The thyrotropin receptor in thyroid carcinoma

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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.