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Immunopathogenesis of Graves' ophthalmopathy: the role of the TSH receptor

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Abstract

Graves' ophthalmopathy is an inflammatory autoimmune disorder of the orbit. The close clinical and temporal relationships between Graves' hyperthyroidism and ophthalmopathy have long suggested that both conditions derive from a single systemic process and share the thyrotropin receptor as a common autoantigen. This receptor is expressed not only in thyroid follicular cells, but also in orbital fibroblasts with higher levels measured in orbital cells from ophthalmopathy patients than in cells from normal individuals. Recent studies from several laboratories have shown that thyrotropin receptor activation in orbital fibroblasts enhances hyaluronic acid synthesis and adipogenesis, both cellular functions that appear to be upregulated in the diseased orbit. The phosphoinositide 3-kinase/Akt signaling cascade, along with other effector pathways including adenylyl cyclase/cAMP, appears to mediate these processes. Future therapies for this condition may involve inhibition of thyrotropin receptor signaling in orbital fibroblasts.

Keywords

Graves' orbitopathy; Graves' disease; thyrotropin receptor; thyrotropin receptor autoantibodies; autoimmune disease

A. Introduction

Graves' orbitopathy (GO) is an inflammatory autoimmune disorder of the orbit (1). The immune basis of the disease is suggested by a perivascular and diffuse infiltration of CD4+ and CD8+ T cells, B cells, plasma cells and macrophages (2). In addition, the connective tissues are extensively remodeled with enlargement of the extraocular muscles and orbital adipose tissues (3, 4-6). Underlying these changes are excessive production of hyaluronic acid (HA) and new fat cell development. While GO affects primarily patients with a history of Graves' hyperthyroidism, it is also encountered in euthyroid and hypothyroid individuals with laboratory evidence of autoimmune thyroid disease. While the onset of GO occasionally precedes or follows that of hyperthyroidism by several years, these conditions most commonly occur simultaneously or within 18 months of each other (7). Owing to the close clinical and temporal relationships between Graves' hyperthyroidism and GO,

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investigators have long hypothesized that both autoimmune conditions derive from a single systemic process and share the thyrotropin receptor (TSHR) as a common autoantigen. In this review, we will explore current evidence that autoimmunity directed against TSHR on orbital cells sets in motion the connective tissue changes within the orbit that lead to the clinical disease.

B. The target cell in GO

Evidence from several laboratories suggests that orbital fibroblasts are the autoimmune target cells in GO (8-11). Early studies found that orbital-infiltrating CD8⁺ T cells recognize orbital fibroblasts and not eye muscle extracts, and that they respond by proliferation via major histocompatibility complex (MHC) class II and CD40 signaling (9). Unlike eye muscle cells, orbital fibroblasts express human leukocyte antigen (HLA)-DR, suggesting that they may act as antigen-presenting cells (12).

Orbital fibroblasts are heterogeneous and may be classified based on the presence or absence of the cell surface glycoprotein CD90, also known as thymocyte antigen-1 (Thy-1) (13, 14). This antigen contains a variable region-like immunoglobulin domain and may play a direct role in immune responses. While Thy-1 is found on essentially all fibroblasts investing the extraocular muscles, only about 30% of fibroblasts found within the orbital adipose tissues are Thy-1 positive (13). It has been proposed that the Thy-1 positive subset of orbital fibroblasts responds to the orbital immune process by augmenting HA secretion, whereas those not expressing the antigen are capable of undergoing adipogenesis when suitably stimulated. While adipogenesis itself does not appear to impact the expression of Thy-1, Thy-1 is more highly expressed in cultured orbital fibroblasts from GO patients than in normal orbital cells (15).

C. TSHR as autoantigen in GO

TSHR on thyroid follicular cells serves as the autoimmune target in Graves' hyperthyroidism and antibodies directed against this cell surface receptor stimulate the overproduction of thyroid hormones (16). Clinical observations suggesting that the same receptor may be the primary target in GO include that TSHR-directed autoantibodies (TRAb) can be detected in essentially all patients with GO, including euthyroid patients (17), that levels of TRAb correlate with the severity and clinical activity of the disease (18, 19) and with disease prevalence in untreated patients with Graves' hyperthyroidism (20). In addition, higher titers of these antibodies portend a worse prognosis (21). Laboratory studies have shown that while TSHR is expressed in orbital fibroblasts and tissues from both normal individuals and patients with GO (18, 22-25), significantly higher levels are measurable in GO tissues (26). Further, orbital adipose tissues from patients with active GO express higher levels of the receptor than do tissues obtained from patients with inactive disease (27). Orbital fibroblasts, when cultured under adipogenic conditions, increase TSHR expression as they differentiate into mature adipocytes (25, 28). This suggests that enhanced adipogenesis within the GO orbit may lead to increased expression of the autoantigen, which may in turn further drive the local autoimmune process, thus establishing a positive feedback loop that acts to propagate the disease.

D. TSHR structure and function

TSHR is a glycoprotein hormone receptor which, along with luteinizing hormone receptor (LHR) and follicle-stimulating hormone receptor (FSHR), is a member of the G protein-coupled receptor (GPCR) family (16). TSHR contains a large extracellular domain (ectodomain) that is mainly responsible for recognition and binding to the ligand, a seven-transmembrane domain, and an intracellular domain (endodomain) bound to G-protein

subunits, mainly the G α s and G α q. Upon stimulation, both subunits trigger signaling cascades that result in often overlapping down-stream effects. TSHR undergoes several post-translational modifications producing a wide diversity of receptors expressed on the cell surface (29). Unlike LHR and FSHR, TSHR is prone to spontaneous ectodomain mutations that contribute to the receptor's constitutive activity (30). A post-translational intramolecular proteolytic cleavage at the hinge region of the extracellular domain is unique to TSHR and may explain why it alone among the GPCR family is involved in autoimmunity (16). This cleavage divides the receptor into two disulfide-linked subunits; subunit A consists solely of the 367 amino terminal amino acids while subunit B contains the remaining portion of the ectodomain closest to cell membrane, as well as the transmembrane and intracellular domains. The disulphide bonds between subunits A and B are prone to cleavage by matrix metalloprotease and protein disulphide isomerase, resulting in shedding of the A subunit. It has been shown that TRAb preferentially recognize shed A subunits, rather than attached A subunits (31). In addition, studies of an adenovirus-mediated mouse model of Graves' disease demonstrate that goiter and hyperthyroidism occur to a much greater extent when the adenovirus expresses the free A subunit than a genetically modified TSHR that cleaves only minimally (32). These data suggest that shed A subunits may be responsible for the induction or amplification of the immune response leading to Graves' hyperthyroidism (31). The uncleaved or B subunits on the cell membrane have a tendency to dimerize or multimerize among themselves, thus modulating TSHR signaling cascades (33).

Most TSHR signaling in thyrocytes is mediated through the G α s protein subunit which activates the adenylyl cyclase/cAMP signaling cascade (34). This effector pathway is known to be constitutively active, meaning that there is receptor signaling in the absence of ligand (35). In the presence of ligand, increased cAMP activates phosphokinase A (PKA) and cAMP response element binding protein (CREB) as well as the PKA-independent MEK (MAPK kinase) pathway (34, 36). Both TSH and some TRAb also activate a cAMP-independent cascade that increases phosphoinositide 3-kinase (PI3K) activity with subsequent phosphorylation of Akt. This pathway is mediated through the G α q effector protein that activates phospholipase C (PLC)- β and the G $\beta\gamma$ subunit. Upon activation, PLC triggers PI3K which results in phosphorylation of Akt and activation of the serine/threonine kinase mammalian target of rapamycin (mTOR). This in turn activates p70 S6 kinase (p70S6K) either by direct phosphorylation or inhibition of a phosphatase. TSH also activates PKC by stimulating PLC and increasing intracellular calcium, a pathway that requires Rho activation and by-passes PKA. In addition to these pathways, each G α effector is impacted by growth factors that signal via MAPK pathways to regulate thyrocyte proliferation, differentiation and survival (37).

E. TSHR antibodies

Sera from individual patients with Graves' disease contain a mixture of TRAb (34, 38). The ultimate clinical expression of the disease appears to be influenced by the particular varieties and affinities of TRAb present. The production of specific human, mouse and hamster monoclonal TSHR antibodies has allowed their use as probes to better understand TRAb binding sites and signaling pathways. TRAb can be classified as stimulating (TSAb), blocking (TBAb), or neutral depending on their respective abilities to induce cAMP generation and thyrocyte proliferation, block TSH from inducing the same, or bind to the receptor without impacting cAMP generation or TSH binding of TSH (16, 38). Monoclonal TSAb, including M22, RSR-12 and MS1, activate both G α s and G α q signaling cascades, thereby increasing not only cAMP production, but also activating the Akt and PKC pathways (34). TSAb from human or animal sources recognize binding sites in the leucine-rich region on the TSHR ectodomain (16, 39). These antibodies activate similar signaling

cascades to TSH, albeit with differences in the strength of the signals owing to different TSHR binding properties and binding domains. M22, a monoclonal antibody with high affinity for TSHR, effects greater stimulation of cAMP/PKA/CREB than does TSH. Unlike TSH, it also stimulates the phosphorylation of p90RSK (34). TBAbs, such as RSR-B2, Tab-8 and 5C9, do not activate adenylate cyclase to any great extent. However, some TBAbs do exhibit weak TSHR agonistic effects impacting primarily Gαq cascades. Because these antibodies are heterogenous in nature and have epitopes ranging throughout the TSHR structure, they activate or inhibit a variety of pathways. For instance, cRaf/ERK/CREB signaling is activated by RSRB2, but not by Tab8.

Neutral TRAb, including Tab-16 and 7G10, are classified thus because they bind to TSHR without blocking TSH binding or impacting the production of cAMP. However, these ligands appear not to be “neutral” with respect to other signaling cascades (34, 40). While 7G10 suppresses multiple signaling pathways, Tab-16 has been shown at high concentration to activate several pathways, including PI3K/AKT/mTOR/S6, NF-κB/STAT, and p38MAPK/c-Jun N-terminal kinases (JNK). Unlike TSAbs and TBAbs, some neutral TRAbs recognize epitopes in the cleaved region of the receptor and may thereby prevent receptor cleavage. Others activate the NFκB pathway that in turn stimulates the release of various cytokines and immuno-modulators. Neutral TRAbs are demonstrable in the majority of patients with Graves’ disease and evidence suggests that they may augment inflammation through oxidative stress-induced apoptosis (40). The frequency of neutral TRAb in the sera of patients with GO is unknown, as is their possible role in the development of the disease.

F. The potential role of TRAb in orbital tissue remodeling

Signs and symptoms experienced by patients with GO include proptosis (forward protrusion of the eyes), conjunctival and eyelid swelling and erythema, diplopia, and ocular pain. These features derive from expansion of the orbital adipose tissues and extra-ocular muscle bodies within the inflexible bony orbit. The resulting increase in orbital pressure displaces the orbit forward and hinders venous drainage, facilitating the accumulation of inflammatory mediators (1). Orbital inflammation in GO appears to be initiated by infiltrating T-helper cells. This sets in motion the local production of cytokines, including interferon-γ (IFN-γ), interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α), oxygen free radicals, and TRAb, although the latter may also reach the orbit via the circulation (41).

Gene expression profiling of GO orbital adipose tissues reveals over-expression of a number of adipocyte-related genes, suggesting that new fat cell development is responsible for the increase in orbital adipose tissue volume in GO (42, 43). In order to examine whether TSHR activation by TRAb or TSH might impact adipogenesis in GO orbital fibroblasts, we treated cultures lacking the pro-adipogenic factors insulin and insulin-like growth factor-1 (IGF-1) with the high-affinity human stimulatory monoclonal antibody M22 or bovine TSH (44). We found that both agents stimulate cAMP production and phosphorylation of Akt in these cells. In addition, both were found to act as pro-adipogenic factors, as shown by an increase in late adipocyte genes (adiponectin and leptin) with an accumulation of lipid as assessed by oil-red O staining. The adipogenesis promoted by M22 was abrogated by treatment of orbital cultures with the PI3-kinase inhibitor LY294002, suggesting that the effect of M22 is mediated via PI3K activation. In another study, we demonstrated that M22 also stimulates the secretion of interleukin-6 (IL-6), a cytokine synthesized by mature adipocytes (45). Zhang and colleagues performed a series of experiments in which TSHR was activated in orbital preadipocytes not by TRAb, but by the introduction of an activating mutant receptor (46). This led to an increase in early adipocyte differentiation, as demonstrated by 2-8 fold elevations in levels of the early to intermediate adipocyte markers, CCAAT/enhancer binding protein β (C/EBPβ) and peroxisome proliferator-activated receptor-γ (PPARγ)

(47). Oil red-O staining revealed measureable lipid production with somewhat higher levels in the mutant TSHR than in the WT-TSHR transfected cells. However, while the basal lipid content of activated cells was higher, it failed to increase in response to a PPAR γ agonist, suggesting that TSHR activation in these transfected orbital cells renders them refractory to later stages of adipogenesis. While the cell systems used in the 2 sets of experiments differ, our studies and those of Zhang and colleagues point towards TSHR activation as a contributor to the enhanced adipogenesis that occurs in the GO orbit.

The over-production and accumulation of HA is another histologic hallmark of GO (3). HA production is regulated by hyaluronan synthases 1, 2 and 3, of which HAS 1 exhibits a tissue specific expression in fibroblasts (48), HAS 2 is inducible by IL-1 β in orbital fibroblasts (49) and HAS 3 is constitutively present in most cells (50). There is clear evidence that IGF-1R, MAPK (p38) and Akt pathways are involved in positive regulation of HAS 1 and HAS 2 (51). A recent study demonstrated the possible regulation of HAS 2 via the PI3K-Akt-mTOR-p70S6K pathway in breast cancer cell lines (52).

Smith and colleagues studied the effect of purified immunoglobulins from patients with Graves' disease (GD-IgG) on HA synthesis in cultured orbital fibroblasts that had not undergone adipocyte differentiation (53). They found that GD-IgG (by definition containing TRAb as well as other IgGs), but not human recombinant TSH (hrTSH), increases HA production in GO cells. In contrast, the group of van Zeijl and found that neither GD-IgG nor hrTSH increases HA production in undifferentiated GO orbital fibroblasts (54). In a subsequent study, this time using GO orbital fibroblasts that had undergone adipocyte differentiation, van Zeijl and colleagues found that GD-IgG, but not hrTSH, stimulates HA synthesis (55). We recently performed studies using undifferentiated GO orbital fibroblasts and found both bovine TSH and M22 to stimulate HA production in these cells (56). A related study by Zhang demonstrated stimulation of HA synthesis by bovine TSH as well as by 2 monoclonal TRAb (one stimulatory and one neutral) in normal undifferentiated orbital fibroblasts, but not in GO fibroblasts (46). They also demonstrated that transfection of GO orbital fibroblasts with an activating mutant TSHR induces hyaluronan synthases 1 and 2 and increases HA production over that found in control transfected cells. The differences between the 4 studies described above may be reconciled by the significantly greater affinity and potency of the monoclonal TRAbs used in our study and the Zhang study, in comparison with the heterogeneity of the circulating TRAb (stimulating, blocking and neutral of various affinities) present in the pooled GD-IgG used in studies by Van Zeijl and Smith. In addition, both we and Zhang used bovine TSH, known to have greater affinity for human TSHR and greater signaling activity than hrTSH (57, 58), while Smith and van Zeijl used hrTSH in their studies. Finally, it appears that relatively high levels of TSHR expression, or potent TSHR activation, is necessary for the enhancement of HA production in orbital fibroblasts. Preadipocyte fibroblasts that have undergone adipocyte differentiation, as were used in the 2011 Zeijl study, exhibit fairly abundant TSHR expression (25,28), and cells containing the transfected TSHR-activating mutation, as were used by Zhang, would have both high expression levels and potent receptor activation. It may be that the very high affinity and potency of the M22 antibody or bovine TSH used in our studies allowed stimulation of HA production in our undifferentiated cells with relatively low TSH expression.

G. Possible interactions between TSHR and IGF-1R

The IGF-1R, as well as the structurally related insulin receptor (IR), contains two α - and 2 β -chains that form a heterotetrameric structure linked by covalent disulphide bridges (59, 60). These receptors may also exist as hybrid dimers consisting of one α - β -dimer of each receptor type. Binding of either IGF-1 or insulin initiates dimerization, autophosphorylation,

and activation of the receptor complex. Subsequent phosphorylation of tyrosine substrates leads to binding and activation of the regulatory subunit of PI3K. This stimulates PI3K activity and increases phosphatidylinositol 3,4,5-triphosphate (PIP3) levels with recruitment of Akt to the membrane where it can be phosphorylated and activated. The PI3K/Akt pathway mediates many cellular functions, including cell survival through its inhibition of apoptosis (61) and the promotion of adipogenesis (62).

The study by Smith and colleagues discussed above also demonstrated that IGF-1 stimulates HA production in GO orbital fibroblasts (53). They further showed that an IGF-1R blocking monoclonal antibody (1H7) completely attenuates GD-IgG- or IGF-1-induced HA synthesis. They concluded that HA synthesis in the GO orbit may be mediated by a subset of circulating autoantibodies that target IGF-1R, rather than TSHR. However, attempts to measure specific IGF-1R antibodies in GO sera have been unsuccessful (63). In another study, the same group immunoprecipitated both receptors from orbital fibroblast or thyrocyte preparations using specific monoclonal antibodies directed against either receptor and also demonstrated co-localization of the receptors in the perinuclear and cytoplasmic compartments (64). They suggested, as had earlier investigators (65, 66), that TSHR and IGF-1R may interact as physical and/or functional complexes. Earlier studies by other investigators also pointed towards an interaction between these receptors. This might take the form of physical association between the receptors and/or be manifest through indirect modulation of common downstream signaling cascades. In recent studies, we found that 1H7 blocks both M22-induced phosphorylation of Akt and M22- or IGF-1-induced HA synthesis in GO orbital fibroblasts (56, 67). Whether these results reflect the presence of physical or functional TSHR/IGF-1R interactions, or suggest a lack of receptor specificity in 1H7, awaits further study.

IGF-1R signaling is influenced not only by insulin and the IR, but also through cross-talk with other signaling pathways, including epidermal growth factor receptor pathways (64, 68). A recent review by Wiersinga suggests that TSHR signaling pathways within the GO orbit may be influenced by IGF-1 itself, rather than by IGF-1R autoantibodies, as well as by other growth factors acting in an autocrine or paracrine fashion (69).

H. Novel TSHR-directed therapy for GO

Recent information concerning the structure of TSHR and its similarities to LHR and FSHR has led to the development of a generation of small molecule ligands (SML) of TSHR. Drs. Susanne Neumann and Marvin Gershengorn (Clinical Endocrinology Branch, National Institutes of Health, Bethesda, Maryland) have used molecular modeling, high-throughput screening and functional experiments to identify SML that inhibit TSH- and TSAb-stimulated signaling, as well as constitutive signaling of thyrocyte TSHR (70). Functioning as allosteric modulators, these molecules position themselves within the transmembrane helices binding pocket and prevent contact with deeper residues essential for agonist activity. Thus, their action does not involve competition for extracellular TSH or TRAb binding sites. These SML have been shown to inhibit cAMP production in human thyrocytes stimulated either by TSH or IgG from each of 30 patients with Graves' hyperthyroidism (71). Whether these SML are also capable of inhibiting TSHR signaling pathways, HA synthesis, cytokine secretion or adipogenesis in human orbital fibroblasts is at present unknown. Because SML can be produced in large quantities and are not degraded in the GI tract, they may represent potential novel oral therapy for Graves' hyperthyroidism and/or GO.

I. Summary

We present evidence that autoimmunity directed against TSHR on orbital cells sets in motion connective tissue remodeling within the orbit that leads to the various clinical expressions of GO. HA accumulation, expansion of orbital adipose tissues and local inflammation appear to be the salient histologic features of the disease. Orbital fibroblasts express functional TSHR and are considered to be the target cells. Several laboratories have explored the impact of TSHR activation in these cells on signaling and cellular functions relevant to the orbital tissue changes. Both HA synthesis and new fat cell development appear to be enhanced by activation of orbital fibroblast TSHR, whether effected through ligation of the receptor by TSH or monoclonal TRAb, or by the introduction of an activating mutant TSHR. The phosphoinositide 3-kinase/Akt signaling cascade appears to be the primary effector of the processes, with input from adenylyl cyclase/cAMP and other signaling pathways. Circulating TRAb in patients with Graves' hyperthyroidism are heterogeneous with differing potency and affinities. These antibodies activate various TSHR signaling cascades in thyrocytes, resulting in the over production of thyroid hormones. It appears likely that they similarly activate orbital fibroblast TSHR to modulate HA synthesis and adipogenesis (Fig.1). While IGF-1 and other growth factors may act in an autocrine or paracrine fashion to impact TSHR signaling, little evidence supports a role in GO for circulating autoantibodies directed against IGF-1R. Future therapies may involve inhibition of TSHR signaling in orbital fibroblasts, perhaps using SML recently developed to block thyrocyte TSHR effector pathways.

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Research Agenda

- Signaling cascades activated in orbital fibroblasts by stimulatory, blocking and neutral TRAb should be investigated.
- The impact of various TRAb on adipogenesis, HA synthesis and cytokine secretion in orbital fibroblasts should be assessed.
- Studies to determine whether SML targeting TSHR block the effects of monoclonal TRAb or Graves' IgG on orbital fibroblast functions are needed.
- Further studies to clarify possible physical and functional interactions between IGF-1R and TSHR should be performed.

Figure 1.

Role of the TSH receptor in the immunopathogenesis of Graves' ophthalmopathy. Circulating TSH receptor autoantibodies of differing potency and affinities recognize the receptor on fibroblasts residing within the orbit. Ligation of the receptor results in activation of the phosphoinositide 3-kinase/Akt signaling cascade, as well as others including the adenylyl cyclase/cAMP pathway. This results in increased production of hyaluronic acid by these cells, with a subset exhibiting enhanced adipogenesis. IGF-1 and other growth factors within the orbit may act on the IGF-1 receptor to modulate these processes within the orbital fibroblasts. This results in accumulation of HA within the orbital tissues and expansion of the orbital fat volume, leading to the varied clinical expressions of the disease.