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TSH RECEPTOR AUTOANTIBODIES

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Abstract

Thyrotropin receptor autoantibodies (TSHR-Abs) of the stimulating variety are the hallmark of Graves' disease. The presence of immune defects leading to synthesis of TSHR-Abs causes hyperthyroidism and is associated with other extrathyroidal manifestations. Further characterization of these antibodies has now been made possible by the generation of monoclonal antibodies with this unique stimulating capacity as well as similar TSHR-Abs not associated with hyperthyroidism. Their present classification divides TSHR-Abs into stimulating, blocking (competing with TSH binding) and neutral (no signaling). Recent studies using monoclonal TSHR-Abs has revealed that stimulating and blocking antibodies bind to the receptor using mostly conformational epitopes, whilst neutral antibodies utilize exclusively linear peptides. Subtle differences in epitopes for stimulating and blocking antibodies account for the diversity of their biological actions. Recently non-classical signaling elicited by neutral antibodies has also been described, raising the need for a new classification of TSHR-Abs.

Keywords

thyroid autoimmunity; TSH receptor; Graves' disease; TSHR antibodies; antibody epitopes; signal transduction

Take-Home messages

- TSHR is the main autoantigen in Graves' disease
- TSHR is constitutively dimerized and undergoes complex post-translational modifications: glycosylation, cleavage and shedding
- TSHR antibodies of the stimulating, blocking and neutral variety can be found in patients with autoimmune thyroid disease.
- Monoclonal antibodies against the TSHR have been raised in hamsters, mice and humans with similar stimulating, blocking and neutral activity.
- Stimulating and blocking antibodies utilize mostly conformational epitopes, whilst neutral antibodies are restricted to linear peptides

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- Some neutral antibodies are not neutral and have the ability to signal through non-classical signaling cascades

1. The TSH Receptor antigen

The thyroid stimulating hormone receptor (thyrotropin receptor - TSHR) belongs to the large family of G-protein coupled receptors with seven transmembrane spanning domains (class 5 or E, the cAMP generators). The holoreceptor has 764 residues which is cleaved post-translationally into α (or A) and β (or B) subunits (Figure 1)(1). The physiologic agonist, TSH, is secreted by the anterior pituitary gland and acts as the main regulator of thyroid function, stimulating thyroid growth and function and the synthesis and secretion of thyroid hormones (1). The TSHR has gained much attention as a primary autoantigen in thyroid autoimmune disease, especially in Graves' disease.

Expression of the TSHR is not confined to the thyroid gland. The presence of biologically active TSHRs has been confirmed in a variety of human and animal cells tissues since the first attempts at probing the TSHR (2), including adipocytes & fibroblasts, bone cells (osteoblasts and osteoclasts), bone marrow cells, cardiomyocytes and more (1). Moreover, the TSHR is expressed early in development (3) and in embryonic stem cells (ES-cells) (4). The widespread and early expression profile of the receptor indicates that the TSHR plays additional roles rather than solely regulating thyroid metabolism, and likely modulates the development of various tissues and organs as seen in the bones of the TSHR knock out mouse (5).

Another distinct and not fully understood feature of the TSHR, is a complex series of posttranslational modifications. The TSHR undergoes N-glycosylation in 6 defined sites on its ectodomain (residues 77, 99, 113, 177, 198 and 302) and after being transported to the surface, the receptor molecule is subjected to intra-molecular cleavage, leading to the removal of a 50 amino acid sequence between residues 316 and 366. As a result, the receptor consists of the two subunits bound together with disulfide bonds, α or A – consisting entirely of the ectodomain structure, and β or B – the transmembrane and a short intracellular domain (6;7). Several membrane based enzymes have been proposed to be responsible for the cleavage, including ADAM10 (8). In subsequent steps, the α subunits shedding, leaving an excess of ectodomain-deprived β subunits on the cell membrane. Interestingly, TSHR antibodies are directed almost exclusively to the α domain suggesting their immune processing outside the thyroid gland (9).

The TSHR constitutively oligomerizes, and combined with the process of intramolecular cleavage, this introduces the presence of multiple oligomerized receptor forms including uncleaved receptors and β subunits only. Apart from a possible influence on TSHR physiology, the presence of cleaved and unshed receptors introduces additional forms of the receptor to be recognized by immune competent cells. The TSHR life cycle also includes internalization and further intracellular trafficking, but the full-length uncleaved receptors are known to undergo recycling and return to the cell membrane intact (10).

2. TSH receptor antibodies

Graves' disease is a leading cause of hyperthyroidism in the US and worldwide with an annual incidence of 30 to 200 per 100,000 (11;12). Classical biochemical features of hyperthyroid Graves' disease (elevated thyroid hormone levels and low to undetectable TSH) arise from the action of TSHR stimulating autoantibodies which can be detected in virtually all untreated patients using third generation immunometric receptor assays (13). The majority of stimulating TSHR antibodies are oligoclonal and belong to the IgG₂ class,

and their action is to stimulate the synthesis of iodinated thyroglobulin with subsequent release of thyroid hormones (7). In spite of actions similar to TSH, TSHR autoantibodies harbor some distinct features in addition to the different pharmacodynamics of IgG molecules, which includes their prolonged action when compared to TSH itself and hence their original name of long-acting thyroid stimulators (LATS) (14).

Bioassay analysis of Graves' disease patients' sera has revealed that not all TSHR-Abs present are thyroid stimulators (15). According to their ability to induce the generation of intracellular thyrocyte cAMP, TSHR-Abs have been classified as stimulating – increasing cAMP concentrations, blocking – reducing TSH effects, and neutral – with no effect on TSH binding and no effect on cAMP levels (Figure 2).

3. T cells and Graves' disease

Despite the fact that Graves' disease appears superficially to be a Th2 driven pathology, with activation of TSHR-specific B cells and subsequent production of TSHR-Abs, T cells are also involved in disease pathogenesis. Thyroid tissues obtained from Graves' disease patients, exhibit various levels of infiltration by both CD4+ and CD8+ T cells. It is known that activated autoreactive CD4+ T cells which must have escaped central deletion or are not effectively suppressed by peripheral tolerance mechanisms are associated with B-cell activation. CD4+ CD25+ T cells (Tregs) would be expected to fail to suppress expansion of effector CD4+ T cells and fail to suppress effector cells in all autoimmune disorders including Graves' disease although findings in patients are disparate (16). In contrast, involvement of Tregs in animal models of Graves' disease has been shown in thyroiditis-resistant C57BL mice which were able to be successfully immunized with a TSHR adenovirus construct when depleted of CD4+ CD25+ cells (17). Moreover, mutations of the X-chromosome FOXP3 gene, responsible for the development of Tregs, leads to systemic autoimmune disorders in mice (18) and has been reported to be associated with Graves' disease by some investigators (19), the finding has not been confirmed by others (20). Genetic variation in the gene has been shown to correlate with the risk of juvenile autoimmune thyroid disease in a Caucasian population study (21).

4. Monoclonal antibodies to the TSHR

All three types of TSHR-Abs found in patients with AITD have been modeled in laboratory animals. Immunization of mice with recombinant TSHR proteins and peptides produced only TSHR blocking antibodies (22;23). All TSHR stimulating antibodies have been raised against natural full-length receptor or natural ectodomain using TSHR expressing cells or genetic immunizations or infections. The first reported stimulating monoclonal antibody against the TSHR (Mount Sinai-1, or MS-1) was raised in hamsters using TSHR expressing CHO cells (24) rapidly followed by a number of other laboratories (25;26), and the finding of a highly potent human TSHR-mAb (27). Stimulating TSHR-mAbs bind only to native TSH receptor retaining its tertiary conformation, as shown by binding assays on fixed and unfixed cells expressing TSHR. Classically, these antibodies elicit intracellular cAMP generation but also compete with TSH binding and block the activation of the receptor caused by its physiologic ligand. Blocking antibodies have similar features but fail to initiate cAMP stimulation. Since they may interfere with TSH binding and may not elicit signal transduction by themselves, true blocking antibodies are able to induce hypothyroidism by compromising TSHR driven thyroid metabolism as described above.

5. TSHR-mAb induced signal transduction

The TSH receptor is constitutively active, and its signals are further enhanced by stimulating agonists, TSH and stimulating TSHR-Abs. Intracellular signal propagation is mediated by

classical GPCR effector proteins, with Gαq and Gαs directly shown to interact with the receptor.

Docking of Gαs to the activated receptor leads to an increase in adenylate cyclase activity which generates cAMP, thus directly activating protein kinase A (PKA) and cAMP response element-binding protein (CREB). Docking of Gαq involves formation of PI3 and DAG which are involved in the activation of Ca²⁺ and protein kinase C (PKC) and subsequently Erk1/2 and p90RSK. In our recent studies, stimulating TSH-mAb were shown to act through the Gαq-PKC-Akt cascade in a dose-dependent and time-dependent manner, while PKA-dependent signaling was apparently found unchanged in the rat thyroid cell model (FRTL-5 cells) utilized (28;29). In the same study, some monoclonal antibodies regarded as blocking, actually disclosed some agonistic activity, stimulating either typical TSH-related pathways, or they induced more non-classical signaling events. Moreover, some neutral antibodies did not increase cAMP, but were able to signal through Akt, c-Raf/ERK1/2/p90RSK, PKC, and PKA/CREB (29). These observations lead to a proposed revision of the classification of TSHR antibodies which simply reflects their potential heterogeneity (Table 1).

6. TSHR antibody epitopes

Since only the antibodies raised against native TSHR were able to induce cAMP generation *in vitro*, and hyperthyroidism *in vivo*, it became clear that the maintenance of the normal structure of the receptor was essential. It had been shown previously that antibodies that failed to bind to fixed cells expressing TSHRs utilized conformational epitopes (24;25). Typically, conformational epitopes are discontinuous, and as far as the TSHR is concerned consist of multiple short peptides averaging from 5 to 12 aminoacids, scattered across a TSH binding pocket on the TSHR (22). Recently, a part of the TSHR ectodomain (residues 1-260) with a bound stimulating antibody Fab fragment (M22-Fab) has been crystallized (30). According to these data, residues scattered on the concave surface of the leucine-rich repeat region (LLR) of the TSHR ectodomain were crucial to the binding of the monoclonal Fab (30). Six residues were found to have the strongest interaction with the antibody: residues 38, 80, 109, 129, 183, and 255. In a subsequent study, several mutations were performed to examine their influence on TSHR biological activity. Three aminoacids in the heavy chain and one in the light chain of the mAb IgG were crucial in eliciting a stimulating signal (31) and four of these residues (80, 109, 185 and 255) were those previously found to have a strong association with the antibody. However, mutation of these aminoacids did not hamper cAMP generation elicited by native TSH indicating their specificity for the TSHR-mAb. This suggested that although the crystal structure of TSHR-M22-Fab resembled the aminoacid rearrangements of native TSH with its receptor, minor differences in the structure of the ligand may influence signaling events. We have recently found evidence that the majority of aminoacids interacting with TSHR stimulating mAbs are common (32). However, subtle differences can be found, suggesting that small differences in epitopes may be responsible for the diversity of biological actions seen with TSHR-mAbs. For example, in our own ongoing studies (32) conformational epitopes for two stimulating mAbs (our hamster derived MS-1 and the human-derived M22) had epitopes which consisted of at least five small peptides in the TSHR leucine rich repeat domain and no significant differences between the antibodies examined was found (Figure 2). However, in our study of stimulating TSHR-mAbs, we have shown variations in signaling pathways utilized by these antibodies highlighting the possibility that differences in affinity and concentration may also be paramount in signal transduction (29).

TSHR blocking antibodies can be raised in animal models against a variety of TSHR antigen preparations suggesting that their epitopes are less conformationally dependent and perhaps more widely distributed than stimulators. TSHR-mAbs of the blocking variety were

subsequently found to utilize both conformational and linear epitopes (22;33). Therefore, such antibodies do not have a “good enough fit” in the TSH binding pocket. They could prevent TSH or other antibodies from binding and from interacting with crucial signaling residues and thus failed to initiate intracellular cAMP generation. Epitopes for blocking antibodies have been reported on both α and β subunits of the receptor (22;23;33;33). Ectodomain binding sites have included the LRR region and more downstream residues in the proximity of the hinge region.

By definition, true neutral antibodies do not propagate any signal through classical or non-classical signaling pathways in addition to allowing TSH to bind and signal. Since their neutrality applies to a lack of competition with TSH binding sites their epitopes must be located away from the TSH or stimulating antibody binding pockets. These antibodies exclusively utilize linear epitopes, which are on average 5 to 20 consecutive residues in the receptor structure. We have shown that majority of neutral TSHR-mAbs bind to the N-terminus portion of the TSHR ectodomain or its cleavage region (residues 337-356)(22)

7. Summary

Since no TSHR-Abs can be found in sera of healthy individuals, their presence is seen as a hallmark of autoimmune thyroid disease. TSHR-Abs are easily found in most patients with Graves' disease and in 10-15% of patients with Hashimoto's thyroiditis and the level of TSHR-Abs correlates with the severity of the disease (degree of hyperthyroidism), and with the degree of ophthalmic involvement (34). The recent characterization of TSHR-Abs has been made possible by the generation of a variety of TSHR-mAbs with differing biological activity: stimulating, blocking and neutral. Our data presenting non-classical signaling by some neutral TSHR-mAbs has raised the question of whether these antibodies may also influence thyroid cell growth and function. Given the increased risk for thyroid carcinoma widely reported in Graves' disease patients (35), the further description of TSHR-Ab initiated signaling cascades involved in cell proliferation is potentially important. In addition, the detailed characterization of epitopes for blocking the TSHR may contribute to the development of novel therapeutic strategies.

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Figure 1.

TSH receptor structure – the model of full-length TSHR. TSHR has a large extracellular domain (α or A subunit) with nine leucine-rich repeat domains and transmembrane / intracellular domain (β or B subunit). After cleavage of residues 316-366, subunits remain covalently bound and are subjected to shedding. Adapted from Ando T et al. (2004, *Endocrinology* **145**: 5185–5193).

Figure 2. Comparison of peptides originating from the TSHR ectodomain subjected to protease digestion (Trypsin and AspN endopeptidase) under protection by monoclonal TSHR antibodies, both of stimulating variety. Panel **A** illustrates SELDI mass-spectrometry peaks for MS-1 antibody (hamster derived) and **B** – for M22 (human derived). X-axis represents MW, Y-axis peak intensity related to amount of protein.

Table 1

Proposed neoclassification of TSHR autoantibodies. The classification is based upon the potential to induce cAMP production (classical signaling pathway), stimulate non-classical signaling cascades and to block TSH binding.

Antibody Class	cAMP signalling /classical/	Non-cAMP signaling /non-classical/	TSH Binding
Stimulators	+++	++	Block
True Blockers	-	+/-	Block
False Blockers	+/-	++	Do Not Block
True Neutrals	-	-	Do Not Block
False Neutrals	-	+	Do Not Block