TBII and TSAb may occur shortly after RaI in TMNG and that pre-existing autoimmunity may not be a requirement for the induction of TBII, as evidenced by the absent effects of RaI in Graves disease.

INTRODUCTION

Several studies report the occurrence of Graves like hyperthyroidism in 1-5% of patients who have been treated with radioiodine (RaI) for toxic multi-nodular goiter (TMNG) (301-307). This phenomenon appears to be associated with the induction of thyrotropin receptor (TSHR) binding antibodies (TBII). TBII are a generic term for both thyroid stimulating antibodies (TSAb) and thyroid blocking antibodies (TBAb). Hyperthyroidism in Graves disease is caused by TSAb, which bind and activate the TSHR (308-310). It has been postulated that the induction of Graves like hyperthyroidism after RaI in TMNG is associated with pre-existing thyroid auto-immunity as indicated by the presence of thyroid peroxidase antibodies (TPO-antibodies)(311;312) or low titers of TBII in TMNG (313). If pre-existing autoimmunity is associated with the induction of TBII after RaI, then it would be expected that RaI in patients with Graves disease would also increase TBII levels, which is indeed reported (314;315).
The disadvantage of commercially available TBII assays is the inability to detect the biological activity of the antibodies. Therefore, to test whether RaI induces TSAb in TMNG, a highly sensitive functional TSAb assay is required. In addition, to test the hypothesis that pre-existing autoimmunity enhances the induction of TSAb, ideally a group of patients with Graves disease undergoing RaI should be included as a control group.
A number of studies have been published on bioassays for TBII. Initially, radioimmunoassays were used to measure cAMP activity in FRTL-5 cells or cell-lines stably transfected with the TSHR (259;316-319). More recently, bioassays have been developed based on the incorporation of a luciferase construct in TSHR transfected cell-lines (320-323). However, the threshold of the luciferase based assays published is relatively low, ranging from 1 mU/l bovine TSH (324) to 100 mU/l (325). We have developed a luciferase-based bioassay for TSAb and found a lower TSH threshold than in previously published assays (326).
Some studies have been published on the induction of TSAb by RaI in TMNG using a functional TSAb assay. In one study, (327) a bioassay with limited sensitivity was used as reflected by the absence of TSAb and TBAb in patients with positive TBII. In addition, in this study, only TMNG patients were studied whereas a control group of Graves disease was lacking. In another study, a small number (n=6) TMNG patients and Graves disease patients were studied, using a luciferase based assay (328).
We investigated whether RaI in TMNG induces TSAb on the short term using a new highly sensitive luciferase based bioassay (326). To study the concept that preexisting autoimmunity induces TSAb after RaI, we included a group of patients with Graves diseases as well.

MATERIALS AND METHODS

PATIENTS

The following patient categories were selected from the outpatient clinic of the Department of Endocrinology and Metabolic diseases of the Leiden University Medical Centre

(Table 1). Thirty-one patients with Graves disease as confirmed by elevated serum free T4 and suppressed TSH levels and intense diffuse uptake of Tc-pertechnetate at thyroid scintigraphy. The radioactivity applied was based on a volume based dosing regimen (329) and ranged from 200-833 MBq. Seventeen patients with TMNG were selected as diagnosed by elevated serum free T4 and suppressed TSH levels and typical focal accumulation of radioactivity at scintigraphy (Table 1): all TMNG patients had well localized autonomous tissue and there were no patients with disseminated autonomous tissue The radioactivity applied ranged from 600-3218 MBq. Patients who used thyreostatic medication before RaI continued this medication until 6 months after RaI.
Serum was taken both before and 6 weeks after radioactive iodine treatment Six weeks was chosen, because in earlier reports the induction of TBII after RaI in Graves disease appears to be a rapid phenomenon (330;331).

TABLE 1. Clinical Data of Patients who underwent Radioiodine (RaI) for Graves Disease or Toxic Multinodular Goiter

Characteristic		
Diagnosis	Graves disease	Toxic Multinodular Goiter
	N=31 N	=17
Gender(male/female)	1/30	4/13
Age (years)	46.8 ± 14.0	66.2 ± 9.6
Activity (MBq)	288.2 ± 133.0	656.9 ± 89.2
Medication post RaI:		
Thyreostatics (N, % of patients)	27 (87)	7 (33)
Thyroxine (N, % of patients)	20 (65)	2 (13)
Outcome 1 year after RaI (%):		
Euthyroid	21 (68)	14 (82)
Hypothyroid	10 (32)	3 (18)
Relapse	0 (0)	0 (0)

LUC BIOASSAY FOR TSH RECEPTOR ACTIVATION

The generation of the TSH receptor bioassay has been described (326). In short, the JP26-26 cell-line (kindly donated by Dr. G. Vassart, Service de Génétiques, ULB, Campus Erasme, Brussels 1070, Belgium (332) was transfected with a cAMP Responsive Element-luciferase construct (kindly provided by Himmler A, Ernst Boehringer Institute, Bender + Co. GmbH, Vienna, Austria). We selected the clone with the highest stimulated/non-stimulated ratio. This clone, named B1, was used for further experiments. Luminescence was detectable at

0.2 mU/l bovine TSH (bTSH) in minimal medium supplemented with 5% dextran T-70 and a linear relationship between bTSH and luminescence was detected. B1-cells were stimulated with 200µl patients serum in 200µl minimal medium supplemented with 5%PEG-6000 (MERCK-Schuchardt, Hohenbrunn bei München, Germany) to improve TSHR binding. Luminescence was measured after 20h with the Luciferase Reporter assay system (Promega, Madison, USA) according to the protocol. Ten µl of cell lysate was assayed for firefly luciferase using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin–Elmer, Boston, MA, USA). To define a cut-off value for TSAb, 27 control subjects were studied. These subjects were patients with post-surgical hypopituitarism for pituitary adenoma and stably substituted with hydrocortisone, recombinant human growth hormone (rhGH), thyroid hormone and/or sex steroids when appropriate. The luminescence in these controls ranged from 1845 to 3052 luminescence units (LU). The cut-off value was therefore defined as 3052 LU. All serum samples were measured in triplicate.

LABORATORY ASSAYS

Free thyroxin (T4) was measured on a Modular Analytics E-170 (Roche Diagnostic Systems, Basel, Switzerland; intra-assay variability: 2.47-7.57%, inter-assay variability: 5.6-12.4% at different levels). TSH was determined with on a Modular Analytics E-170 (Roche Diagnostic Systems, Basel, Switzerland), intra-assay variability: 0.88-10.66%, intra-assay variability: 0.91-12.05%). Anti-Tg was determined with an anti-TPO RIA kit (Brahms, Berlin Germany, functional assay sensitivity < 50 U/ml). Anti-TPO was determined with an anti-TPO RIA kit (Brahms, Berlin Germany, functional assay sensitivity < 30 U/ml) and Tg was determined with an Immuno-radiometric assay (IRMA) (Brahms, Berlin Germany, functional assay sensitivity=0,3ng/ml) on the Wizard γ-counter, 1470 automatic gamma counter (Perkin Elmer). TBII were measured with the TRAK assay on the scintiscan (TRAK RIA Kit, Brahms, Berlin Germany 10 U/l, intra-assay variability: 5.1-6.8%; intra-assay variability 10.2-13.2%). Sera that were TBII negative according to the TRAK assay were re-measured with the medizym T.R.A. assay (Medizym T.R.A.kit, Medipan, Berlin Germany).

STATISTICS

Data are expressed as mean $\pm$ standard deviation for normally distributed data. Non-normally distributed data were expressed as median and range. Differences within and between groups were performed by unpaired or paired students T-test for normally distributed data. Non normally distributed data were analyzed with the Mann-Whitney or Wilcoxon test. Proportional data were compared by Chi-square analysis. A p value of <0.05 was considered significant. All statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL).

RESULTS

Biochemical data are given in Table 2 and Figure 1.

GRAVES DISEASE

At baseline, 97% of the patients had measurable TBII. Sixty percent of the patients had measurable TPO antibodies and 47% had measurable Tg antibodies. In 64% of the patients TSAb were measurable with the bioluminescence assay. RaI therapy lead to a significant increase in serum Tg levels, indicating a thyrotoxic effect of RaI. However, the number of patients with measurable Tg antibodies significantly decreased after RaI. No RaI induced changes were observed in the median titres and the proportion of patients with measurable TPO, TBII and TSAb. Interestingly, one patient with a low concentration of TBII (5.79 kU/l) before RaI had extremely high TSAb levels (luminescence 12.628 LU), which almost doubled after RaI to 22.361 LU. In contrast, 2 patients with high TBII levels after RaI (246

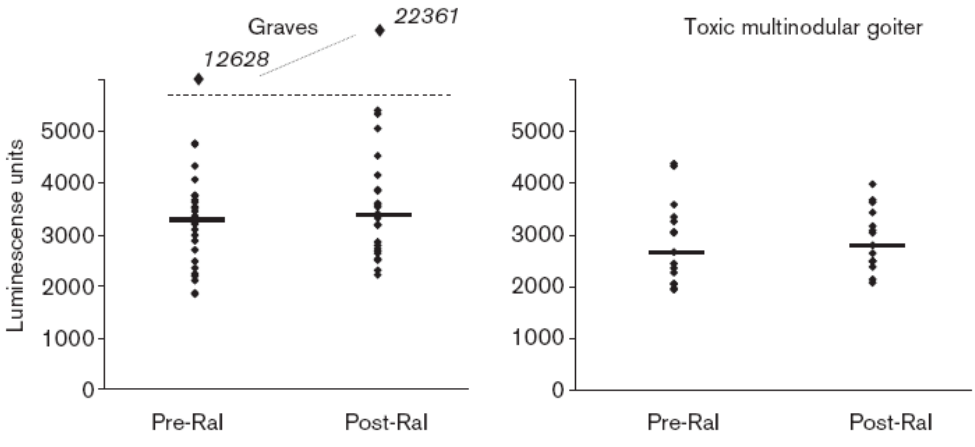

FIGURE 1. TSH-receptor activating antibodies as measured with the TSAb bioassay before and after radioactive iodine therapy (RaI) in Graves (left) and Toxic Multinodular Goiter (TMNG, right), expressed as luminescence units.
No significant differences in pre- and post RaI levels of luminescence (LU) were observed in patients with Graves disease and TMNG. Median levels are indicated with ——

TABLE 2. Effects of Radioiodine (RaI) Therapy for Graves and Toxic Multinodular Goiter (TMNG) on Thyroid Parameters

	Graves disease			TMNG		
Parameter:	Baseline	Post-RaI	p	Baseline	Post-RaI	p
Free T4 *(pmol/l)(Mean ± SD)*	24.91 ± 13.58	17.35 ± 6.61	0.024	21.94 ± 4.98	22.97 ± 14.58	0.309
TSH *(mU/l)(Mean ± SD)*	0.889 ± 1.391	1.919 ± 4.097	0.022	0.304 ± 0.674	0.527 ± 1.347	0.496
Thyroglobulin (Tg) *(µg/l) (Median, Range)*	69 (0.6 – 238)	117 (<0.3 – 1038)	0.028	34 (<0.3 – 634)	50 (3.9 – 865)	0.332
Anti Tg Antibodies *(Ku/l) (Median, Range)*	<50 (<50 – 12170)	<50 (<50 – 15450)	0.345	50 (50 - 12380)	50 (50 – 2057)	0.655
Negative / Positive *(N, % of Patients)*	16 / 15 (53 / 47)	21 / 10 (68 / 32)	0.030	8 / 8 (50 / 50)	14 / 3 (83 / 17)	<0.001
TSH Receptor Binding Antibodies *(U/l) (Median, Range)*	10 (<1 – 129)	9 (<1 - 246)	0.559	<1 (<1 – 20)	<1 (<1 - 37)	0.893
Negative / Positive *(N, % of Patients)*	1 / 30 (3 / 97)	3 / 28 (10 / 90)	0.040	14 / 3 (82 / 18)	11 / 6 (65 / 35)	0.040
anti-TPO *(kU/l) (Median, Range)*	125 (50 – 10310)	158 (50 – 14700)	0.910	<50 (<50 –2588)	<50 (<50 – 1300)	0.249
Negative / Positive *(N, % of Patients)*	12 / 19 (40 / 60)	10 / 21 (32 / 68)	0.239	10 / 7 (60 / 40)	10 / 7 (60 / 40)	1.000
Luminescense *(LU)*	3289 (1855 – 12628)	3373 (2216 – 22361)	0.130	2670 (1855 - 4372)	2792 (2216 – 3395)	0.701
<3052 *(N, % of Patients)*	11 (36)	13 (42)	0.780	12 (73)	11 (67)	0.355
>3052 *(N, % of Patients)*	20 (64)	18 (58)		5 (27)	6 (33)	

and 223 kU/l) had luminescence levels only marginally elevated above the threshold level of 3052 LU: 3576 and 3192 LU respectively.

TMNG

In the TMNG group, at baseline 50% of the patients had measurable Tg antibodies and 40% measurable TPO antibodies. In 3 patients TBII were measurable. It was carefully verified by reviewing scintigraphies that the diagnosis of TMNG was correct. Interestingly, in 5 of the patients, TSAb appeared to be present before RaI, whereas TBII were unmeasurable in 3 of these 5 patients.
RaI therapy did not lead to a significant increase in serum Tg levels, and the number of patients with measurable Tg antibodies significantly decreased. No RaI induced changes were observed in the median values and number of patients that were positive for TPO antibodies.
Interestingly, RaI lead to induction of measurable TBII in 3 patients. In one of these patients, the induction of TBII was accompanied by the appearance of TSAb (luminescence level before RaI <3052 LU and after RaI 3672 LU). In the other 2 patients with newly measurable TBII, luminescence was <3052 LU, thus suggesting that the TBII were TBAb.
In the 5 patients with measurable TSAb before RaI, luminescence levels decreased in all patients, in 2 patients below the threshold.
Clinical outcome of TMNG and Graves patients after RaI was evaluated after 1 year. None of the patients had a relapse, 3 TMNG patients and 10 Graves patients became hypothyroid after 1 year. No relation was found between initial and post RaI levels of antibodies and outcome of RaI. However, in the Graves patients, the proportion of patients with a rise in TSAb after RAI was significantly lower in patients who became hypothyroid (3/10) than in the patients who became euthyroid (16/5, p=0.02).

DISCUSSION

We performed the present study to investigate whether RaI induces TSAb in TMNG using a new highly sensitive luciferase based bioassay. To study the concept that preexisting autoimmunity induces TSAb after RaI, we included a group of patients with Graves disease as well. Earlier studies addressing this issue were limited by the fact that no functional TSAb assays were used (333-337). Two studies (338;339) did use a functional TSAb assay, however, the sensitivity of these assays were limited, one study did not include Graves disease patients (340) and the other study was small (341).
We used a luciferase based TSAb bioassay with a functional sensitivity of 0.2 mU/l bTSH. In the group of Graves disease patients, we did not find evidence for RaI induced TSAb, although a significant rise in Tg was observed, thus indicating the release of thyroid antigens. Although TBII were present in 97% of the patients, TSAb were present in 64%, indicating that TSAb may disappear spontaneously. Three TMNG patients had measurable TBII. It is a subject of debate whether the diagnosis TMNG is valid in these patients or that they have Graves disease despite the typical scintigraphical pattern (342). Some authors adopt the

concept of subclinical autoimmunity in TMNG (343-345). Even if the diagnosis of TMNG might be withdrawn, TSAb were present in 5 patients, 4 of whom had immeasurable TBII by non-functional assays. This underscores the high sensitivity of our TSAb bio-assay, but also the fact that thyroid autoimmunity may be more common in TMNG than previously appraised. The exact significance of this finding and more specifically, whether TSAb play a causative or enhancing role in the pathogenesis of TMNG remains to be clarified. We found induction of TBII after RaI in 3 TMNG patients. In only one of the patients with de-novo TBII, TSAb were present. The proportion of patients with TSAb was not influenced by RaI in TMNG.

It can be questioned if the findings in our study may have been influenced by thyreostatic medication. There have been conflicting studies about the immunosuppressive effects of thyreostatic drugs and the clinical relevance, some studies clearly showing immunosuppressive effects (346-349), others not so clearly (350-353). We believe that the potential confounding effects of medication are prevented by the fact that medication before and after RaI was identical in all patients.
In the study of Chiovato et al (354) high TSAb levels in patients with Graves disease before RaI were related with resistance to therapy. This was not the case in our study. They also found that a post RaI increase in TSAb was related to the development of hypothyroidism. In contrast we found that a post RaI rise in TSAb in patients with Graves disease was associated with a lower proportion of hypothyroidism. In the study of Michelangeli et al (355), hypothyroidism after RaI for Graves disease was mainly observed in patients with a post RaI rise in TBII which was attributable to both TSAb and TBAb. In patients with only TSAb, no hypothyroidism developed, which is thus in line with our observation.
We conclude from the present study that induction of TBII and TSAb may occur shortly after RaI in TMNG, that pre-existing autoimmunity is not a requirement for the induction of TBII (as evidenced by the absent effects of RaI in Graves disease) and that TSAb measured with a high sensitivity bio-assay may be present in TMNG patients with TBII below the detection threshold.

Chapter 7

Summary & Discussion

INTRODUCTION

Existing therapies, consisting of surgery and radioiodine (RaI) therapy for Differentiated thyroid carcinoma (DTC) are highly effective for most patients. However, the therapeutic arsenal in DTC is limited. Once distant metastases have occurred, usually in the lungs or bones, the prognosis is worse, because the results of radioiodine (RaI) therapy, which is virtually the only curative treatment, are moderate. A major problem in this category of patients is the diminished, or lost, ability of thyroid cancer cells to accumulate RaI, indicated by negative post-therapeutic whole body scintigraphy. In these cases the prognosis is poor, as alternative treatment options (external radiotherapy or chemotherapy) have limited success. Therefore, the improvement of conventional therapy by increasing RaI uptake and the development of new innovative therapies is needed. In this thesis, we explored new approaches, focussing on the thyroid thyrotropin receptor (TSHR) as a target for therapy. In addition, we also explored the possibilities of redifferentiation therapy.

TSH SUPPRESSION IN DIFFERENTIATED THYROID CARCINOMA (DTC)

In conventional therapy and follow-up of thyroid cancer further improvements in the treatment of patients may be achieved by fine-tuning existing therapies. TSH suppression by slightly overdosing of L-thyroxine substitution is common practice in patients with DTC to prevent recurrence. In **Chapter 2**, we studied the correlation between TSH levels and recurrence of DTC as this relation has only been studied to a limited extent focusing on the optimal levels of TSH suppression..

In patients with DTC, suppression of TSH levels by thyroxine replacement is common practice. The rationale for this treatment is based on the observation that TSH has proliferative effects on thyroid carcinomas in vivo and in vitro. However, the induced hyperthyroidism has adverse effects on bone mineral density and cardiac function. Therefore an optimal level of TSH suppression should be maintained, enough to prevent recurrence of thyroid tumors, while minimizing side effects. Despite these observations, observational clinical studies on the effect of thyroxine-induced TSH suppression on the prevention of DTC recurrence or DTC-related death remain scarce. Previous studies differed from our study in the homogeneity of the patient groups with respect to initial therapy, the study size and the duration of follow-up. To further rationalize recommended TSH levels we studied the association between serum TSH concentrations in patients during follow-up for DTC with thyroid carcinoma specific mortality and risk for recurrence in more detail in a group of 366 consecutive DTC patients. We found positive associations between serum TSH concentrations and risk for thyroid carcinoma related death and relapse. In a multivariate Cox-regression analysis model, in which tumor stage and age were also included, this association remained significant in patients who have been cured 1 year after initial therapy. The median of the TSH concentrations in each patient appeared to be the best predictor for thyroid carcinoma related death and relapse. However, subsequent analyses revealed that this effect became apparent at higher median TSH values (cut-off level of 2 mU/l). No dif-

ferences in risks for thyroid carcinoma related death and relapse were observed between suppressed TSH levels (both TSH < 0.4 mU/l and <0.1 mU/l) and unsuppressed TSH levels (TSH levels within the reference range). Interestingly, this association between TSH levels and risk for relapse or thyroid carcinoma related death was present both in patients with initial stages T1-3 and M0 and with stages T4 or M1. Even for initial tumor stage T1-3 and M0, median TSH was an independent predictor for thyroid carcinoma related death. These results differ from earlier studies the studies of Mazzaferri et al and Cooper et al., which did not report an independent relation between TSH and prognosis. Our patient group is comparable with the study of Pujol et al. They found a difference in relapse between the extremes of TSH suppression (continuously undetectable vs. continuously unsuppressed). Pujol et al, however, did not report the relation between TSH levels and DTC-related death. Our study results are in line with the recent report of Jonklaas et al., which demonstrated that the degree of TSH suppression is a predictor of DTC-specific survival in high risk patients, independently of radioiodine ablation therapy and the extent of thyroid surgery. Our analysis extends their findings in the respect that in patients who received total thyroidectomy and radioiodine ablation, and who were cured 1 year after initial therapy, TSH remains an independent predictor for disease specific survival. Our study confirms the findings of Jonklaas et al. that this relation is only present at TSH levels in the higher normal range, so that sustained TSH suppression is not recommended in low risk patients. The results of our study, i.e. the deleterious effects of TSH on thyroid carcinoma recurrence or thyroid carcinoma related death become apparent above a median TSH of 2 mU/l, provide a rationale for the advice in the recently published European and United States guidelines for the follow-up of thyroid carcinoma to aim at TSH levels in the lower normal range (0.4 – 1 mU/l) in low-risk DTC patients, as unnecessary TSH suppression is associated with lower bone mineral density and cardiac dysfunction. Although the relation between TSH levels and risk for DTC-related death or recurrence was also present in non-cured patients and patients with an initially high risk, subgroup analysis did not reveal a safe TSH threshold in these patients. Because we found indications that the hazard of elevated TSH levels for DTC-related death is especially important in non-iodine accumulating metastases, and taking the findings of Jonklaas et al into consideration we advice to maintain suppressed TSH levels (<0.1 mU/l) in patient categories with initial high risk and/or recurrent tumor.

SYNERGISM OF TROGLITAZONE AND LOVASTATIN IN DTC TREATMENT

Statins and thiazolidinediones are not primarily used in the treatment of malignities. The primary clinical indication of statins is to treat hypercholesterolemia and prevent cardiovascular disease, whereas the indication of thiazolidinediones is to improve insulin sensitivity in patients with type 2 diabetes mellitus. Studies have shown that in addition to these effects the statins like lovastatin and thiazolidinediones, like troglitazone, are also effective inhibitors of growth and invasion of tumor cells of various origins. In vitro statins are effective drugs against various cancers e.g. anaplastic thyroid cancer, melanoma, prostate cancer and pancreatic cancer. Thiazolidinediones have also been shown to be effective in a range of different cancer cell-lines in vitro, e.g. breast cancer, hepatocellular

carcinoma, pancreatic cancer, ovarian carcinoma, melanoma, lung carcinoma, and lymphoma cells. An additional effect of these compounds is their capacity to promote cellular differentiation. Recently, Yao et al. found, that a combination of lovastatin and troglitazone can produce a dramatic synergistic effect against human glioblastoma and CL1-0 human lung cancer cells lines in vitro by inducing apoptosis at low concentrations which are clinically achievable. We hypothesized, that this combinational therapy may also be beneficial in thyroid cancer not only by inducing apoptosis in tumor cells, but also by redifferentiation of the thyroid tumor cells and thus sensitizing these cells to conventional RaI therapy. To test our hypothesis, we evaluated in **Chapter 3**, whether this combinational therapy was effective in inhibiting cell growth and differentiation in vitro, in the human follicular thyroid carcinoma cell-line FTC-133.

The combination of troglitazone and lovastatin resulted in a remarkable synergistic effect on morphology and cell density in the FTC-133 cell-line. This effect was previously reported by Yao et al. at similar low concentrations. They explained the effects on growth, at least in part, by the inhibition of the mevanolate pathway by counteracting the effects of the combined therapy with the addition of mevalonolactone. We could mimic the effect on cell growth and morphology of the troglitazone/lovastatin combination by the combination of the geranylgeranylation blocker GGTI with 10M troglitazone, whereas GGTI alone had no effect. This indicates, that inhibition of geranylgeranylation is sufficient for the effects observed on growth and morphology. This points to a Rho related mechanism rather then Ras.

In order to explore if the impaired cell growth and detachment of the cells was due to apoptosis or only to cell arrest shown by the phosphorylation state of Rb, we performed FACS analysis using the cell surface apoptosis marker ANNEXIN V. None of the treatments resulted in an increased expression of the apoptosis marker Annexin on the cell surface, indicating cells were in growth arrest rather then apoptotic. Additionally, most cells were still viable and resumed normal growth and morphology after transfer to normal medium, indicating that the cells appear to arrest rather than move into apoptosis after receiving the troglitazone/lovastatin combination treatment. Higher doses of lovastatin do appear to cause apoptosis, as Wang et al. observed apoptosis in ARO-cells with a lovastatin dose of 50 μM.

One possible explanation for the observed growth inhibition may lay in Rho-related inhibition via p27, an inhibitor of CDK4/6 cyclinD complex assembly. Geranylgeranylation of Rho is essential for degradation of this inhibitor and facilitates progression of G1 to S phase. To initiate this degradation, Rho needs to be activated by geranylgeranylated during the G1 phase, a process blocked by lovastatin and GGTI. Geranylgeranylation enables RhoA to be positioned at the inner face of the plasma membrane where it serves as a switch in cytoplasmic cascades by switching between an active(GTP) and inactive state(GDP).

Troglitazone also appears to have an effect on several cell cycle regulators, including an increase of p21 and p27 levels and reduction in phospho-Rb in several cell lines such at the mRNA and protein level in rat and human hepatoma cells. Furthermore, forced expression of p27 results in G1 phase cell-cycle arrest in most cell-lines. On the protein level Yao et al. observed this effect on p27 when using the combination treatment.

In addition to the known effects on degradation of p27 via Rho, we observed a 12-fold in-

crease in p16 expression and an almost 10-fold increase of P15 expression, when troglitazone and lovastatin were combined. P15INK4b and P16INK4a are members of INK4b-ARF-INK4 a tumor suppressor locus. An excess of these inhibitors can cause G1 cell-cycle arrest by blocking the assembly of the catalytical active CDK4/6 cyclinD complex which facilitates Rb phosphorylation.
P15 and p16 are more primarily associated with growth arrest, whereas p21 and p27 are more associated with apoptosis. This seems to correspond with our findings that the FTC-133 cells only experience growth arrest and no apoptosis after treatment. So an accumulation of these CDK inhibitors is likely to result in G1 phase cell-cycle arrest. The effects on p15 and p16 give at least a partial explanation for the inhibitory effects of the troglitazone/ lovastatin treatment but multiple pathways may be involved.
There is hope that this combination can induce this effect in vivo, because the effects were found at clinically achievable concentrations of lovastatin and troglitazone. In addition, both lovastatin and troglitazone have been shown to have re-differentiating properties. The observed effects on growth of the combined troglitazon/ lovastatin treatment seem to be universal for cancer cell-lines, as Yao et al. discovered similar effects in human glioblastoma, lung-, prostate-, pancreatic- and cervical cancer cells lines. Although the synergism of troglitazone and lovastatin is dramatic in vitro, these observations require confirmation in patients in vivo.

STRATEGIES TO IMPROVE RAI THERAPY

One of the approaches to further improve thyroid cancer therapy for DTC has been the attempt to reintroduce, or boost, RaI-uptake by re-activation or upregulation of NIS by various strategies, such as epigenetic therapies and retinoids.
In vitro, epigenetic therapies have led to the re-introduction of NIS mRNA expression and RaI uptake in DTC. However, a mayor drawback is toxicity, as non-target genes may also be subjected to these interventions. A second approach has been the use of retinoids (derivatives of vitamin A), which actions are mediated through two families of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). In thyroid cancer cell-lines, retinoids increase mRNA NIS expression in FTC-133 and FTC-238 cell-lines, but down-regulated NIS mRNA in FRTL-5 cells. Clinical studies measuring the effect of I-uptake in aggressive DTC have reported an increase in I-uptake in 20-42% of patients.
We focused on two other compounds, lovastatin and troglitazone, which also have been shown to promote cellular differentiation (Chapter 3). Frohlich et al. investigated the effects of troglitazone, rosiglitazone and pioglitazone on differentiation in normal porcine thyrocytes and in follicular carcinoma cell-lines FTC-133 and FTC-238. Troglitazone was most effective of the tested thiazolidinediones in re-differentiating the carcinoma cell-lines as demonstrated by significantly increased I-uptake and apoptosis and decreased cell-number.
In addition to decreased survival rates at doses ranging from 10-75µM, Wang et al. also found a significant effect of lovastatin on differentiation of the anaplastic thyroid cancer ARO cell-line. At a dose of 25 µM, lovastatin was able to significantly increase thyroglobulin

levels in the culture medium in a time dependant manner.
An additional effect of these agents is their capacity to induce apoptosis in tumor cells. Therefore, a combination of these components may be beneficial on two fronts in thyroid cancer, by simultaneously enhancing the effects of conventional RAI therapy and by induction of apoptosis.
We found an increase in NIS and TSHR expression after 2 days of treatment with troglitazone and lovastatin. In addition we showed that the combination of troglitazone and lovastatin treatment resulted in a remarkable synergistic effect on morphology and cell density in the human follicular thyroid carcinoma cell-line, FTC-133. Therefore we believe that a combined troglitazone/lovastatin treatment may proof to be beneficial in patients with DTC as remarkable reduction of growth coincides with increased NIS expression.

MEMBRANE RECEPTOR TARGETED THERAPY

As thyroid cancers progress, thyroid-associated proteins, such as NIS and the TSHR, may gradually be lost. Loss of NIS greatly impairs conventional RAI therapy, as the ability to accumulate iodine is dependant on NIS expression. A major approach to overcome this problem has thus far been increasing NIS expression in these de-differentiated tumors. The TSHR may prove to be a more rewarding target in these tumors as TSHR expression is much longer maintained in de-differentiated tumors. The use of the TSHR as a target for these higher tumor stages thus eliminates the need for re-differentiation.
Our ultimate goal would be to direct toxins exclusively to TSHR-expressing thyroid cancers. As TSH is the natural binding agent of the TSHR, we tried to recombinantly modify TSH in such a way, that it is capable of carrying fused proteins exclusively to TSHr expressing cells. If the activating properties of the TSH fusion protein are maintained, it would in theory be capable of transporting the fused protein into the cell. This transport is crucial for applications, such as a TSH-toxin fusion protein, which needs internalization of the toxin to send cells into apoptosis.
In order to convert TSH into a viable protein for guiding proteins to TSHR expression cells, we modified wild type human TSH (Chapter 4) in three ways:
1. We increased stability by fusing the beta and alpha chain of TSH.
2. We introduced mutations to improve binding to and activation of the TSHR
3. We fused a short protein to our modified single chain TSH to test, whether it is possible to use modified TSH as a vehicle for therapeutic proteins

AD 1: IMPROVING TSH STABILITY

Single chain TSH (scTSH) should in theory be more stable than TSH, which consists of 2 separate chains. Fusion of the beta and alpha chain of TSH improves stability of TSH and bypasses the rate limiting assembly step, which is essential for secretion and hormone specific glycosylation of TSH. Previously, stability was determined by using an immunoassay which is specific for heterodimeric TSH. Grossmann et al. showed that single chain TSH as well as hTSH were stable at 37C for at least 21 days, while scTSH was significantly more sta-

ble than hTSH at 55C. In contrast, we found degradation of both the rhTSH and single chain TSH at 37ºC, when using our TSHR activation assay (Chapter 5). As we anticipated, the scTSH constructs displayed a higher stability than rhTSH, as approximately 50% of mscTSH and 25% of rhTSH activity remained after 48h. In contrast to the effect we found at 37ºC, we saw a sharp decrease of activity at 56ºC with approximately 25% of activity remaining in all TSH constructs after 24h, whereas the activity was almost completely abolished after 48h. This contradictionary effect of temperature on stability between Grossmann's and our study may be due to the different methods used for measuring stability. The method used by Grossmann is based on an immunoassay specific for heterodimeric TSH. Thus, Grossmann et al. did not assess the biological activity, whereas our method is based on actual TSHR activating properties. This suggests that loss of activity may not be directly linked to dissociation of the subunits, but may occur prior to this event.

AD 2: IMPROVING TSH ACTIVITY

In order to create a super-active scTSH, we introduced several mutations in single chain TSH known to improve rhTSH binding to the TSHR. We tested the properties of our modified scTSH (mscTSH) for binding to and activation of the receptor and the relevant biological outcome in the form of iodine uptake. Both binding to and activation of the TSHR by mscTSH were improved when compared to commercially available rhTSH by respectively 10- and 20-fold.
One possible application of super agonistic TSH analogues may lie in improved RaI treatment. RaI is routinely used in the management of thyroid cancer for treatment and diagnostic purposes. As TSH stimulates RaI uptake, patients used to be treated with thyroid withdrawal protocols to increase TSH levels. In recent years recombinant hTSH has become an alternative and phase III trials have demonstrated that rhTSH treatment is nearly or as effective in stimulating RaI uptake as traditional methods. In vitro our mscTSH was almost twice as effective in RaI uptake as rhTSH using FRTL-5 cells, making it a potential candidate for more efficient 131I uptake in vivo. Direct labelling of mscTSH with a radioactive ligand may be another feasible application, especially when distant metastases are involved which sometimes lose RaI uptake but maintain TSHR expression.

AD 3: FUSION PRODUCTS OF TSH

We wanted to know whether it would be possible to fuse a protein to mscTSH, while maintaining biological activity of TSH. As a model for mscTSH fusion proteins we fused a 6xhistidine tag with flexible linker alone and in combination with a six amino-acid sequence to the N terminus of mscTSH since the α-carboxy terminus (α 88-92) is unavailable for binding due to its crucial role in TSHR binding and activation. Use of a nickel gel purification step confirmed the presence of the 6xHis tag and the accessibility of it. We subsequently tested the biologic potential of the 6xHis tagged mscTSH constructs with our bio-luc assay (Chapter 5) and found that the full TSHR stimulating potential was maintained. Furthermore, the addition of a His tag to the mscTSH construct did not impair the stability when compared to the scTSH thus suggesting that the conformation of mscTSH was not dramatically influ-

enced by the additional extension on the N-terminus.
The maintained TSHR-activating potential of TSH is essential for a TSH-TSHR complex to be internalized into the TSHR bearing cell, an essential step for functionality of immunotoxins. As the His-mscTSH and His-13X-mscTSH fusion products still possess the full potential of the modified single chain TSH, it is feasible that our mscTSH is able to guide proteins into the thyroid and thyroid tumors *in vivo*.
After TSHR activation, the normal route of TSH leads to the lysosymes as the TSH-TSHR complex is internalized through clathrin-coated vesicles. This is followed by the recycling of the majority of receptors to the surface and degraded of TSH by lysosomes. In theory, this mechanism would enable TSH-bound components, e.g. toxins, to enter thyroid (tumor) cells, expressing the TSHR because various toxins of bacterial origin (e.g. pseudomonas exotoxin(PE), Diphteria toxin (DT), Ricin, Shiga toxin) use this lysosomal route to kill eukaryotic cells. It is likely that these toxins, when fused to mscTSH, would be able to follow their normal route into the target cell .
The normal cell binding domain of these toxins can be replaced with a different binding domain and possibly with our mscTSH. Within the group of toxins the ones with a cell binding domain on the carboxy terminal side will be best compatible with our mscTSH as the carboxy terminus (α 88-92) of mscTSH is unavailable due to its crucial role in TSHR binding and activation.
Other applications of a TSH fusion protein may lie in the field of diagnostics. Our mscTSH may be able to guide markers towards TSHR bearing cells. However, for diagnostic purposes internalization of mscTSH may not be needed or will even be undesired. A blocking TSH may therefore be more favourable. One way to achieve this could be the introduction of novel mutations which abolish oligosaccharide chain formation. In this way it could be possible to attach markers to the surface of TSHR bearing cells without risking degradation.

Recently, Ochiai et al. reported an additional effect of an EGFRvIII-targeted immunotoxin. This toxin not only had a direct cytotoxic effect by killing tumor cells expressing the mutated EGF-receptor target cells but was also capable of inducing an immune response against tumor cells. After EGFRvIII-targeted immunotoxin treatment the mice not only developed long lasting immunity to EGFRvIII expressing tumor cells, but also to tumor cells lacking EGFRvIII expression. This effect may also prove to be beneficial in the treatment of TSHR expressing thyroid tumors with a TSHR based immunotoxin. Due to the effect of cross immunity shown by Ochiai et al. even highly dedifferentiated thyroid tumors that have lost expression of the TSHR may still be treatable by a TSHR-toxin fusion protein.

In addition to the induction of immunity by TSHR-immunotoxins other strategies that induce an immune response against the TSHR may also prove to be beneficial in treating advanced thyroid tumors. In mice attempts have been made to induce anti-TSHR responses to mimic Graves disease by vaccination with TSHR preparations, TSHR expressing cells and DNA-based vaccines with various results. Similar techniques may well prove to be beneficial in treating advanced thyroid tumors still expressing the TSHR. Recently, the feasibility of this strategy was successfully demonstrated in a mouse model using NIS which is another thyroid specific protein. DNA vaccination using the MIDGE/hNIS vector was able to

induce h-NIS associated immune responses in mice which resulted in a remarkable inhibition of tumor formation after the mice were challenged with NIS transfected tumor cells. These results make it feasible that a TSHR based vaccination approach will have a beneficial effect on thyroid cancers that often still possess the TSHR. In the clinic, NIS vaccination may not be as relevant as TSHR vaccination because the administration of RaI is already a very effective therapy for less advanced thyroid tumors that retain NIS expression.

An alternative for TSH-toxin fusion proteins may consist of antibody-based toxin fusion products. For TSHR binding immunotoxins to be effective, the TSHR has to be internalized together with the toxin, a process which is normally induced by activation of the TSHR by TSH. Thus, when antibodies are used in TSHR binding immunotoxins, a special subgroup of TSHR antibodies is required which not only bind but also activate the receptor. There have been many attempts to produce potent TSHR stimulating monoclonal antibodies in the past using animal models of Graves disease, but monoclonal antibodies with potent stimulating activity remain scarce. Recently, a few attempts have led to monoclonal antibodies with full agonistic activities that have a potential use in immunotoxins. Ando et al. was the first to clone a fairly potent TSHR stimulating Ab isolated from hamsters immunized with the adenovirus construct AdTSHR. Their monoclonal antibody was capable of stimulating the TSHRr at a dose of 20ng/ml. Almost simultaneously, Sanders et al. also were ably to produce TSHr stimulating monoclonal antibodies after immunization of a mouse with hTSHR cDNA. These antibodies were also capable of stimulating the TSHr at a dose of 20ng/ml.
Recently more potent monoclonal antibodies have been reported from mouse and human origin. Gilbert et al. have succeeded in cloning these potent TSHR stimulating monoclonal Ab from mice immunized with hTSH cDNA containing recombinant adenoviruses. Their monoclonal antibodies named KSAb1 and KSAb2 were capable of TSHR stimulation at a concentration of only 1,2 and 2,2 ng/ml, respectively
Most attempts to produce monoclonal TSHR stimulating antibodies from the blood of patients with Graves' hyperthyroidism have been fruitless so far. However, one attempt to produce a monoclonal antibody from a Graves' patient has been successful. Sanders et al. succeeded in producing a potent human TSHR stimulating antibody based on isolated human lymphocytes from the blood of a Graves patient capable of stimulation the hTSHr in the 1ng/ml range.
Whether Ab-fragments based or TSH based immunotoxins will prove to be more effective in targeting DTC, will have to be a subject of further study. However, a mayor advantage of Ab over TSH is the possibility to produce these in E.coli as mscTSH production in CHO did not have a high yield in our production system.
In a commercial setting low levels of protein may prove to be a problem to make production commercially viable. The use of more efficient vectors, optimized secretion and an extensive selection of highly producing cell-lines may result in higher levels of secreted TSH constructs.

THE TSHR IN OTHER TISSUES

A potential problem for TSHR guided toxins may be the non-exclusiveness of the TSHR to thyroidal tissues. A number of papers have reported the prevalence of TSHR mRNA and/or protein in non-thyroid tissues such as lymphocytes, thymus, pituitary, testis, kidney, heart and orbital tissues. However TSHR levels are very low in these tissues are very low and may be due to 'leaky' transcription which presumably occurs incidentally rather than intentionally implicating a lack of function of the TSHR in the extra thyroidal expression. However, recently some papers reported an active role of the TSHR in bone remodelling. It has been shown that osteoblasts express TSH receptors and display increased levels of cAMP when exposed to TSH. However, Tsai et al. concluded that given the low levels of expression, specific binding and cAMP signalling that it is unlikely that TSH plays a physical role in bone remodeling. Furthermore, Abe et al. showed that TSHr-/- mice developed severe osteoporosis and heterozygous TSHr+/-mice with normal T3, T4 and TSH levels still developed bone loss indicating a critical role for TSH in bone remodelling. In contrast, Bassett et al. found that TSH levels did not influence bone remodeling by the use of thyroid hormone receptor knock out mice which demonstrated bone loss despite elevated TSH levels.
So, the importance of the TSHR in non-thyroidal tissues remains inconclusive and the TSHr may have no physiological function in these tissues. However, the use of mscTSH-toxin constructs and subsequent destruction of TSHr bearing tissues may cause problems if the TSHr really plays a role in other tissues. But taken into consideration that only one in a hundred toxic domains reach their cytosolic target the therapeutic window between the TSHr rich thyroid cells and other TSHR expressing tissues should be sufficient for therapeutic applications, but of course should be a subject of further study.
When using mscTSH constructs for visualization of the thyroid or thyroid derived tumors the presence of the TSHR in other tissues is unlikely to interfere due to the high levels of TSHR present in the thyroid when compared to other tissues.

BIOASSAY FOR TSHR ACTIVATION

In our attempts to produce functional scTSH based conjugates (*see* **Chapter 4**) we were in need of an effective assay to test TSHR stimulation. Commercial TSH assays are not suited for this purpose, as they measure the capacity for binding to the TSHR but not actual stimulation of the TSHR. In order to test the TSHR activating potential of our mscTSH based conjugates we developed a bioassay based on cAMP induced luciferase expression. Stimulation of this assay with bTSH and hTSH resulted in a near linear increase in luminescence up to a TSH concentration of 50mU/l. After validating the assay we used it to test the activity of our mscTSH constructs described in Chapter 4.
In addition to the testing of the bioactivity of our mscTSH constructs, we realized the potential of using our assay in studying groups of patients with autoimmunity to the TSHR. These TSHR binding Ab can either stimulate the TSHR or block access of TSH causing hyperthyroidism or hypothyroidism, resp.. The autoimmune disease Graves' disease (GD) is the most prevalent cause of hyperthyroidism and is characterized by the presence of autoanti-

bodies against the TSHR that are referred to as TRAb (TSH receptor antibodies) or TBII (TSH receptor binding inhibiting immunoglobulins). TBIIs are a generic term for both thyroid stimulating antibodies (TSAb) and thyroid blocking antibodies (TBAb). Hyperthyroidism in GD is caused by TSAb, which bind to, and activate, the TSHR.

There are several commercial tests for these TBII but the obvious disadvantage of these tests is their inability to detect the biological activity of the antibodies. Consequently, it is not possible to correlate the test results with the degree of hyperthyroidism. This is particularly important in pregnancy, where the distinction between TSAb and TBAb, rather than the demonstration of TBII, has clinical consequences. Our assay enabled the measurement of direct stimulation of the TSHr by TSAb in sera of patients with GD and we correlated our TSHr activation with serum free T4 levels as a clinical in vivo end-point of TSH receptor activation. The results of our assay revealed a strong correlation between TSHR activation and serum free T4 levels in the 35 untreated GD patients. In contrast, TBII titres did not correlate with serum free T4 levels. In addition, we found that high TBII titres were associated with weak TSHR activation.

Bioluminescence assays published so far have demonstrated the feasibility of this approach. These studies gave a good indication of the spectrum of TSHR activation in these patients. The purpose of our study was to develop a test with a higher in vitro sensitivity for TSH than those previously published and to study the direct correlation between in vitro TSHR stimulation and serum free T4 levels as a clinical end-point of TSHR stimulation. This correlation could not be studied in earlier studies because of the fact that *de novo*, untreated GD patients as well as treated patients were studied.

We found a strong and highly significant correlation between the *in vitro* TSHR-stimulating activity of GD patient sera and their serum free T4 levels, in contrast to the absence of a relationship between TBII levels as assessed by TRAK and serum free T4 levels. To our knowledge, only one animal study has been published demonstrating a relationship between the TSHR-stimulating hamster antibody MS-1 and free T4 levels in mice. In our study, we found a strong correlation between activating properties of TSAb in patients with GD and serum free T4 levels, irrespective of TBII titres.

Another category of patients, in which the determination of TSAb or TBAb could be helpful are patients which received radioiodine treatment. RaI in toxic multinodular goitre (TMNG) has been associated with the occurrence of Graves'-like hyperthyroidism and it has been postulated that pre-existing autoimmunity may contribute to this phenomenon. To study whether RaI induces TSAbs in the short term in TMNG and whether pre-existing autoimmunity is relevant, we tested TMNG patients with our bioassay and included a group of patients with Graves' disease in Chapter 6.

Earlier studies addressing this issue were limited by the fact that no functional TSAb assays were used. Two studies did use a functional TSAb assay but the sensitivity of these assays was limited; one study did not include Graves' disease patients and the other study was small.

We used a luciferase-based TSAb bioassay with a functional sensitivity of 0.2 mU/l bTSH. In the group of Graves' disease patients, we did not find evidence for RaI-induced TSAbs, although a significant rise in Tg was observed, thus indicating the release of thyroid antigens. Although TBIIs were present in 97% of the patients, TSAbs were present in 64%, indicating

that TSAbs may disappear spontaneously. Three TMNG patients had measurable TBIIs. It is a subject of debate whether the diagnosis TMNG is valid in these patients or that they have Graves' disease despite the typical scintigraphic pattern. Some authors adopt the concept of subclinical autoimmunity in TMNG . Even if the diagnosis of TMNG might be withdrawn, TSAbs were present in five patients, three of whom had TBIIs that were not measurable by non-functional assays. This underscores the high sensitivity of our TSAb bioassay, but also the fact that thyroid autoimmunity may be more common in TMNG than previously thought. The exact significance of this finding and, more specifically, whether TSAbs play a causative or enhancing role in the pathogenesis of TMNG, remains to be clarified. We found induction of TBIIs after RaI in three TMNG patients. TSAbs were present in only one of the patients with de-novo TBIIs. The proportion of patients with TSAbs was not influenced by RaI in TMNG.

In the study by Chiovato et al. high TSAb levels in patients with Graves' disease before RaI were related to resistance to therapy which was not the case in our study. These authors also found that a post-RaI increase in TSAbs was related to the development of hypothyroidism. In contrast, we found that a post-RaI increase in TSAbs in patients with Graves' disease was associated with a lower proportion of hypothyroidism. In the study by Michelangeli et al., hypothyroidism after RaI for Graves' disease was mainly observed in patients with a post-RaI rise in TBIIs which was attributable to both TSAbs and TBAbs. In patients with only TSAbs, no hypothyroidism developed, which is thus in line with our observation.

We conclude that the newly developed B1-TBII bioassay has several advantages: The use of the bioassay enables an insight into the degree of TSHR activation in contrast to the standard TRAK assay, which only determines antibody binding to the TSHR. This is illustrated by the strong correlation between the free T4 levels and the luminescence. However, additional analyses in frozen plasma from patients with hyperthyroidism showed no significant correlation between luminescence and free T4 levels. Possible explanations for this may be partial loss of function of TBII due to long storage or repetitive freezing alternatively unknown factors in the serum may influence the bioassay by increasing intracellular cAMP levels independently of TSHr stimulation.

Furthermore, we invested the hypothesis that pre-existing autoimmunity contributes to RaI induced Graves'-like hyperthyroidism. From the present study we conclude that TBIIs may be present before RaI in TMNG, that RaI may induce TBIIs shortly after RaI but that this induction is not accounted for by TSAbs only and that pre-existing autoimmunity is not a requirement for the induction of TBIIs (as evidenced by the lack of effect of RaI in Graves' disease). In addition, TSAbs measured with a high sensitivity bioassay may be present in TMNG patients with TBIIs below the threshold of detection.

OVERALL CONCLUSION

Existing therapies for thyroid cancer are highly effective in the majority of patients. However, a subgroup of patients (10-15% of patients with DTC) with distant metastases have high remission rates after conventional RaI-treatment. Therefore improvement of conventional therapy or new innovative therapies are needed. We have explored several

routes which in time may help to improve the prognosis for this subset of patients, focussing on the TSHR.

The combination of troglitazone and lovastatin may have potential use in DTC as we observed a strong reduction of growth and distinct changes in morphology in the follicular thyroid carcinoma cell-line FTC-133 at clinically achievable concentrations. Furthermore, the combination of troglitazone and lovastatin was able to increase the expression of NIS and the TSHR which may prove to be beneficial in sensitizing thyroid tumor cells to conventional RaI therapy. Therefore a combination of these components may be beneficial on two fronts in thyroid cancer by simultaneously enhancing the effects of conventional RaI therapy and by growth reduction.

Secondly, we explored the possibility of thyroid specific membrane associated therapy by using the TSHR as a target. The TSHR may prove to be a more rewarding target in DTC as TSHR expression is much longer maintained than NIS in de-differentiated tumors. We succeeded in modifying TSH into a potential vehicle for toxins by converting it into a single chain protein with improved binding to the TSHR. The fusion of short proteins to our modified single chain TSH did not impair binding thus confirming the potential in using modified TSH as a vehicle for therapeutic proteins.

Tumors derived from thyroid cells potentially offer unique opportunities for treatment due to their unique nature. One of these features, the accumulation of RaI has been used for many decades and its success may be enhanced by the upregulation of NIS, possibly via troglitazone and/or lovastatin treatment. In addition to NIS, other unique proteins may be used for treatment. We believe the TSHR is a prime candidate for the specific targeting of thyroid derived tumor cells by modified TSH- or Ab-conjugates. Besides the options discussed in this thesis other innovative thyroid specific therapies may further improve thyroid cancer treatment, alone or as a supplement to existing therapies.

The TSHR may not only be a target for therapies, but also mediate the growth promoting effects of TSH. TSH suppressing thyroxine replacement therapy has therefore always been an important element in the clinical follow-up of DTC patients. We have demonstrated in our studies that a balanced attitude is feasible, in which complete TSH suppression in cured patients is not necessary, thus preventing those patients from the potential negative effects of long term TSH suppression on other organs.

Chapter 8

Samenvatting & Discussie

INTRODUCTIE

Conventionele therapieën voor gedifferentieerd schildklier carcinoom (DTC) bestaan uit een chirurgische ingreep (totale schildklierverwijdering) en de toediening van radioactief jodium (RAI). Deze behandelingen zijn effectief in de meerderheid van de patiënten, maar dit beperkte therapeutische arsenaal schiet vaak tekort zodra zich afstandsmetastasen hebben gevormd. Deze metastasen die merendeels in de longen en botten optreden, blijken het vermogen tot radioactief-jodium opname vaak geheel of gedeeltelijk te verliezen zoals aangetoond kan worden met totale lichaam scintigrafie. waardoor RAI therapie hier slechts in een beperkt aantal gevallen leidt tot genezing. Aangezien alternatieve opties, zoals radiotherapie en chemotherapie, weinig succesvol blijken, zijn de vooruitzichten voor deze patiëntengroep slecht. Daarom is het wenselijk dat de conventionele therapie wordt verbeterd door het vermogen tot radioactief jodium opname te verbeteren of geheel nieuwe vormen van therapie te ontwikkelen. In dit proefschrift richten wij ons met name op nieuwe therapieën die aangrijpen op de receptor voor het schildklierstimulerend hormoon (TSHr). Tevens hebben wij nieuwe mogelijkheden onderzocht voor therapie gebaseerd op re-differentiatie.

TSH SUPPRESSIE IN GEDIFFERENTIEERD SCHILDKLIER CARCINOOM (DTC)

Binnen de conventionele therapie en follow-up voor de behandeling van schildkliertumoren is het wellicht mogelijk om een verdere verbetering te behalen in de behandeling van schildkliertumoren door processen te optimaliseren. Het is gebruikelijk om serum TSH spiegels te onderdrukken door overdosering van thyroxine in DTC patiënten om recidieven te voorkomen: door de hoge doses thyroxine wordt het serum TSH onderdrukt, wat nuttig is omdat TSH een groeifactor is voor DTC. In **hoofdstuk 2** hebben wij de relatie onderzocht tussen serum TSH spiegels en de kans op recidief en sterfte bij DTC aangezien dit slechts in beperkte mate is onderzocht.

Bij patiënten met DTC is suppressie van TSH spiegels door middel van thyroxine overdosering gebruikelijk. De achterliggende gedachte voor deze behandeling is de observatie dat TSH de proliferatie stimuleert van schildklier carcinoma *in vivo* en *in vitro*. Een nadeel van deze praktijk zijn de nadelige effecten op botdichtheid en hartfunctie. Hierdoor is het van belang om een TSH niveau te handhaven, waarbij terugkeer van tumoren voorkomen wordt, maar waarbij de nadelige effecten tot een minimum beperkt blijven. Ondanks het feit dat de bestaande praktijk gebaseerd is op deze observaties zijn er slechts weinig klinische, observationele studies uitgevoerd naar de effecten van thyroxine-geïnduceerde TSH suppressie op DTC ontwikkeling en DTC gerelateerde mortaliteit.
Eerdere studies verschillen van onze studie als we kijken naar homogeniteit van de patientengroepen (in relatie tot initiële therapie), het aantal patiënten en de duur van de follow-up. Teneinde een rationele keuze te kunnen maken voor een optimaal TSH niveau hebben wij de relatie onderzocht in patiënten gedurende de follow-up tussen TSH

concentraties en DTC gerelateerde mortaliteit en recidive in een groep van 366 patiënten met DTC. Hierbij hebben wij positieve associaties gevonden tussen TSH concentraties in serum en het risico tot DTC gerelateerde mortaliteit en recidieven. In een univariate Cox-regressie analyse waarbij tumor stadium en leeftijd werden meegenomen als covariabelen bleek er een significante relatie te zijn tussen serum TSH spiegels, sterfte en recidief.
De mediaan van de TSH concentratie van iedere patiënt lijkt de beste indicator voor schildkliercarcinoom gerelateerde sterfte en recidief. Uit een verdere analyse van de data bleek dat dit effect een rol speelt vanaf hogere TSH waardes (2 mU/L). Tussen onderdrukte TSH concentraties (zowel TSH < 0.4 mU/L en <0.1 mU/L) en TSH concentraties binnen de referentie-range werden geen verschillen gevonden in relatie tot de risico's op schildkliercarcinoom gerelateerde dood en terugval.
Het is interessant dat deze associatie tussen TSH niveaus en het risico op terugval of schildkliercarcinoom gerelateerde dood aanwezig was in patiënten met zowel de beginstadia T1-3 en M0 als de stadia T4 of M1. Zelfs bij de initiële tumorstadia T1-3 en M0 bleek de TSH mediaan een onafhankelijke voorspeller te zijn voor schildkliercarcinoom gerelateerde sterfte. Deze uitkomsten verschillen van eerdere studies door Mazzaferri et al. en Cooper et al. waar geen onafhankelijke relatie werd gevonden tussen TSH en prognose. Onze patiëntengroep is vergelijkbaar met de studie van Pujol et al. Zij vonden een verschil in terugval tussen de extremen in TSH suppressie (totale suppressie versus geen onderdrukte TSH niveaus). Pujol et al. konden echter geen relatie aantonen tussen TSH niveaus en DTC-gerelateerde dood. De resultaten van onze studie corresponderen ook met een recente studie van Jonklaas et al. die aantoonden dat de mate van TSH suppressie een voorspeller is voor DTC specifieke overleving van patiënten met een hoog risico onafhankelijk van radioactief jodium therapie of de omvang van schildklier chirurgie. Onze analyse breidt deze bevindingen verder uit naar patiënten die na 1 jaar genezen waren na totale schildklierverwijdering en behandeling met radioactief jodium. Bij deze patiënten blijft TSH een onafhankelijke voorspeller voor ziektevrije overleving. Verder bevestigt onze studie de bevindingen van Jonklaas et al. die stelden dat deze relatie alleen aanwezig is bij TSH niveaus in het hogere normale bereik. Daarom is permanente TSH suppressie niet aan te raden bij patiënten met een laag risico.

Uit onze studie blijkt dat TSH een effect heeft op terugkeer van schildkliercarcinoom of schildkliercarcinoom gerelateerde dood vanaf de 2mU/L TSH mediaan. Dit geeft een rationele onderbouwing voor het advies dat recentelijk werd gepubliceerd in de Europese en Amerikaanse richtlijnen voor de follow-up van DTC, waarbij werd aangeraden TSH niveaus voor laag risico DTC patiënten binnen de range van 0.4-1mU/L te houden, omdat onnodige TSH suppressie wordt geassocieerd met lage minerale bot dichtheid en cardiaal disfunctioneren. Hoewel de relatie tussen TSH niveaus en DTC gerelateerde dood of recidieven ook aanwezig was bij niet genezen patiënten of patiënten met een hoog initieel risico kon hier na subgroep analyse geen veilige TSH grenswaarde gevonden worden. Aangezien we indicaties gevonden hebben dat verhoogde TSH niveaus vooral een gevaar opleveren voor DTC gerelateerde sterfte bij metastasen die geen jodium accumuleren en als we de resultaten van Jonklaas et al. in ogenschouw nemen adviseren wij om TSH te onderdrukken (<0.1 mU/L) in patiënten met een hoog initieel risico en/of terugkerende tumoren.

SYNERGIE VAN TROGLITAZONE EN LOVASTATINE BIJ DTC BEHANDELING

Maligniteiten zijn niet het primaire doel van statines en thiazolidendiones. Statines worden gebruikt als behandeling van hypercholesterolemie ter voorkoming/behandeling van cardiovasculaire ziekten. Thiazolidendiones worden ingezet om insuline sensitiviteit te verhogen in patiënten met type 2 diabetes mellitus.
Verscheidene studies hebben echter aangetoond dat zowel statines (b.v. lovastatine) en thiazolidinediones (b.v. troglitazone) ook effectieve remmers zijn van groei en invasie van tumorcellen van verschillende origine. *In vitro* is de effectiviteit van statines op tumoren van verschillende origine aangetoond bij anaplastisch schildklierkanker, melanoma, prostaat kanker en pancreas kanker. Ook thiazolidinediones hebben in *in vitro* systemen bewezen effectief verscheidene tumorcellijnen te kunnen bestrijden b.v. borstkanker, hepatocellular carcinoom, pancreas kanker, ovarium carcinoom, melanoom, long carcinoom en lymphoma cellen. Een additioneel effect van deze stoffen is hun vermogen om cellulaire differentiatie te stimuleren. Yao et al. hebben recentelijk aangetoond dat een combinatie van lovastatine en troglitazone een dramatisch synergistisch effect heeft op humane glioblastoma en humane CL1-0 long kanker cellijnen *in vitro* door apoptose te induceren, bij klinisch haalbare lage concentraties. Hypothetisch gezien zou deze combinatietherapie een gunstig effect kunnen hebben op schildkliertumoren door de inductie van apoptose, maar wellicht ook door redifferentiatie van schildkliertumoren welke de gevoeligheid voor conventionele radioactief jodium therapie zou kunnen verhogen. Om onze hypothese te testen hebben wij in **Hoofdstuk 3** geëvalueerd of deze combinatietherapie effectief is in de remming van celgroei en reedifferentiatie van de folliculaire schildklier tumorcellijn FTC-133.
De combinatie van troglitazone en lovastatine resulteerde in een opmerkelijk synergistisch effect op morfologie en celdichtheid van de FTC-133 cellijn. Dit effect op groei dat eerder is geobserveerd door Yao et al. bij vergelijkbaar lage concentraties, werd door hen (in ieder geval ten dele) toegeschreven aan een remming van de mevanolaat transductie route aangezien remmende effecten teniet werden gedaan door de toevoeging van mevalonolactone. Wij konden het gecombineerde effect van troglitazone/lovastatine op groei en cel morfologie nabootsen door de geranylgeranylatie blocker GGTI te combineren met troglitazone waarbij GGTI zonder troglitazone geen effect had. Dit wijst erop dat remming van geranylgeranylatie voldoende is om het geobserveerde effect van troglitazone/lovastatine op groei en cel morfologie te bereiken.
Om uit te zoeken of de remming op celgroei en het latere loskomen van de cellen het gevolg is van apoptose of cel arrest, hebben wij een FACS analyse uitgevoerd met als doel om de apoptose marker phosphatidylserine (PS) op te sporen via het phosphatidylserine bindende ANNEXIN V-FITC. Geen van de behandelingen resulteerde in een verhoogde expressie van phosphatidylserine op het celoppervlak van FTC-133, wat er op wijst dat de cellen meer in groei arrest zijn dan apoptotisch. Daarnaast bleken de meeste cellen nog levensvatbaar na 2 dagen van troglitazone/lovastatine behandeling en gingen ze over tot normale groei na overbrenging in medium zonder troglitazone/lovastatine. Hogere doses van lovastatine lijken wel in staat om apoptose te veroorzaken, Wang et al. toonden apoptose aan na behandeling van ARO cellen met 50 µM lovastatine.
Een mogelijke verklaring voor de geobserveerde remming op groei ligt mogelijk bij Rho

gerelateerde remming via p27 (een remmer van CDK4/6 cyclineD complex vorming). Geforceerde overexpressie van deze remmer zorgt voor groei arrest in de G1 fase in het merendeel van geteste cellijnen. Geranylgeranylatie van Rho is essentieel bij de degradatie van deze remmer en zorgt voor progressie van G1 naar de S fase. Voor initiatie van deze degradatie moet Rho geactiveerd worden door geranylgeranylatie in de G1 fase. Dit proces van geranylgeranylatie kan geblokkeerd worden door lovastatine en GGTI. Geranylgeranylatie resulteert in de migratie van RhoA naar de binnenkant van het plasmamembraan waar het cytoplasmatische cascades aanstuurt door te schakelen tussen een actieve (GTP) en inactieve (GDP) vorm.
Troglitazone lijkt ook een effect te hebben op verscheidene cel regulatoren wat aangetoond is in rat en humane cellijnen op mRNA en eiwit niveau. Aangetoonde effecten waren een toename van p21 en p27 niveaus en een reductie van gefosforyleerd Rb. Yao et al. hebben deze effecten op p27 aan kunnen tonen op eiwit niveau na behandeling met de troglitazone/lovastatine combinatie.
Naast deze effecten van de troglitazone/lovastatine combinatie op p27 via Rho zagen wij een 12 voudige toename van p16 expressie en een bijna 10 voudige toename van p15 expressie. $p15^{INK4b}$ en $p16^{INK4a}$ behoren tot het tumor suppressor locus INK4b-ARF-INK4 en een overmaat van deze remmers kan G1 arrest veroorzaken door de vorming van een actief CDK4/6 cyclinD complex dat zorgt voor blokkering van Rb fosforylatie.
p15 en p16 worden meer geassocieerd met groei arrest terwijl p21 en p27 meer geassocieerd worden met apoptose. Dit lijkt in overeenstemming met de observatie dat de FTC-133 cellen in onze experimenten tot groei arrest komen en niet overgaan tot apoptose na de behandeling met troglitazone en lovastatine.
Kortom, een accumulatie van deze CDK remmers zal waarschijnlijk leiden tot G1 fase cel-cyclus arrest en de geobserveerde effecten op p15 en p16 geven in ieder geval een gedeeltelijke verklaring voor het remmende effect van de troglitazone/lovastatine behandeling op de groei maar mogelijk zijn er meerdere transductieroutes bij betrokken.
Mogelijk zal de troglitazone/lovastatine behandeling toepasbaar zijn *in vivo* aangezien de geobserveerde *in vitro* effecten optraden bij klinisch haalbare concentraties van lovastatine en troglitazone. Naast de effecten op groei is bij lovastatine en troglitazone ook aangetoond dat ze aan kunnen zetten tot redifferentiatie. Hoewel de synergie van lovastatine en troglitazone een dramatisch effect heeft in vitro zal verder onderzoek moeten uitwijzen of deze toepassing in vivo tot reductie van tumoren zal leiden.

STRATEGIEËN VOOR DE VERBETERING VAN RADIOACTIEF JODIUM THERAPIE

Een van de invalshoeken voor verbetering van therapie voor schildklierkanker is de herintroductie of stimulatie van het vermogen tot radioactief jodiumopname via de natrium-jodide symporter (NIS). Hiertoe zijn pogingen gedaan met verscheidene strategieën zoals epigenetische therapieën en retinoiden. *In vitro* hebben epigenetische therapieën ertoe geleid dat NIS mRNA expressie werd herintroduceerd en het vermogen tot radioactief

jodium opname hersteld werd in DTC. Er kleeft echter een groot nadeel van deze aanpak, namelijk toxiciteit. Deze kan optreden doordat genen die niet het doelwit zijn ook kunnen reageren op deze interventies.
Een andere strategie voor herstel van jodiumopname is het gebruik van retinoiden (derivaten van vitamine A). Retinoiden kunnen een effect bereiken door binding aan twee families van nucleaire receptoren: retinoic acid receptoren (RAR) en retinoid X receptoren (RXR). In schildkliertumor cellijnen kunnen retinoiden resulteren in een effect op NIS expressie. In de schildklier tumorcellijnen FTC-133 en FTC-238 resulteert toediening van retinoiden in een toename van NIS mRNA maar in FRTL-5 cellen waren retinoiden in staat om NIS mRNA te reduceren. Klinische studies hebben een effect laten zien in patiënten met agressieve DTC. In deze patiënten werd verhoogde jodiumopname gevonden in 20-42% van de patiënten.
Wij hebben ons gericht op twee andere stoffen, lovastatine en troglitazone, welke hebben bewezen herdifferentiatie in cellen te kunnen bevorderen **(Hoofdstuk3)** Frohlich *et al.* heeft de effecten van troglitazone, rosiglitazone enand pioglitazone op de differentiatie van normale varkens thyrocyten en de folliculair carcinoom cellijnen FTC-133 en FTC-238 onderzocht. Binnen deze studie bleek troglitazone het meest effectief te zijn van de geteste thiazolidinediones in de inductie van herdifferentiatie in de carcinoma cellijnen. Deze herdifferentiatie kon worden aangetoond door een significante toename van I-opname en afname van celgroei. Ook lovastatine lijkt een redifferentiërend effect te hebben, Wang et al. vonden naast een afnemende overleving van anaplastische schildklier tumorcellen (ARO) bij doses van 10-75µM lovastatine ook effecten op redifferentiatie van deze cellijn *in vitro*. Bij een dosis van 25 µM was lovastatine in staat om thyroglobuline niveaus in het kweek medium significant tijdsafhankelijk te verhogen. Een additioneel effect van deze stoffen is hun vermogen om apoptose te induceren in tumor cellen. De eigenschappen van deze stoffen maken het wellicht mogelijk te schildkliertumoren te behandelen door simultaan het effect van conventionele radioactief jodium (RAI) therapie te verhogen en daarnaast apoptose te induceren in tumorcellen.
Wij vonden een toename van NIS en TSHr expressie na 2 dagen behandeling met troglitazone en lovastatine. Verder zagen wij dat de combinatie van troglitazone en lovastatine resulteerde in een opmerkelijk synergetisch effect op morfologie en celdichtheid van de folliculaire schildkliercarcinoma cellijn FTC-133. Gelet op deze resultaten lijkt het ons mogelijk dat een behandeling met troglitazone en lovastatine een gunstig effect kan hebben bij patiënten met DTC aangezien de combinatie therapie *in vitro* resulteert in zowel groeireductie als verhoogde NIS expressie van tumoren.

MEMBRAAN RECEPTOR GERICHTE THERAPIE

Tijdens dedifferentiatie van schildklierkanker kunnen specifieke schildklier gerelateerde eiwitten zoals NIS en de TSHr geleidelijk verloren gaan. Het verlies van NIS heeft een sterk negatief effect op de conventionele RAI therapie, daar het vermogen tot jodiumopname direct gerelateerd is aan NIS expressie. Pogingen om het effect van bestaande RAI therapie te vergroten zijn tot dusver voornamelijk gericht geweest op het verhogen van NIS expressie

in deze ge-dedifferentieerde tumoren. Een andere therapeutische invalshoek, waarbij de behandeling gericht is op de TSHr i.p.v. NIS, heeft wellicht een grotere kans van slagen, omdat de TSHr langer tot expressie blijft komen dan NIS in ge-dedifferentieerde tumoren. Een dergelijke therapie zou de noodzaak voor reedifferentiatie elimineren.

Ons uiteindelijke doel is het sturen van toxines naar schildklier tumoren die de TSHr tot expressie brengen. Gezien het feit dat TSH de natuurlijke ligand is van de TSHr hebben wij geprobeerd om TSH zodanig te modificeren dat deze kan fungeren als drager van gefuseerde eiwitten naar cellen die de TSHr tot expressie brengen. Als de TSHr activerende eigenschappen behouden blijven, zou het in theorie mogelijk moeten zijn om gefuseerde eiwitten in de cel te brengen. Een dergelijk transport is cruciaal voor de werking van TSH-toxine fusie-eiwitten omdat deze opgenomen moeten worden door de cel om de cel naar apoptose te leiden.

Om TSH geschikt te maken voor het transport van eiwitten naar schildklier tumoren die de TSHr tot expressie brengen hebben we TSH op 3 manieren gemodificeerd:

1. het verhogen van de stabiliteit door de fusie van de alpha en beta keten.
2. De introductie van mutaties, die de binding aan en activatie van de TSHr verhogen
3. De fusie van een korte eiwitsequentie aan het gemodificeerde TSH om de hypothese te onderzoeken of TSH kan fungeren als drager van therapeutische eiwitten zonder functie verlies

AD 1: VERHOGEN VAN TSH STABILITEIT

TSH bestaande uit een enkele keten (scTSH) zou in theorie stabieler moeten zijn dan wild type (wt) TSH dat uit 2 losse ketens bestaat, aangezien dissociatie dan voorkomen wordt. Daarnaast wordt de productie wellicht geoptimaliseerd, omdat de snelheidsbepalende productiestap die essentieel is voor secretie en hormoon specifieke glycosylering, wordt overgeslagen.
Recentelijk is de stabiliteit van scTSH bepaald door Grossmann et al. met behulp van een immunoassay dat specifiek is voor het heterodimere TSH. Zij toonden aan dat zowel TSH dat bestaat uit een enkele keten als recombinant humaan (rh) TSH stabiel waren gedurende minimaal 21 dagen bij 37^{O}C, terwijl scTSH significant stabieler was bij 55^{O}C. Onze data lijken te contrasteren met deze bevindingen aangezien wij bij zowel rhTSH als scTSH degradatie zagen bij 37^{O}C na activiteitsmeting met een TSHr activatie assay (Zie Hoofdstuk 5). Hoewel beide TSH's in activiteit afnamen bleek scTSH een hogere activiteit te behouden (respectievelijk 50% en 25% activiteit na 48h voor mscTSH en rhTSH). Bij 56^{O}C zagen wij een scherpe activiteit afname na 24h tot 25% van de initiële activiteit bij beide TSH's gevolgd door een totaal verlies van activiteit na 48h. De verschillen tussen de studie van Grossmann et al. en onze resultaten zijn mogelijk te verklaren door de gebruikte meetmethoden voor de stabiliteitsmetingen. De methode van Grossmann et al. is gebaseerd op een immunoassay dat specifiek is voor heterodimeer TSH en meet niet de daadwerkelijke activiteit terwijl onze assay directe TSHr activatie kan aantonen. Dit lijkt erop te wijzen dat verlies van activiteit niet direct gerelateerd is aan dissociatie van de subunits maar al eerder optreedt.

AD 2: VERBETEREN VAN DE TSH ACTIVITEIT

Met als doel een superactief scTSH te creëren hebben wij verscheidene mutaties aangebracht om binding aan de TSH receptor te verbeteren. Het aldus ontstane gemodificeerde scTSH (mscTSH) hebben wij getest op het bindingsvermogen aan, en activatie van, de TSHr en op relevante biologische output in de vorm van jodiumopname. Zowel binding als activatie door mscTSH lieten een verbetering zien in vergelijking met commercieel verkrijgbaar rhTSH van respectievelijk 10 en 20 keer. Een mogelijke toepassing van super agonistische TSH analogen zou een verbetering kunnen zijn van de radioactief-jodiumbehandeling. Een behandeling met radioactief-jodium wordt routinematig gebruikt bij schildkliertumor management voor zowel behandeling als diagnostiek. Omdat TSH radioactief jodium opname stimuleert, werden patiënten in het verleden behandeld met stopzetting van schildklierhormoon therapie om de endogene TSH niveaus te verhogen. De laatste jaren is rhTSH beschikbaar gekomen als alternatief en fase III trials hebben laten zien dat rhTSH behandeling bijna of net zo effectief is als traditionele methode van stimulatie van radioactief jodium opname. *In vitro* was ons mscTSH bijna 2 maal zo effectief als rhTSH in het stimuleren van jodiumopname in FRTL-5 cellen. Dit maakt mscTSH tot een potentiële kandidaat om de efficiëntie van ^{131}I opname *in vivo* te verhogen. Directe labelling van mscTSH met een radioactieve ligand is wellicht een andere bruikbare applicatie, vooral wanneer zich metastasen hebben ontwikkeld die het vermogen tot ^{131}I opname verloren hebben maar de TSHr nog tot expressie brengen.

AD 3: TSH FUSIE-EIWITTEN

Wij wilden weten of het mogelijk zou zijn om een eiwit te fuseren met mscTSH terwijl de biologische activiteit gehandhaafd blijft. Als een model voor mscTSH fusie eiwitten hebben wij een 6xHIS tag met een flexibele linker gefuseerd met mscTSH. Verder hebben wij hetzelfde construct nogmaals verlengd door een korte sequentie (6 aminozuren) tussen de 6xHIS tag en mscTSH te zetten. Deze extensies zijn toegevoegd aan de N-terminus van mscTSH daar de COOH terminus (α 88-92) niet gebruikt kan worden omdat deze een cruciale rol speelt bij TSHr binding en activatie. Succesvolle opzuivering van de nieuwe eiwitten met een nikkel gel kolom bevestigde de aanwezigheid en beschikbaarheid van de 6xHis tag. Vervolgens hebben wij getest of het mscTSH gefuseerd met de korte extensies zijn biologische activiteit behouden had met de TSHr activatie assay **(Hoofdstuk 5)**. Na enkele testen bleek dat het volledige TSHr stimulerend potentieel is behouden en verder bleek dat de stabiliteit van het scTSH niet beïnvloed werd door de aanwezigheid van de HIS tag. Dit alles suggereert dat de conformatie van mscTSH niet dramatisch is veranderd door de additionele extensies aan de N-terminus. Het behoud van dit TSHr-activerend potentieel van TSH is essentieel voor internalisatie van een gevormd TSH-TSHR complex. Deze internalisatie is een essentiële stap wanneer de TSHr gebruikt gaat worden om TSH-fusie-eiwitten de cel binnen te loodsen. Aangezien de HIS-mscTSH en HIS-13X-mscTSH(mscTSH met een extra extentie van 13 aminozuren) fusie-eiwitten nog volledig biologisch actief zijn zal het wellicht mogelijk zijn om mscTSH in de toekomst te gebruiken om eiwitten *in vivo* naar de schildklier danwel schildkliertumoren te brengen.

Na TSHr activatie en na internalisatie van het TSHr-TSH complex via clathrine-gecoate vesicels

wordt TSH naar het lysosoom geleid. Dit wordt gevolgd door recycling van het merendeel van de receptoren naar het celmembraan en degradatie van TSH in de lysosomen.
In theorie zou dit mechanisme het mogelijk maken om aan TSH gebonden eiwitten (b.v. toxinen) de cel binnen te loodsen. In de praktijk gebruiken meerdere toxinen (b.v. Pseudomonas exotoxine (PE), Diphteria toxine (DT), Ricine en Shiga toxine) deze route (via een andere receptor) om de cellen binnen te dringen en de cel te doden. Het is aannemelijk dat deze toxinen nadat ze zijn gefuseerd met mscTSH, cellen die deze receptor tot expressie brengen via hun normale route kunnen binnendringen en doden.
Om toxinen geschikt te maken voor dit doel kan het natieve cel bindende domein vervangen worden door een ander domein, in dit geval TSH. Binnen de groep van toxinen zijn wij op zoek gegaan naar toxinen die aan de N-terminus van mscTSH gekoppeld kunnen worden omdat de COOH-terminus belangrijk is voor binding en activatie van de TSHr.
Verder zijn er nog applicaties denkbaar binnen de diagnostiek, waarbij mscTSH markers naar cellen brengt die de TSHr tot expressie brengen. Voor deze toepassing is internalisatie over het algemeen niet noodzakelijk of zelfs ongewenst en een blokkerende TSH variant verdient hier wellicht de voorkeur.

Buiten de directe inductie van celdood hebben immunotoxinen wellicht nog andere effecten. Recentelijk hebben Ochiai et al. melding gemaakt van een additioneel effect van een EGFR gericht immunotoxine. Buiten het directe cytotoxisch effect op cellen die de EGF-receptor bezitten, was het immunotoxine ook in staat om een immuunrespons op te wekken tegen de tumorcellen. Na behandeling met immunotoxinen gericht tegen de EGF-receptor ontwikkelden muizen immuniteit tegen tumorcellen die deze receptor tot expressie brengen maar ook tegen tumoren waarbij deze receptor ontbrak. Dit additionele positieve effect zal mogelijk ook optreden na behandeling van tumoren die de TSHr tot expressie brengen met een TSHr gericht immunotoxine, zodat ook sterk gededitferentieerde tumoren die TSHr expressie verloren hebben nog behandeld kunnen worden met op de TSHr gerichte immunotoxinen.

Naast de inductie van een immuunrespons door TSHr-immunotoxinen zijn er andere strategieën denkbaar die een immuunrespons tegen schildkliertumoren kunnen induceren. Er zijn verscheidene pogingen gedaan om een immuunrespons op te wekken tegen de TSHr in muizen door vaccinatie met TSHr preparaten, TSHr tot expressie brengende cellen en DNA vaccins met als doel een muizenmodel voor de ziekte van Graves te ontwikkelen met wisselend succes. Vergelijkbare technieken zullen mogelijk toepasbaar zijn bij de behandeling van schildkliertumoren waar de TSHr expressie gehandhaafd is. Recentelijk is de haalbaarheid van een dergelijke strategie succesvol aangetoond in een muismodel met NIS (een ander schildklierspecifiek eiwit) als doelwit. DNA vaccinatie bij muizen met de MIDGE/hNIS vector resulteerde in een immuunrespons die een opmerkelijke reductie in tumorvorming tot gevolg had na injectie met NIS getransfecteerde tumoren. Een dergelijke benadering met de TSHr als doelwit zal mogelijk een vergelijkbaar effect hebben en in de praktijk wellicht een grotere relevantie hebben omdat NIS afhankelijke RaI therapie al heeft bewezen effectief te zijn in tumoren die NIS tot expressie brengen.

Een alternatief voor TSH-toxinen zijn immunotoxinen die gebaseerd zijn op TSHr bindende antilichamen. Deze antilichamen zullen niet alleen aan de TSHr moeten binden maar deze ook moeten activeren om noodzakelijke internalisatie van het immunotoxine-TSHr complex te induceren.
Er zijn in het verleden vele pogingen gedaan om sterk TSHr stimulerende antilichamen te produceren maar overwegend met weinig succes. Daarom zijn monoklonale antilichamen met sterke TSHr stimulerende activiteit een zeldzaamheid. Recentelijk hebben enkele pogingen echter geresulteerd in de productie van monoklonale antilichamen met volledige agonistische activiteit die wellicht bruikbaar zijn in immunotoxinen. Ando et al. waren de eersten die erin slaagden om een redelijk potent monoklonaal TSHr stimulerend antilichaam te isoleren uit hamsters geïmmuniseerd met het adenovirus construct AdTSHr. Dit monoklonaal antilichaam is in staat om de receptor te stimuleren vanaf een dosis van 20ng/ml. Vrijwel tegelijkertijd slaagden Sanders et al. erin om via DNA-vaccinatie van muizen een monoklonaal te genereren dat ook in staat is om de TSHr te activeren vanaf 20ng/ml.
Recentelijk is het gelukt om TSHr gerichte monoklonale antilichamen te genereren met een sterker activerend vermogen uit zowel muizen als mensen. Gilbert et al. zijn erin geslaagd om monoclonale antilichamen te maken na immunisatie van muizen met recombinant adenovirus waarin hTSH cDNA geïncorporeerd is. Deze monoklonale antilichamen genaamd KSAb1 en KSAb2 zijn in staat om de TSHr te stimuleren bij concentraties van respectievelijk 1,2 en 2,2 ng/ml.
De meeste pogingen om monoclonale antilichamen uit bloed van patiënten met de ziekte van Graves te isoleren zijn vruchteloos gebleven op een poging na. Sanders et al. zijn erin geslaagd om een potent TSHr stimulerend monoklonaal antilichaam te maken na isolatie van humane lymfocyten uit het bloed van Graves patiënten dat in staat is om de TSHr te stimuleren vanaf 1ng/ml.
Verdere studies zullen moeten uitwijzen of op antilichaam of TSH gebaseerde immunotoxinen het meest effectief zullen zijn om patiënten met DTC te behandelen. Een groot voordeel van antilichamen is dat de productie van antilichamen in *E.coli* over het algemeen een goede opbrengst geeft terwijl de mscTSH productie in CHO een lage opbrengst gaf in ons productie systeem. In een commerciële setting zullen dergelijke lage productieniveaus een mogelijke belemmering vormen bij het winstgevend maken van een dergelijk medicijn. Het gebruik van efficiëntere vectoren, geoptimaliseerde secretie en een uitgebreide selectie van cellijnen met een hogere productie zullen mogelijk leiden tot hogere opbrengsten van mscTSH

DE TSHR IN ANDERE WEEFSELS

Een potentieel probleem voor TSHr gerichte therapie van schildkliertumoren is de aanwezigheid van deze receptor in andere weefsels. Een aantal artikelen heeft melding gemaakt van TSHr mRNA en/of eiwit in andersoortige weefsels zoals lymfocyten, de thymus, hypothalamus, testes, nieren, hart en orbitale weefsels. De TSHr niveaus zijn echter erg laag en worden mogelijk veroorzaakt door “lekkende expressie” i.p.v. functionele expressie
Recentelijk zijn er echter een paar artikelen verschenen waar de TSHr een actieve rol wordt toebedeeld in de modellering van bot. Osteoblasten bleken TSH-receptoren tot expressie te brengen en te reageren met verhoogde cAMP niveaus na stimulatie met TSH. Tsai et al.

concludeerden echter, dat gezien de lage expressie niveaus van de TSHr, deze waarschijnlijk geen actieve rol speelt bij de remodellering van bot.
Naast deze bevindingen hebben Abe et al. aangetoond dat $TSHr^{-/-}$ muizen ernstige osteoporose ontwikkelen en dat $TSHr^{+/-}$ muizen met normale T3, T4 en TSH niveaus bot verliezen wat wijst op een rol van de TSHr op bot modellering. In contradictie met deze studie zijn de bevindingen van Bassett et al. die geen effect van verhoogde TSH concentraties kon vaststellen in muizen die geen schildklierhormoon receptor tot expressie kunnen brengen.
Kortom, het belang van de TSHr in andere weefsels dan de schildklier blijft onduidelijk en de receptor vervult wellicht geen functie in deze weefsels maar dit kan niet geheel uitgesloten worden. Mocht de TSHr een functie hebben in andere weefsels dan zal dit waarschijnlijk geen problemen opleveren voor toxines gekoppeld aan TSH omdat het verschil in de hoeveelheid TSHr expressie tussen schildkliertumoren en andere weefsels waarschijnlijk voldoende ruimte laat voor het bepalen van een geschikte therapeutische dosis. Maar dit zal uiteraard verder onderzocht moeten worden.

EEN BIOASSAY VOOR TSHR ACTIVATIE

Gedurende onze pogingen om functionele mscTSH conjugaten te produceren (Hoofdstuk 4) bestond de noodzaak voor een effectieve assay voor het bepalen van TSHr stimulatie. Commerciële TSH assays zijn niet bruikbaar voor dit doel aangezien deze alleen het vermogen tot binding aan de TSHr meten en niet de daadwerkelijke activatie van de receptor.
Om onze gemodificeerde scTSH constructen te kunnen testen op TSHr activatie hebben wij een bioassay ontwikkeld dat gebaseerd is op cAMP geïnduceerde luciferase expressie. In dit assay resulteerde stimulatie door bovine (b)TSH en hTSH in een bijna lineaire toename in luminescentie tot een TSH concentratie van 50 mU/L. Na validatie hebben wij dit assay gebruikt om de activiteit te meten van de constructen die beschreven worden in **Hoofdstuk 4**. Naast het testen van de bioactiviteit van onze mscTSH constructen realiseerden wij ons dat onze bioassay wellicht mogelijkheden zou bieden voor het bestuderen van groepen patiënten met auto-immuniteit gericht tegen de TSHr. De TSHr bindende antilichamen in deze patiënten kunnen zowel stimulerend als blokkerend zijn, wat resulteert in hyperthyreoidie danwel hypothyreoidie. De auto-immuun ziekte Graves (GD) is de meest voorkomende oorzaak van hyperthyreoidie en wordt gekarakteriseerd door de aanwezigheid van auto-antilichamen die zijn gericht tegen de TSHr. Deze antilichamen staan bekend als TRAb (TSHr antilichamen) of TBII ("TSH receptor binding inhibiting immunoglobulins"). TBII is een overkoepelende naam voor zowel stimulerende (TSAb) als blokkerende (TBAb) antilichamen. Hyperthyreoidie in Graves wordt veroorzaakt door TSAb die aan de TSHr binden en deze activeren.
Er zijn verscheidene commerciële testen beschikbaar die de hoeveelheid TBII kunnen bepalen maar een nadeel van deze testen is hun onvermogen om TSHr activatie te meten. Daarom is het niet mogelijk om de testuitslagen te correleren met de mate van hyperthyriodie. Dit is voornamelijk van belang bij zwangerschap waar het verschil tussen TSAb en TBAb klinische consequenties heeft. Onze assay maakt het mogelijk om de directe stimulatie van de TSHr te meten door TSAb in sera van patiënten met Graves. Vervolgens konden we de door ons gemeten TSHr stimulatie correleren met vrij T4, welke een klinisch eindpunt is voor TSHr

activatie. De resultaten die wij verkregen hebben met onze assay laten een sterke correlatie zien tussen TSHr activatie en vrije T4 niveaus in serum van 35 onbehandelde GD patiënten. Bij de gemeten TBII titers ontbrak de relatie met vrij T4 en hoge TBII titers waren vaak geassocieerd met lage TSHr activatie.

Deze studies hebben een goede indicatie gegeven van het spectrum van TSHr activatie in deze patiënten. Het doel van onze studie was de ontwikkeling van een test met een hogere *in vitro* sensitiviteit voor TSH dan eerder gepubliceerde studies en om de directe correlatie te onderzoeken tussen in vitro TSHr stimulatie en vrije schildklierhormoon (vrije T4) niveaus in serum (welke een klinisch eindpunt zijn van TSHr stimulatie). Deze correlatie kon in eerdere studies niet bestudeerd worden omdat zowel onbehandelde als behandelde patiënten meededen aan de studies.

Wij vonden een sterke significante correlatie tussen *in vitro* TSHr-stimulerende activiteit van GD sera en de vrije T4 niveaus in deze sera terwijl er geen relatie was tussen TBII niveaus (gemeten met het TRAK-assay) en de vrije T4 niveaus. Zover wij na konden gaan is er slechts een *in vivo* studie gepubliceerd die een direct verband aantoont tussen het TSHr stimulerende hamster antilichaam MS-1 en vrije T4 niveaus in muizen. In onze studie hebben wij een sterk verband gevonden tussen het TSHr activerend vermogen van GD serum en de vrije T4 niveaus onafhankelijk van TBII titers.

Een andere categorie van patiënten waar het verschil tussen TSAb en TBAb een rol zou kunnen spelen zijn patiënten die behandeld zijn met radioactief jodium. RaI in toxisch multinodulair struma (toxic multinodular goiter, TMNG) is wel geassocieerd met de ontwikkeling van een op Graves gelijkende hyperthyreoidie en het is geopperd dat al bestaande autoimmuniteit bijdraagt aan dit fenomeen.

Om te bestuderen of een RaI behandeling TSAb's induceert in TMNG op de korte termijn en of bestaande autoimmuniteit van belang is, hebben wij TMNG patiënten getest met ons bioassay in **Hoofdstuk 6** evenals een groep Graves patiënten.
Eerdere studies over dit onderwerp hadden hun beperkingen omdat geen functionele TSAb assays werden gebruikt. Twee studies hebben wel gebruik gemaakt van dergelijke assays maar de sensitiviteit van deze assays was lager dan ons assay. Binnen een van deze studies is geen gebruik gemaakt van Graves patiënten terwijl de andere studie slechts een laag aantal Graves patiënten bevatte. Binnen de groep van Graves patiënten hebben wij geen bewijs gevonden voor RAI geïnduceerde TSAb's hoewel wij wel een significante toename van serum thyroglobuline (Tg) spiegels zagen, die wijst op het vrijkomen van schildklier specifieke antigenen. Hoewel TBII's aanwezig waren in 97% van de patiënten waren TSAb's aanwezig in slechts 64% wat erop wijst dat TSAb's spontaan kunnen verdwijnen. Van de TMNG patiënten hadden er drie meetbare TBII's. Het is onderwerp van debat of de diagnose TMNG de juiste is voor deze groep van patiënten of dat Graves meer van toepassing is ondanks het typische scintigrafisch patroon. Sommige auteurs hebben het idee geopperd dat autoimmuniteit subklinisch aanwezig is in deze TMNG patiënten. Dus hoewel de TMNG diagnose wellicht moet worden verworpen waren TSAb's aanwezig in 5 patiënten waarbij er

3 onmeetbaar waren met conventionele assays.

Dit onderstreept de hoge sensitiviteit van onze bioassay en toont ook aan dat schildklier auto-immuniteit meer algemeen lijkt te zijn bij TMNG patiënten dan voorheen gedacht werd. Het belang van deze bevinding, en meer specifiek, in hoeverre TSAb's een actieve rol spelen bij de ontwikkeling van het ziektebeeld bij TMNG, zal verder onderzocht moeten worden. Wij konden de inductie van TBII's na RaI aantonen bij drie TMNG patiënten, terwijl TSAb's verschenen bij slechts een patiënt met nieuw ontwikkelde TBII's. Het aandeel van patiënten met TSAb's werd niet beïnvloed door RAI in TMNG.
In de studie door Chiovato et al. konden hoge TSAb niveaus voor de start van RAI therapie bij patiënten met Graves gerelateerd worden met resistentie tegen de therapie. Dit was niet het geval was in onze studie. Verder vonden deze auteurs dat een post-RAI toename van TSAb gerelateerd was met de ontwikkeling van hypothyreoidie. Onze bevindingen contrasteren hiermee aangezien in onze studie een post-RAI toename van TSAb geassocieerd was met een lagere mate van hypothyreoidie. In de studie van Michelangeli et al. werd hypothyreoidie na RAI behandeling van Graves geassocieerd met een post RAI toename van TBII bestaande uit zowel TSAb en TBAb. In patiënten met alleen TSAb ontwikkelde zich geen hypothyreoidie wat in lijn is met onze observaties.
Wij concluderen dat onze nieuw ontwikkelde B1-TBII bioassay verscheidene voordelen heeft: Het gebruik van het bioassay maakt het mogelijk om inzicht te krijgen in de mate van TSHr activatie terwijl de standaard TRAK assay alleen binding aantoont. Deze directe meting van activatie wordt verder geïllustreerd door de sterke correlatie tussen vrije T4 niveaus en luminescentie. Een verdere analyse van ingevroren plasma van patiënten met hyperthyroïdie liet echter geen significante correlatie zien tussen luminescentie en vrije T4 niveaus. Mogelijke verklaringen hiervoor zijn een verlies van binding door TBII door lange opslag of herhaaldelijk invriezen of onbekende factoren in het serum die invloed hebben op cAMP niveaus in de cellen van de assay, onafhankelijk van TSHr stimulatie.
Verder hebben wij de hypothese onderzocht dat bestaande autoimmuniteit bijdraagt aan RAI geïnduceerde Graves-achtige hyperthyroïdie. Uit onze studie concluderen wij dat TBII's mogelijk aanwezig zijn voor behandeling met RAI van TMNG en dat RAI mogelijk TBII kan induceren vlak na RAI behandeling. Deze inductie resulteert echter niet uitsluitend in TSAb's. Daarnaast is bestaande autoimmuniteit is niet noodzakelijk voor de inductie van TBII (aangetoond door het ontbreken van de vorming van TBII na RAI behandeling van Graves patiënten).

EIND CONCLUSIE

Bestaande therapieën voor DTC zijn effectief in een meerderheid van de patiënten. Er is echter een subgroep van patiënten (10-15% DTC patiënten) met metastasen bij wie conventionele RAI behandeling niet effectief is. Binnen deze groep is het noodzakelijk dat conventionele therapieën geoptimaliseerd- of nieuwe innovatieve therapieën ontwikkeld kunnen worden. Wij hebben verschillende nieuwe mogelijkheden onderzocht, met een focus op de TSHr, die de vooruitzichten van deze subgroep van patiënten mogelijk kunnen verbeteren

De combinatie van troglitazone en lovastatine is een interessant uitgangspunt voor het ontwikkelen van therapieën voor DTC gezien het opmerkelijk synergetisch effect op morfologie en celdichtheid van de folliculair schildkliercarcinoom cellijn FTC-133 *in vitro* bij klinisch haalbare concentraties. Buiten dit effect op groei was de combinatie van troglitazone en lovastatine in staat om de expressie van NIS en de TSHr te verhogen. Dit effect zal wellicht bijdragen om de gevoeligheid van schildkliertumorcellen voor conventionele RAI te vergroten. Gelet op deze resultaten lijkt het ons mogelijk dat een behandeling met troglitazone en lovastatine een gunstig effect kan hebben op twee fronten door zowel groeireductie als verhoogde NIS expressie van tumoren te induceren.

Een tweede mogelijkheid die wij hebben onderzocht is specifieke membraan geassocieerde therapie door de TSHr te gebruiken als doelwit. De TSHr zal wellicht een beter doelwit zijn voor de behandeling van DTC zijn dan NIS omdat TSHr expressie veel langer behouden blijft in de-differentierende tumoren dan NIS. Wij zijn erin geslaagd om TSH door modificatie om te vormen tot een singlechain TSH met verbeterde binding aan de TSHr. Dit gemodificeerd single chain TSH is in potentie geschikt om toxines specifiek naar schildkliercellen te brengen. Korte eiwitsequenties gefuseerd aan ons gemodificeerde TSH gaven geen inhibitie van binding waaruit blijkt dat TSH geschikt is om therapeutische eiwitten te vervoeren naar cellen die de TSHr tot expressie brengen.

Tumoren die afkomstig zijn van schildklier cellen bieden unieke mogelijkheden voor behandeling door hun schildklier specifieke eigenschappen. Een van deze eigenschappen, de mogelijkheid om RAI te accumuleren, wordt al tientallen jaren gebruikt voor behandeling en zal mogelijk effectiever kunnen worden door een toename van NIS expressie mogelijk door een troglitazone en/of lovastatine behandeling. Naast het gebruik van NIS zullen andere unieke eigenschappen wellicht ook gebruikt kunnen worden voor behandelingen. Wij denken dat de schildklier specifieke TSHr een ideaal 'handvat' zou kunnen zijn om tumoren afkomstig van de schildklier te bereiken met gemodificeerd TSH- of antilichaam conjugaten.
Naast de mogelijkheden die worden aangedragen in dit proefschrift zullen ook andere innovatieve behandelingen, zoals de recente successen met tyrosine kinase remmers, alleen of in combinatie met bestaande therapieën, hopelijk bijdragen aan een verdere verbetering bij de behandeling van schildkliertumoren.

De TSHr heeft buiten een mogelijke rol als doelwit voor therapieën waarschijnlijk ook een andere functie die van belang is bij de behandeling van schildkliertumoren, namelijk stimulerende effecten op de celgroei na activatie van de receptor door TSH. Om deze reden is TSH onderdrukkende thyroxine substitutie therapie een belangrijk deel in de klinische follow-up van DTC patiënten. Wij hebben aangetoond dat, uitgaande van onze studies, totale suppressie van TSH niet noodzakelijk is en het is daarom raadzaam om te zoeken naar een balans waarbij remissie van schildklier kanker voorkomen word maar het TSH niveau hoog genoeg is om negatieve lange termijn effecten op andere organen te voorkomen.

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Nawoord

Het moment dat aan de start van het AIO-schap nog zo ver weg leek is ineens daar. Het proefschrift is af!
Achteraf lijkt de tijd omgevlogen maar in de afgelopen jaren heb ik toch heel wat nieuwe ervaringen en inzichten gekregen. Zo blijkt wetenschap vaak een kwestie van lange adem in plaats van snelle sprongen. Het spreekt voor zich dat het voltooien van een proefschrift niet mogelijk is zonder goede begeleiding en een stimulerende omgeving. Tal van technieken heb ik geleerd van de postdocs, analisten, mede AIO's en andere collega's om me heen, die niet op de laatste plaats ook garant stonden voor een goede sfeer, bedankt hiervoor!

Vrienden, hoewel het er steeds meer op lijkt dat de meesten van jullie liever in het buitenland wonen dan in Nederland hoop ik dat we elkaar regelmatig blijven zien om mijn frustraties en momenten van euforie tijdens het leven als onderzoeker kwijt te kunnen maar natuurlijk vooral voor wat diepzinnige gesprekken of zwetspraat.

Pap, ich wil dich hiel erg bedanke veur alle sjteun die ich mien ganse laeve al van dich heb gekrege. Doe en mam hebbe altied veur mich klaorgesjtaon.

Lieve Marjolein, een promotie gaat altijd gepaard met ups en downs, maar jij hebt mij altijd in balans gehouden. Nu wordt het hoog tijd om de promotieperikelen achter ons te laten en te gaan genieten van ons nieuwste gezinslid.

Guido Hovens

Curriculum Vitae

Guido Christiaan Johannes Hovens werd op 15 Augustus 1976 geboren te Tegelen waar hij vervolgens de kleuterschool en basisschool met succes doorliep. De schoolcarriere werd voortgezet op het Marianumcollege in het naburige Venlo dat hij in 1995 verliet met een VWO diploma op zak.

Vanuit de al lang aanwezige interesse in de biologie en het 'willen weten hoe al dit leven nu in elkaar steekt' koos hij voor de studie bioprocestechnologie in Wageningen. Na enkele jaren liet hij het proceskundige deel achter zich en koos hij voor een moleculair/cellulaire specialisatie. Binnen deze specialisatie heeft hij afstudeervakken gevolgd bij het laboratorium voor microbiologie en bij de vakgroep immunologie waar hij onderzoek heeft gedaan naar respectievelijk de thermofiele archaea bacterie *Pyrococcus furiosis* en de immuunrespons tegen *Trypanosoma carassii* in de karper. Daarna volgde een buitenlandse stage van 6 maanden bij de Universiteit van Aberdeen waar hij onderzoek deed naar DNA vaccinatie bij forellen, dit onderzoek resulteerde in een wetenschappelijk artikel.

Na zijn afstuderen in 2002 aan de Wageningen Universiteit vertrok hij naar Leiden om een promotie onderzoek te doen binnen de groep van prof dr. Hans Romijn. Hier heeft hij onderzoek gedaan naar gedifferentieerd schildklier carcinoom veelal gecentreerd rondom de TSHreceptor onder begeleiding van prof. dr. Jan Smit. De resultaten van dit promotie onderzoek hebben geresulteerd in een aantal wetenschappelijke artikelen welke gebundeld zijn in dit proefschrift.

Thans bouwt hij voort op dit onderzoek binnen het LUMC waar hij sinds april 2007 in dienst is als wetenschappelijk onderzoeker binnen de afdeling Endocrinologie. Hier doet hij onderzoek naar fusie-eiwitten die mogelijk bruikbaar zullen zijn in de diagnostiek of behandeling van gedifferentieerd schildklier carcinoom.

List of Publications

1 Collet B, **Hovens GCJ**, Mazzoni D, Hirono I, Aoki T, Secombes CJ: Cloning and expression analysis of rainbow trout Oncorhynchus mykiss interferon regulatory factor 1 and 2 (IRF-1 and IRF-2). Developmental and Comparative Immunology (2003), 27:111–126

2 **Hovens GCJ**, Buiting AMJ, Karperien M, Ballieux BEPB , van der Pluijm G, Pereira AM, Romijn JA and Smit JWA: A bioluminescence assay for thyrotropin receptor antibodies predicts serum thyroid hormone levels in patients with de novo Graves' disease. Clinical Endocrinology (2006),64: 429–435

3 **Hovens GCJ**, Heemstra KA, Buiting AMJ, Stokkel MP, Karperien M, Ballieux BEPB, Pereira AM, Romijn JA and Smit JWA: Induction of stimulating thyrotropin receptor antibodies after radioiodine therapy for toxic multinodular goitre and Graves' disease measured with a novel bioassay. Nuclear Medicine Communications (2007), 28:123–127

4 **Hovens GCJ**, Stokkel MP, Kievit J, Corssmit EP, Pereira AM, Romijn JA, and Smit JWA: Associations of serum thyrotropin concentrations with recurrence and death in differentiated thyroid cancer. The Journal of Clinical Endocrinology & Metabolism (2007), 92(7):2610–2615

5 **Hovens GCJ**, Karperien M, Romijn JA and Smit JWA: Treatment of the follicular thyroid carcinoma cell-line FTC-133 with Troglitazone and Lovastatin synergistically increases expression of G1 transition inhibitors and induces re-differentiation. Submitted

6 **Hovens GCJ**, Karperien M, Romijn JA and Smit JWA: Superior thyrotropin receptor binding and activation of a novel, modified, single chain thyroid stimulating hormone. Submitted