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Graves' Orbitopathy Models: Valuable Tools for Exploring Pathogenesis and Treatment

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ABSTRACT

Graves' orbitopathy (GO) is the most common extrathyroidal complication of Graves' disease (GD) and severely affects quality of life. However, its pathogenesis is still poorly understood, and therapeutic options are limited. Animal models are important tools for preclinical research. The animals in some previous models only exhibited symptoms of hyperthyroidism without ocular lesions. With the improvements achieved in modeling methods, some progressive animal models have been established. Immunization of mice with A subunit of the human thyroid stimulating hormone receptor (TSHR) by either adenovirus or plasmid (with electroporation) is widely used and convincing. These models are successful to identify that the gut microbiota influences the occurrence and severity of GD and GO, and sex-related risk factors may be key contributors to the female bias in the occurrence of GO rather than sex itself. Some data provide insight that macrophages and CD8+ T cells may play an important pathogenic role in the early stage of GO. Our team also replicated the time window from GD onset to GO onset and identified a group of CD4+ cytotoxic T cells. In therapeutic exploration, TSHR derived peptides, fingolimod, and rapamycin offer new potential options. Further clinical trials are needed to investigate these drugs. With the increasing use of these animal models and more in-depth studies of the new findings, scientists will gain a clearer understanding of the pathogenesis of GO and identify more treatments for patients.

Introduction

Graves' orbitopathy (GO) is the most common extrathyroidal complication of Graves' disease (GD) [1–3], occurring in 20–50 % of GD patients [4]. Although 5 % of GO patients are euthyroid or even hypothyroid [5, 6], GO usually arises at the same time as or within the first 18 months of hyperthyroidism [4]. Eyelid contracture, proptosis, ocular pain, and ocular movement disorder are the most common clinical signs. Vision decrease or even blindness may occur in severe situations. GO significantly affects the quality of life and psychological health of patients [7–9]. According to a report, patients with GO even have a comparable quality of life to those with diabetes or some malignancies [10].

The available treatment options for GO are limited. Glucocorticoids are currently the mainstay of treatment to relieve symptoms and shorten the course of the condition [11, 12]. However, this treatment has drawbacks, such as unsatisfactory results, significant side effects, and a high recurrence rate following drug cessation [9]. Clinical trials investigating several other medicines, such as teprotumumab [13–15], rituximab [16, 17], tocilizumab [18], and azathioprine [19], have been conducted based on new understandings of the mechanism underlying GO.

Orbital pathology is characterized by lymphocytic infiltration, fibroblast activation, adipogenesis, and glycosaminoglycan accumulation [20, 21]. Thyroid stimulating hormone receptor (TSHR) and insulin like growth factor 1 receptor (IGF1R), which are regarded as the primary and secondary autoantigens in GO, respectively, are overexpressed in the orbital fibroblasts of GO patients [22–26].

The lymphocytes infiltrating orbital tissue are mainly CD4+ T lymphocytes. Early research linked GO to Th1/Th2 balance [27–30], and recent research has shown that Th17 cells can improve the proinflammatory, fibrotic, and adipogenic actions of orbital fibroblasts [31, 32]. Our team also discovered a GO specific cell type CD4+ cytotoxic T cells that featured inflammation, chemotaxis, and toxicity. They localize to ocular tissue and cause orbital inflam-

mation and tissue remodeling and are associated with GO recurrence [33]. Furthermore, autoreactive B cells secrete TSHR antibodies (TRAbs), which are associated with the activity and severity of GO [34–36]. Although GO is mainly related to orbital T cell infiltration and TRAb, the exact pathogenic mechanism is unclear.

Disease models are the foundation of research, and developing appropriate and effective models is critical for studies into the pathogenesis, treatment, and prevention of GO. As a result, in this review, we outline the main GO animal models and the exploratory findings based on them.

Animal models of GO

Since no spontaneous animal models of GO have been discovered thus far, scientists have artificially induced the condition. The methods used to induce GO include pituitary extract injection, cellular immunization, and genetic immunization (including plasmids and adenoviruses), as outlined in ▶ **Table 1** and ▶ **Table 2**.

Basic requirements for an ideal animal model of GO

According to Ludgate and Baker [37], ideal GO model animals should exhibit all or some of the following criteria: 1) increased thyroid hormone levels and/or decreased thyroid stimulating hormone (TSH) levels in the blood; 2) TRAb positivity, at least for TSHR binding inhibitory immunoglobulins (TBII) and preferably TSHR stimulating antibodies (TSAbs); 3) alterations in the structure and size of the thyroid gland; 4) lymphocytic nondestructive thyroiditis; 5) clinical symptoms of hyperthyroidism, such as weight loss; 6) higher incidence in females than in male animals; and 7) orbital changes that are observed during GO, including the disturbance of extraocular muscle structure, oedema, the infiltration of immune cells, and fat neogenesis.

Pituitary extract injection

Smelser et al. [38] reported the first animal model in which some characteristics of GO were observed in 1936. When pituitary extracts were injected into thyroid depleted guinea pigs for 3–6 weeks, 88 % developed proptosis due to the increased volume of fatty connective tissue, dorsal lacrimal glands, and extraocular muscles. Oedema, lymphocyte infiltration, and increased mucopolysaccharide levels in the ocular tissue were observed on pathological examination. However, the high level of TSH and lack of thyroid tissue in this model are inconsistent with the actual status of GD patients; thus, this model is not accepted by scientists. Furthermore, there is no record of these results being repeated.

Cellular immunization

Many et al. [39] reported the first animal model with orbital alterations caused by cellular immunization in 1999. After receiving TSHR activated splenocytes, 68 % of BALB/c mice exhibited muscle fiber dissociation, oedema, adipose tissue accumulation, and inflammatory cell infiltration dominated by mast cells. Unlike the typical thyroid hyperplasia observed in GD patients, the thyroid glands of mice with ocular lesions showed severe thyroiditis. In addition, subsequent studies that replicated the model failed to reproduce the orbital changes [40].

Genetic immunization

Genetic immunization is the most extensively used and effective method for modelling GD and GO. Some scientists have used it to establish stable animal models of GD. With in-depth research, scientists have optimized these GD animal models and established excellent GO animal models.

Immunization with full length hTSHR cDNA

Costagliola et al. [41] intramuscularly injected outbred NMRI mice with cDNA encoding full length human TSHR (hTSHR). TRAb producing mice were found in 97 % of the mice that received the injection. However, only 5 of the 29 female mice and 1 of the 30 male mice developed hyperthyroidism, manifested by elevated TT4 levels and undetectable TSH levels. Extraocular muscle oedema, amorphous material accumulation, and immune cell infiltration dominated by mast cells and macrophages were also observed in the hyperthyroid mice. Although this model showed a sex differentiated influence, the incidence of GO was exceedingly low, and replication in follow up studies has proven difficult [40].

Muscle electroporation with a plasmid encoding the hTSHR A subunit

Some previous studies that immunized animals with plasmids encoding the TSHR without in vivo electroporation failed to induce orbital lesions or did not investigate orbital alterations [42, 43]. TSHR comprises a large extracellular A subunit (amino acid residues 22–289) and a transmembrane/cytoplasmic B subunit [44–46]. The TSHR A subunit was observed to lead to higher GD incidence than the full length TSHR [47]. In addition, electroporation can improve transfection efficiency, induce a powerful and long-lasting antibody response to TSHR, and lead to an increased incidence of hyperthyroidism [48].

Zhao et al. [49] challenged female BALB/c mice with hTSHR A subunit plasmid or human IGF1R A subunit plasmid or both by injection and electroporation. Eight quarters of the TSHR A subunit immunized mice developed hyperthyroidism, and 5/12 showed markedly elevated TSAb levels, orbital tissue changes including fibrosis, and the deposition of collagen and glycosaminoglycan. All IGF1R A subunit immunized mice exhibited no thyroid or orbital changes, although they developed strong antibody responses to the IGF1R A subunit. In addition, the mice challenged with the hTSHR A subunit produced an antibody to the IGF1R A subunit. The reason for this phenomenon is unclear. In the follow up experiment, the researchers improved the experimental method by injecting the plasmid deeper into a larger muscle area. They observed ocular pathology in all mice, including inflammatory cell infiltration and accumulation of adipose tissue. MRI scans provided quantifiable evidence of extraocular muscle hypertrophy and proptosis [50].

However, 75 % of the mice exhibited hypothyroidism with decreased T4 and elevated TSHR blocking antibodies (TSBAbs) [50]. Furthermore, scientists discovered significantly different thyroid function statuses in mice in a subsequent parallel experiment performed in different locations [51]. Most of the mice in Center 1 exhibited hyperthyroidism, while the mice in Center 2 had normal thyroid function. Different gut microbial compositions were later thought to be the cause [52, 53]. Notably, although IGF1R is

▶ **Table 1** Comparison of modelling methods for GO animal models.

Method	Immunization procedure	Strain	Sex	Vector	Time of injection	Disadvantage	Impact	Ref
Pituitary extract injection	Pituitary extract injection and thyroidectomy	Guinea pigs	Both sexes	None	3–9 weeks	Not accepted, single report	First model of GO	[38]
Cellular immunization	hTSHR[a]-activated splenocyte transfer	BALB/c	Female	Splenocyte	Not reported	Severe thyroiditis, not reproducible	First model by cellular immunization	[39]
Genetic immunization	Full-length hTSHR[a] cDNA injection	NMRI	Both sexes	Plasmid	8 weeks	Low incidence, not reproducible	First model by genetic immunization	[41]
Genetic immunization	hTSHRA[b] plasmid injection, electroporation	BALB/c	Female	Plasmid	9–12 weeks	Unstable thyroid function	Widely used and convincing	[49–53, 74–75]
Genetic immunization	hTSHRA[b] adenovirus injection	BALB/c	Female	Adenovirus	30 weeks	Time-consuming	Widely used and convincing	[58, 60–63]

hTSHR[a]: Human thyroid stimulating hormone receptor; hTSHRA[b]: Human TSHR A-subunit.

the second autoantigen of GO, at least by this method, the plasmid encoding the IGF1R A subunit was unable to induce GO.

Immunization with adenovirus encoding the hTSHR A subunit

Genetic vaccination with hTSHR A subunit adenovirus (Ad-TSHRA) in female BALB/c mice is the most commonly used animal model of GD, which has high morbidity and repeatability [54]. However, no orbital lesions were found in experiments using a short-term regimen of 3 immunizations at 3-week intervals, or these experiments did not investigate ocular manifestations [43, 47, 55–57].

Holthoff et al. [58] used a long-term regimen of 3 initial immunizations at 3-week intervals and 6 maintenance immunizations at 4-week intervals to immunize female BALB/c mice. The mice were kept under a long-term, stable hyperthyroid condition. Cardiac changes, including cardiac hypertrophy, tachycardia, and increased ventricular volume, were observed in all mice. Thyroid hormones affect the cardiovascular system directly or indirectly, causing hyperthyroid heart disease [59]. This model is likely useful in research concerning its mechanism and treatment. Unfortunately, in this experiment, the researchers did not investigate orbital lesions.

The researchers subsequently immunized mice using the same method and observed orbital changes in the mice. Their team observed orbital fibrosis in mice in two follow up studies [60, 61] and a significant accumulation of acid mucin and collagen in orbital tissue in another study [62]. These ocular changes, which are similar to those seen in patients, suggest that long-term adenovirus injection can successfully establish a GO model in mice. However, the modeling procedure is quite long, requiring 30 weeks.

We also use this strategy to induce GO. Pathological alterations of retrobulbar adipogenesis and lymphocyte infiltration were observed in 70 % of the mice, and clinical signs of proptosis, conjunctival redness, and eyelid thickness were observed in some mice for the first time [63]. Based on these results, we used this model to investigate GO time related and T cell related mechanisms for the first time. The model replicated features that are observed in GO patients' peripheral blood lymphocytes, such as the Th1 dominance in Th1/Th2 balance and the decrease in the number of Treg cells [63].

Moreover, a group of CD4 + cytotoxic T cells with inflammatory, chemotactic, and toxic functions developed in the orbital tissue and thyroid of mice, which was consistent with the upregulated activities of related functional pathways in splenocytes [64]. Furthermore, we discovered that GD and GO appeared at week 11 and week 23 after modelling, respectively, by observing the animals at different time points during the modelling process. At week 11, splenocytes from GD mice without GO exhibited a trend of upregulated levels of GO specific inflammatory, chemotaxis, and toxicity genes. This finding indicates that the model reproduces the time window from the onset of GD to the onset of GO, and this interval may be the 'latency period' of GO, in which GO specific T cell immunological abnormalities have arisen but GO has not yet occurred. Interventions during this period may interrupt the disease progression of GO, thereby preventing its occurrence (M. Zhang, Z. Y. Chen, B. Y. Shi,Y. Wang, unpublished observations).

Exploration of pathogenesis and treatment based on GO animal models

Gut microbiota influence the disease presentation of GO

Alterations in the gut microbiota composition have been connected to a range of diseases, especially autoimmune diseases, and have been shown to impact disease presentations in animal models [65].

As previously described, mice immunized by plasmid at different experimental sites developed different thyroid function states [49–51]. Subsequently, the investigators immunized two different strains of mice with the same modelling method, but the incidence of GD and GO and the functional status of spleen T cells were inconsistent [66]. Through 16 S rRNA gene sequencing, the investigators found significant differences in the diversity and composi-

▶ **Table 2** Comparison of thyroid and eye changes in animal models.

Method	Immunization procedure	Types of TRAb[a]	Manifestations of thyroid abnormalities	Incidence of thyroid dysfunction	Manifestations of orbitopathy	Incidence of orbitopathy	Ref
Pituitary extract injection	Pituitary extract injection and thyroidectomy	Not reported	Body weight loss, thyroid hyperplasia	Not reported	Proptosis, oedema, mucopolysaccharide increase, lymphocyte infiltration, retrobulbar tissue weight increase	88 %	[38]
Cellular immunization	hTSHR[b]-activated splenocyte transfer	TBII[d]	Thyroiditis	Not reported	Oedema, muscle fiber dissociation, adipose tissue accumulation, lymphocyte and mast cell infiltration	68 %	[39]
Genetic immunization	Full-length hTSHR[b] cDNA injection	TSAb[e], TSBAb[f]	Hyperthyroidism, goiter	Hyperthyroidism: 17 % (f), 3 % (m)	Oedema, amorphous material accumulation, mast cell and macrophage infiltration	17 % (f), 3 % (m)	[41]
Genetic immunization	hTSHRA[c] plasmid injection, electroporation	TBII[d], TSAb[e], TSBAb[f]	Hyperthyroidism, hypothyroidism, normal thyroid function, thyroid hyperplasia	Hyperthyroidism: up to 86 % Hypothyroidism: up to 86 %	Fibrosis, muscle fiber atrophy, adipose tissue accumulation, collagen and glycosaminoglycan deposition, T cell and macrophage infiltration, Treg decrease	Up to 100 %	[49–53, 74–75]
Genetic immunization	hTSHRA[c] adenovirus injection	TBII[d], TSAb[e], TSBAb[f]	Hyperthyroidism, thyroid size increase, thyroid hyperplasia	Hyperthyroidism: up to 89 %	Proptosis, conjunctival redness, eyelid thickness, fibrosis, adipogenesis, acid mucin and collagen accumulation, T cell infiltration	Up to 70 %	[58, 60–63]

TRAb[a]: TSHR antibody; hTSHR[b]: Human thyroid stimulating hormone receptor; hTSHRA[c]: TSHR A-subunit; TBII[d]: TSHR binding inhibitory immunoglobulin; TSAb[e]: TSHR stimulating antibody; TSBAb[f]: TSHR blocking antibody; (f): Female; (m) Male.

tion of the gut microbiota in mice assessed at different sites or between different mouse strains [52, 66].

To verify causality, they altered the gut microbial composition of mice by chronically providing vancomycin, probiotics, or human fecal material transfer (hFMT) [53]. Vancomycin decreased the richness and diversity of the gut microbiota and the incidence and severity of GD and GO. hFMT and probiotics enhanced the severity of GD and GO.

In addition, multiple cross-sectional studies have demonstrated altered gut microbiota diversity and composition in patients with GD or GO [67–69]. These results imply that changes in the gut microbiota affect the occurrence and disease manifestations of GD and GO. The exact mechanism is unclear, but an imbalance of Treg and Th17 cells due to dysbiosis in the gut microbiota is a possible cause [70].

Sex related risk factors determine the female bias in GO

Previous studies have shown that GO often occurs in young women but is more severe in men and older patients [71, 72]. Schluter et al. [73] generated a model by immunizing male and female BALB/c mice with a hTSHR A subunit encoding plasmid and controlled for additional risk factors (such as an advanced age, genetic variation, or smoking status). They compared the features of GO in different sexes and found that the incidence and severity of GO were comparable in both sexes, although the pathogenesis of GO exhibited differences between the sexes. The researchers believed that sex related endogenous and/or exogenous risk factors may be key determinants of the female bias observed in GO, rather than sex itself.

In contrast, Costagliola [41] immunized NMRI mice with cDNA encoding full length hTSHR and showed a differential incidence of GD and GO. Most previous experiments used only female mice for modeling, and there were few relevant results. Experiments that replicate this experiment or that are based on other germlines or modeling approaches are needed to validate this result and to further explore the associated factors.

Macrophages and CD8 + T cells increase during the early stage of GO

Philipp [74] performed orbital tissue immune cell analysis in a mouse model established by electroporation with a plasmid encoding the hTSHR A subunit. The results showed macrophage infiltration, high levels of CD8 + T cell proliferation, and downregulated levels of effector CD4 + T cells and Tregs during the early stage

of the disease. As the disease progresses, the level of orbital brown adipose tissue increases significantly. Previous studies have shown that the immune cells infiltrating the orbit are mainly CD4 + T cells, but in this experiment, macrophages and CD8 + T cells were increased in the early stages of the disease. Macrophages and CD8 + T cells may play an important role in the early pathogenic process of GO, and further studies investigating their functions could help elucidate the onset pathogenesis of GO and identify new treatment options for the condition.

Potential treatment option: TSHR derived peptides

Based on the advantages of antigen specific immunotherapy over nonspecific immunosuppressants and recombinant autoantigens over autoantigen extracts, scientists created TSHR derived peptides. These peptides simulate the cylindrical loops of the TSHR leucine rich repeat domain (LRRD), which contains key amino acid residues for an interaction with TRAb and re-establish tolerance towards TSHR [60–62].

Ad-TSHRA immunized mice were injected intravenously with high doses of peptides. Cyclic peptide 836, the shortened 13-meric cyclic version of cyclic peptide 836, and linear peptide 12 effectively improved GO, as evidenced by a continuous reduction in retroorbital fibrosis [60, 61]. Cyclic peptide 19 significantly reduced the amount of acidic mucin and collagen [62]. However, none of the peptide treated mice reported an improvement in adipogenesis. Cyclic peptide 836 and cyclic peptide 19 reduced thyroid hyperplasia, tachycardia, and cardiac hypertrophy while normalizing the T4 levels [60–62]. The authors also demonstrated that the administration of the peptide did not induce any immune response in immunologically naive mice. Although the actions of these peptides are not identical and the underlying therapeutic mechanisms are not fully understood, TSHR derived peptides are promising new therapeutic options for GO and GD.

Potential treatment option: fingolimod

Since sphingosine-1-phosphate (S1P) signaling is involved in orbital tissue inflammation and remodeling in GO, Plohn [75] used fingolimod, an S1P receptor antagonist and T cell circulation regulator, in a mouse model in which mice were immunized with hTSHR A subunit encoding plasmid to study its efficacy. It was administered orally as a prophylactic during disease onset or therapeutically after disease onset. Prophylactic administration of fingolimod was found to protect animals from hyperthyroidism and orbitopathy, while therapeutic administration limited disease severity. The appearance of GO was improved by reductions in T cell infiltration and adipogenesis.

Furthermore, fingolimod not only relieves hyperthyroidism but also normalizes thyroid dysfunction, which is manifested as T4 levels, heart rate, body temperature, and other variables returning to normal levels. This result is mainly due to the fact that fingolimod reduces the elevated T cell and TSAb levels and increases the levels of Tregs. However, the effect of fingolimod on orbital hyaluronan deposition is difficult to determine due to the low incidence of hyaluronan deposition observed in this experiment, and the experiment did not assess its effect on retroorbital fibrosis.

Potential treatment option: rapamycin

Based on the time window from GD onset to GO onset as observed in the established GO animal model, we investigated methods for the prevention and treatment of GO. The activation of the mTORC1 pathway was found to be upregulated in patients with GO according to a deep analysis of public sequencing data, so we added rapamycin, a mTORC1 inhibitor, to the diet of mice immunized with Ad-TSHRA at week 11. Rapamycin was found to effectively reduce the incidence of GO and improve its pathological manifestations. The frequency of GO was reduced from 70 % to 30 %, and improvements were observed in retrobulbar fibrosis, fat deposition, and lymphocyte infiltration. The thyroid function status also improved, which was mainly manifested in the serum TT4 levels and thyroid pathological manifestations.

Further mechanistic investigations have revealed that rapamycin can significantly ameliorate the imbalance of Th1/Th2 and Th17/Treg cells and dramatically decrease the deposition of CD4 + cytotoxic T cells and the upregulated activation of the corresponding functional pathways in splenocytes. This result suggests that rapamycin could be used not only as an etiological treatment for GO but also as potential prevention for patients with hyperthyroidism who are at risk of developing GO. Of course, more clinical research is needed to confirm these findings [64].

Conclusions

Scientists have established progressive GO models based on the optimization of original models. Intramuscular injection of the hTSHR A subunit encoding plasmid combined with electroporation and intramuscular injection of Ad-TSHRA are the most widely used modeling methods for GO in animals. The disease models established by these two methods have high morbidity and excellent reproducibility, and the disease manifestations are highly similar to those of human diseases.

However, they both have certain drawbacks. It takes too long to immunize animals with Ad-TSHRA. The thyroid function status of mice immunized with the hTSHR A subunit encoding plasmid is highly variable. Therefore, improvement in models of GO should be continued to solve these problems.

These progressive animal models of GO provide valuable tools for understanding the pathogenic mechanisms underlying GO and evaluating new therapies targeting different pathogenic mechanisms. More research is needed to reveal the mechanism through which the gut microbiota affects thyroid function and orbital autoimmunity, the pathogenic role of macrophages and CD8 + T cells during the early stage of GO, and the specific reasons for the female bias in GO. Additionally, these newly discovered drugs could be important strategies for the management of GD/GO, and well-designed clinical trials are needed to confirm this conclusion. With the increasing use of these animal models and more in-depth study of the new findings, scientists will gain a clearer understanding of the pathogenesis of GO and identify more treatments for patients.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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