The thyrotropin receptor in thyroid carcinoma
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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;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-cou-pled receptors and specifically binds the glycoprotein TSH. It is similar to other glycopro-tein 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 extra-cellular 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 cleav-age 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 cleav-age 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 tis-sue (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 com-pared 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, H2O2 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**.