Antigen-specific therapy of Graves´ disease and orbitopathy by induction of tolerance

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1. ABSTRACT

Graves´ disease is an autoimmune disorder, which is characterized by stimulatory antibodies targeting the human thyrotropin receptor (TSHR), resulting in hyperthyroidism and multiple organ damage. The disease can be modelled in mice using adenoviral immunizations with the extracellular A subunit of the TSHR, which induces a long-term stable disease state. TSHR binding cAMP-stimulatory antibodies, thyroid enlargement, elevated serum thyroxin levels, tachycardia, cardiac hypertrophy and orbitopathy are observed in these Ad-TSHR-immunized mice. T cell epitope-derived linear peptides have been identified using immunized HLA-DR3 transgenic mice, which may induce tolerance towards TSHR. A combination of such peptides are being investigated in a first clinical phase I trial in patients with Graves´ disease. Alternatively, intravenous administration of cyclic peptides derived from the interaction site of the TSHR A domain with stimulatory anti-TSHR antibodies can re-establish tolerance towards the antigen in immunized mice, improving symptoms of Graves´ disease within 3 – 4 months after starting these therapies. In immunologically naïve mice, administration of the cyclic peptides did not induce any immune response.

2. INTRODUCTION: CLINICAL FEATURES OF DISEASE

In patients with Graves´ disease, antibodies and other autoimmune markers which target the thyrotropin-TSH receptor (TSHR) in the thyroid gland are observed. This autoimmune condition results in hyperthyroidism (1), with an annual incidence of 15 – 80 per 100,000 persons throughout the world. A quality of life (QOL) assessment showed that the disease is accompanied by a reduced vital and mental QOL for several years despite treatment according to current standards (2). If left untreated, Graves´ leads to significantly increased morbidity and mortality (2).

Graves´ orbitopathy afflicts about 50% of patients and is especially hard to treat - up to 16 per 100,000 women per year (3). Treatments of refractory disease cases and of accompanying ophthalmopathy/orbitopathy are especially challenging. In this condition, anti-TSHR antibody titers and relapse rates are especially high (3).

Another clinical feature is cardiac involvement which is directly caused by the action of thyroid hormones on the myocardium (4). Contractility is increased, and a hypertrophic form of cardiomyopathy (5), as well as tachycardia is frequently seen in these patients. Heart rates at rest are increased and are related to disease severity (4,5). Atrial fibrillation and palpitations are among the most reported symptoms (6). Increased cardiovascular morbidity and mortality have been reported in patients with both, overt or subclinical hyperthyroidism (6-9). Hyperthyroid

patients suffering from Graves´ disease are at especially increased cardiovascular risk (10).

3. ANIMAL MODELS OF DISEASE – THYROID ALTERATIONS AND HYPERTHYROIDISM

Graves´ disease including autoimmune targeting of the thyroid gland and hyperthyroidism can be replicated in BALB/c mice after adenoviral immunisations with the A domain of TSHR (Ad-TSHR) (3,6,7). Earlier short immunization protocols were prolonged by introducing three administrations of recombinant adenovirus and measurements after 20 weeks, which led to reliable disease induction (13). Regular once monthly injections for nine months permanently boosted antibody production in mice (15).

Anti-TSHR antibodies can be determined from mouse serum samples using the gold standard "3rd generation" immunoassay, which detects the ability of the respective sera to inhibit the binding of the monoclonal Graves´ patient antibody M22 to the TSHR (RSR-Cobas Roche), and which is most often used to identify Graves´ disease in humans. In the long term Ad-TSHR-immunization study (15), anti-TSHR antibody titers increased progressively, as determined by this assay. Very comparable results were obtained with a 2nd generation assay using competition against the physiological agonist TSH (15). The TSHR-stimulatory activity of these antibodies was determined as the capacity of mouse serum samples to stimulate TSHR-dependent cAMP levels in test cells (15). This capacity was markedly and consistently increased in serum samples taken from Ad-TSHR-immunized animals.

Macroscopic investigation showed clearly increased thyroid sizes in these mice (confirmed by measuring thyroid volumes from the sums of the areas of standardized sections). In addition, consistent and marked thyroid hyperplasia was observed (15). Histological features included increased thyrocyte length and cuboid epitheloid hyperplasia, and a degenerate image with prominent infoldings of follicles, smaller follicle size and vacuolization (15). Mean T4 levels in the group in Ad-TSHR-immunized animals were consistently and significantly higher than controls (15). Additionally, significant retro-orbital fibrosis was observed (16,17).

4. CARDIAC INVOLVEMENT IN ANIMAL DISEASE MODELS OF GRAVES´ DISEASE

Ad-TSHR immunization in rhesus macaque monkeys and in Ad-TSHR-immunized mice resulted in hyperthyroidism and significant tachycardia (15,18). Upon necropsy, Ad-TSHR-immunized mice revealed significantly increased heart weights and myocardial volumes (adding up digitized cross-section areas of the left ventricles) (15). Histological assessment revealed signs of cardiomyocyte hypertrophy and cardiomyopathy, including increases in cell size and thicker myocardial fibers in the HE-stained sections of the Ad-TSHR-immunized animals.

5. ESTABLISHED THERAPEUTIC APPROACHES

Graves´ disease is often initially treated by giving thyreostatic drugs, such as carbimazol, followed by radioiodine therapy (19) or surgical removal of the thyroid gland. All these treatment options are characterized by relatively high relapse rates, and significant side effect profiles (20). Furthermore, use of selenium has been propagated and is being investigated in clinical studies.

Treatment of refractory disease cases and of accompanying ophthalmopathy/orbitopathy is especially challenging. These patients must frequently be treated with high doses of intravenous corticoids over many weeks, which even incur more side effects (21).

6. RECENT NOVEL EXPERIMENTAL THERAPIES

Therefore, novel treatment options have been explored in recent years. A reduction of B lymphocytic cell counts can be achieved by giving the anti-CD20 antibody rituximab (MabThera[R], anti-CD20 Mab). Driven by the hypothesis that Graves´ disease is mainly a B cell-mediated condition, a recent randomized double-blind trial showed an advantage for the rituximab-treated group (22), whereas another did not (NCT 00595335, ref. 21), perhaps due to frequent side effects of the therapy. Additionally, the anti-CD40 immuno-modulating monoclonal antibody CFZ 533 has been proposed as a treatment of Graves´ disease, and recruitment to a clinical phase II study has been recently completed (23).

The insulin-like growth factor I receptor (IGF-IR) is a co-receptor of TSHR, and involved in a cross-talk with and synergistic enhancement of the activity of TSHR (24). The anti-IGF-IR monoclonal antibody teprotumumab (24) was tested in 88 patients and resulted in improved clinical Graves´ activity scores after 24 weeks (25). However, the side effect profile of this approach remains to be determined in larger studies, e. g. on the interaction with glycemic control (25).

The FIRS lab – RSR, Cardiff, has identified the inhibitory monoclonal TSHR-binding antibody K1-70 from a patient with Graves´ disease, which competes for Graves´ patients´ stimulatory autoantibodies (25). K1-70 can counteract hyperthyroid states in M22-

injected rats (26). A clinical phase I study in Graves´ patients has been started and is currently recruiting participants (27).

In addition, small molecule TSHR antagonists were conceived, but so far only tested *ex vivo* and in healthy or M22-injected mice (28-30). These compounds such as ANTAG3 are specific allosteric regulators of the TSHR hinge region, but seem to act only at fairly high *in vivo* doses. In addition, all suffer from some cross-reactivity with the luteinizing or follicle-stimulating (LH or FSH) hormone receptors at the high doses which are needed for *in vivo* effects, and their toxicological characterizations have so far not been reported.

7. PROSPECT OF ALLERGEN-SPECIFIC IMMUNE THERAPIES IN PATIENTS

In general, treatment with broad-range immunosuppressive drugs may cause serious side effects, so that antigen-specific therapies have been conceived to induce tolerance in a variety of autoimmune conditions. For many decades, specific immune therapies (SIT) have been established successfully for the treatment of allergic-atopic diseases. Recombinant peptides are being increasingly used for such hyposensitization therapies (SIT) which offer significant advantages over the classical raw allergen extracts (31-33).

8. EFFECTS ON T LYMPHOCYTES

In analogy to SIT for allergic diseases, peptides to induce tolerance have been investigated in a variety of other conditions, and been linked to regulatory T cells. Generally, specific immunotherapy for autoimmune diseases lagged behind until the discovery that T helper lymphocytes are activated by peptides bound to major histocompatibility (MHC) class II proteins (34). This led to the design of peptides that selectively target immune cells without risking the activation of self-reactive cytotoxic T or B lymphocytes (33). Exposure to a peptide which stimulates self-reactive T helper lymphocytes can mitigate allergic disease symptoms, which may be explained by the 'two-signal' rule of T-cell activation (34, 35): Self or foreign antigens must be broken down into peptides, which bind MHC class II proteins and must be displayed at the surface of antigen-presenting cells (APCs) to activate effector cells. This step is known as signal 1. The antigen-presenting cells must also upregulate costimulatory molecules, such as CD80 and CD86, to provide the second signal required for T helper cell survival and proliferation.

At conditions when T helper cells receive signal 1, but not signal 2, they induce a state of unresponsiveness known as anergy (35). Starting

from this idea, the group of David Wraith has created "apitopes" to treat Graves´ disease. The concept is based on the observation that some TSHR-derived peptides bind to a MHC class I or II molecule and are presented to a T cell without further processing (36). Such peptides have the capacity to induce tolerance *in vivo* (36). TSHR T cell epitope-derived linear peptides have been identified using immunized HLA-DR3 transgenic mice, which may induce tolerance towards TSHR (37). Subcutaneous administration of the peptide ATX-GD-5D-K was shown to reduce anti-TSHR antibody production after consecutive adenoviral TSHR immunisation in a similar mouse model (37), i. e. in a prophylactic setting. At these conditions, soluble peptides seem to bind to empty MHC receptors and selectively trigger activation of interleukin-10-positive T lymphocytes which specifically suppress pathogenic T helper cells (37,38). The authors have so far not published data on treatment of established disease nor on thyroid size or hyperthyroidism. ATX-GD-59 – probably a combination of several peptides which were selected from this approach is being investigated in a first clinical phase I trial in patients with Graves´ disease (39).

If applied subcutaneously, successful therapy has been related to consecutive antigen dose escalation and to local release of interleukin-10 from lymphocytes (40,41). Although immune-disease-triggered epitope spreading might have occurred at the time when such a therapy is initiated (42), "bystander suppression" of independent epitopes has been implied in therapeutic effects (43). Although these therapies have not all been successful, they are still being pursued by researchers in various fields (44,45). For example, T helper cells treated with nanoparticles which had been coated with a peptide bound to MHC class II proteins (pMHC-NP treatment) triggered signal 1 alone (40,41). This concept has been shown to not only induce anergy, but also to drive T helper cells to differentiation to what resembles regulatory T cells which reduce immune responses (40,41).

9. INTRAVENOUS ADMINISTRATION OF HIGH DOSES OF CONFORMATIONAL ANTIGEN-SPECIFIC CYCLIC PEPTIDES - EFFECTS ON B LYMPHOCYTES

As another option for antigen-specific therapy, the group of Martin Lohse and Roland Jahns has established intravenous administration of fairly high doses of immunogen-mimicking cyclic peptides for the treatment of anti-ß1-adrenergic receptor-mediated autoimmune cardiomyopathy (46-48). The investigation of cyclic peptide-treated animals showed that the beneficial therapeutic effects were related to a marked decrease of antigen-specific pre B lymphocytes in the spleen (48). This phenomenon can be observed
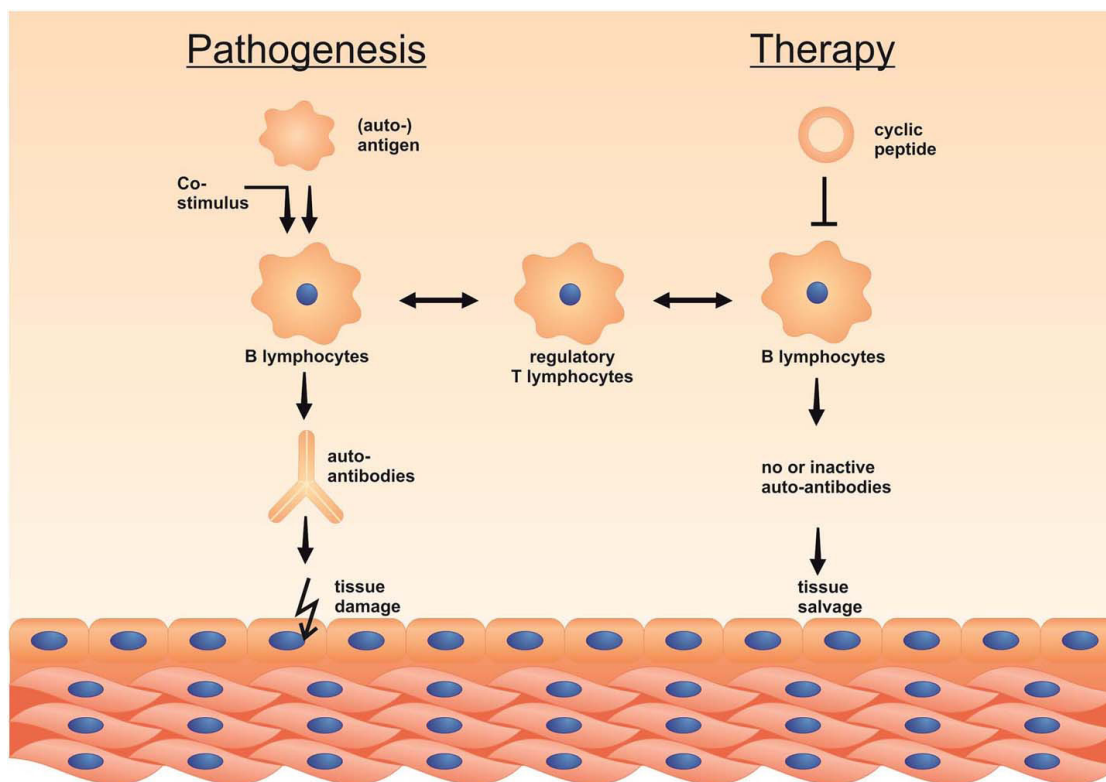
Figure 1. antigen-specific cyclic peptides can induce tolerance to the antigen. The Figure shows that administration of cyclic peptides is thought to induce peripheral B lymphocyte anergy if presented to antigen-presenting immune cells in the absence of a co-stimulatory signal. Regulatory T cell activation might also play a role in this therapeutic effect.

if these cells are exposed to the specific antigen in the absence of a co-stimulatory signal, which again refers to the lack of interaction between CD80/86 – B7.1./2 and CD28.

Figure 1 very schematically shows the possible modes of action of such cyclic peptides, i.e. to induce peripheral tolerance. Cyclic peptides are thought to reduce antibody-producing lymphocytes and pre-B lymphocytes by anergy.

In parallel to T lymphocyte anergy, B cell anergy has been described in several pivotal studies in which it was shown to require constant B cell receptor (BCR) occupancy with rather high levels of (self) antigen (49,50). After initial characterization in rodents, it has also been reconfirmed in human immune cells (51,52), and been explained by chronic low-level BCR cross-linkage (52). In addition, high-level BCR cross-linkage in the absence of co-stimulatory signals leads to clonal B cell deletion after acute high level antigen exposure (52). Originally, clonal anergy was proposed as a way to inactivate B cells stimulated early in development when only autoantigens would be presented (49). Further studies have documented fine-tuning of B lymphocyte responsiveness and inactivation of self-reactive B cell clones by anergy

(53). This phenomenon seems to play a relevant role in a variety of immune phenomena, and seems to be an attractive mechanism for the treatment of several B lymphocyte-mediated diseases.

10. INTRAVENOUS TSHR-DERIVED CON-FORMATIONAL CYCLIC PEPTIDE THERAPY FOR THYROID DISEASE

In parallel to the studies on ß adrenergic receptors (48), cyclic peptides which mimic the tertiary structure of the single cylindrical loops of the TSHR leucine-rich repeat domain (LRD) were tested for their effects in the Graves´disease mouse model. Figure 2 shows the structure of the LRD and indicated how novel peptides were derived from the native structure of the receptor.

These cyclic peptides were tested for their potency to induce tolerance in TSHR-immunized diseased mice after repeated intravenous administration, with a focus on disease manifestations such as thyroid enlargement, hyperthyroidism and retro-orbital fibrosis (17). Therapeutic peptide administrations were started on week 11, when thyroid disease had fully evolved. All cyclic peptides were administered 6 times at 4-weekly intervals (17).
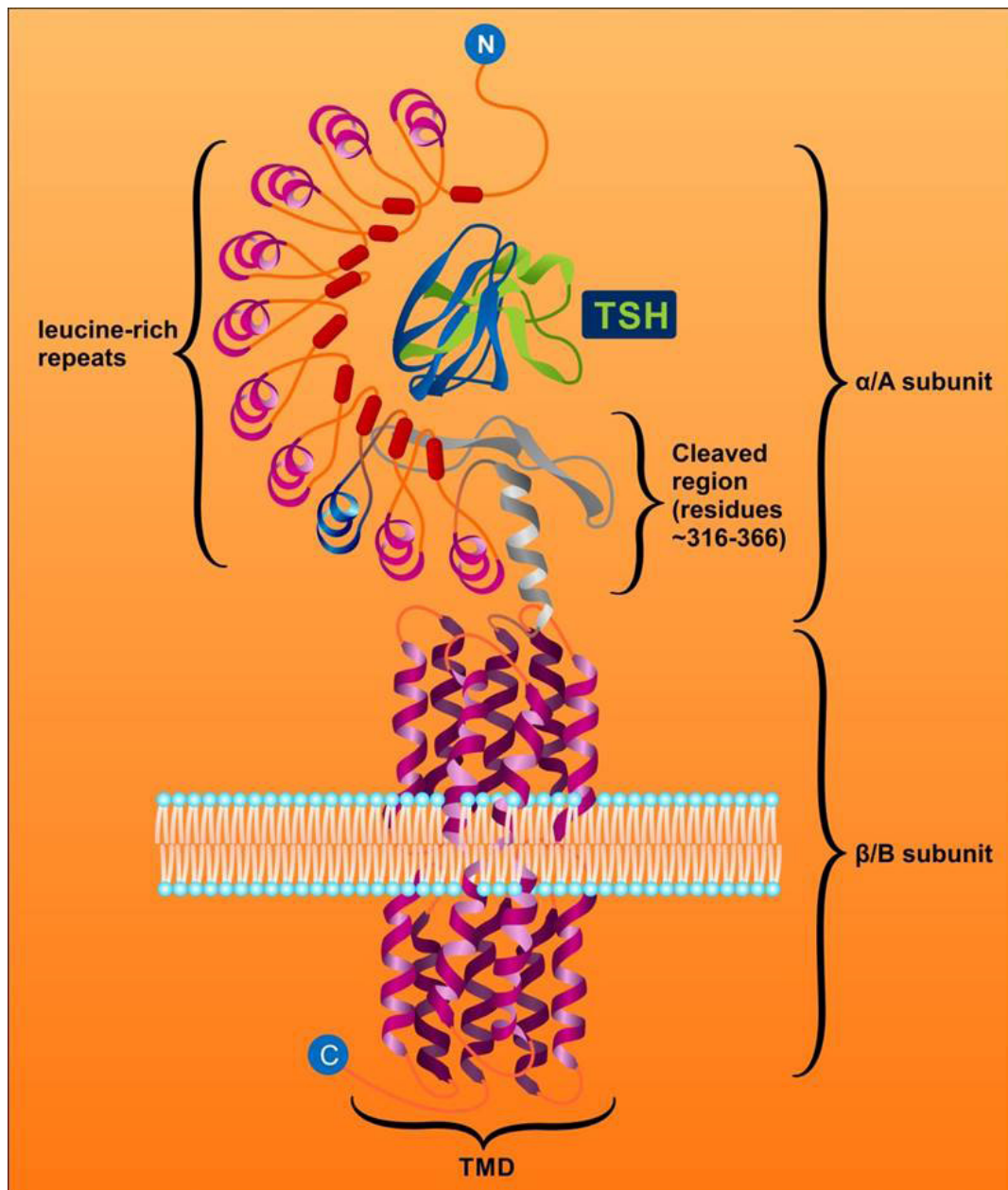
Figure 2. Schematic structure of the thyroid stimulating hormone receptor (TSHR): Peptides were derived from the loop structure of the leucine-rich repeat domain of the extracellular A subunit of the TSHR, as marked in blue colour.

Cyclic peptides which mimic the eighth loop of the leucine-rich repeat domain of TSHR suppressed or at least stabilized the titers of TSH binding antibodies despite continuing immunizations (17).

Thus, established thyroid disease was successfully treated in these animals. Increased thyroid sizes were reduced after 6 months of peptide therapy (17). In parallel, also thyroid hyperplasia and histological alterations were markedly reduced. Elevated thyroxin (T4) levels were reverted to normal values, starting 15 weeks after initiation of peptide therapy (17).

Also retro-orbital fibrosis was reduced, suggesting a positive effect on Graves´ orbitopathy (17). These findings represent an interesting complementation of previous preclinical studies on Graves´ disease models, since such a therapeutic effect has not yet been shown in animal models. On the other hand, patients with Graves´ orbitopathy are especially hard to treat.

Since the investigated peptides were derived from only one of the several possible epitopes of which the LRD of the TSHR is composed, the feasibility to

block polyclonal immune responses in different mice suggests that "linked suppression" (sometimes also termed "bystander suppression") plays a role in the observed effects, although so far only described for T lymphocyte-directed therapies.

In addition, administration of the same peptides at identical dosings (6 monthly administrations) in immunogically naïve mice did not result in any immune reactions. No anti-TSHR titers (as measured with the 3rd generation assay) were observed in these animals at any time during the experiment.

11. SUMMARY AND CONCLUSIONS

In summary, a long-term disease model of Graves´ disease has been established, which stably features many hallmarks of human disease including orbitopathy. Specific linear peptides which mimic TSHR-derived T cell epitopes (e. g. "apitopes") or cyclic peptides derived from B cell epitopes (e.g. loops of the TSHR A domain) hold promise as future treatments of Graves´ disease. Apitopes have been tested in a prophylactic setting in mice, and are currently studied in a first clinical trial. After repeated monthly administrations, cyclic peptides reduced thyroid size, elevated serum T4 levels, tachycardia and retro-orbital fibrosis in TSHR-immunized mice.

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