Review Article

Mouse Models of Graves' Disease

Yuji Nagayama

Department of Medical Gene Technology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Graves’ disease is characterized by overstimulation of the thyroid gland with agonistic autoantibodies against the thyrotropin (TSH) receptor, leading to hyperthyroidism and diffuse hyperplasia of the thyroid gland. Our and other laboratories have recently established several animal models of Graves’ hyperthyroidism with novel immunization approaches, i.e., in vivo expression of the TSH receptor by injection of syngeneic living cells co-expressing the TSH receptor and major histocompatibility complex (MHC) class II or genetic immunization using plasmid or adenovirus vector coding the TSH receptor. This breakthrough has provided important insights into our understanding of the pathogenesis of Graves’ disease. New findings obtained include that (i) professional antigen-presenting dendritic cells appear to be crucial for disease initiation, (ii) the free A subunit of the receptor is likely the main autoimmune for stimulating antibodies, (iii) non-MHC genes as well as MHC genes albeit less significant may be linked to disease susceptibility, (vi) certain infectious pathogens may have a negative impact on disease development, and (v) Graves’ disease is not simplistically a T helper type 2 (Th2)-dominant autoimmune disease as previously considered; indeed TSH receptor-specific Th1 immune response appears to be critical for disease pathogenesis. Further studies with these models will hopefully lead to not only better understanding of the pathogenesis of Graves’ disease but also the development of new approaches for treatment and ultimately prevention of Graves’ disease in the future.

Keywords: Graves’ disease; Thyrotropin receptor; Autoimmunity; Adenovirus; Cytokine

Introduction

Autoimmune thyroid diseases consist of two clinically opposite diseases: Graves’ disease and chronic thyroiditis (also known as Hashimoto thyroiditis). Graves’ disease is well known to be caused by autoantibodies against the thyrotropin (thyroid stimulating hormone, TSH) receptor, called thyroid stimulating antibodies (TSAb), which mimic the agonistic action of its ligand TSH and overstimulate the thyroid gland, resulting in hyperthyroidism and diffuse hyperplasia of the thyroid gland. Occasionally, anti-TSH receptor autoantibodies that block TSH action, thereby called thyroid blocking antibodies (TBAbs), also appear and induce hypothryoidism and thyroid atrophy. Thus it has long been thought that humoral autoimmune response, i.e., T helper cell type 2 (Th2) autoimmune response, is likely involved in disease pathogenesis. In contrast, Hashimoto thyroiditis involves cellular autoimmune response (Th1 autoimmune response) against thyroid specific autoantigens such as thyroglobulin and thyroid peroxidase, or activation of cytokine-regulated apoptotic pathways, leading to thyroid destruction and hypothyroidism.

Although both spontaneous and thyroglobulin- or thyroid peroxidase-inducible animal models of experimental autoimmune thyroiditis, models of Hashimoto thyroiditis in humans, have long been available, no animal model of Graves’ hyperthyroidism has existed until Shimojo et al. established the first inducible mouse model in 1996. Subsequently other three groups including us have generated different mouse models using distinct approaches. I here review and discuss these mouse models of Graves’ hyperthyroidism and the recent data obtained with these models.

Comparison of different models

The first mouse model of Graves’ hyperthyroidism reported by Shimojo et al. as mentioned above involved multiple injections of a fibroblast cell line (L cells) genetically engineered to stably co-express the TSH receptor and major histocompatibility complex (MHC) class II antigen into syngeneic AKR/N mice (H-2k) and other H-2k mouse strains. These cells likely function as non-professional antigen presenting cells because they also express a costimulatory molecule B7-1. However, although reproducibility of this model

Address correspondence: Yuji Nagayama, M.D., Department of Medical Gene Technology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki, 852-8523 JAPAN
TEL: +81-(0)95-849-7173, FAX: +81-(0)95-849-7175, E-mail: nagayama@net.nagasaki-u.ac.jp
was confirmed by other groups.\textsuperscript{11,12} The disease incidence was constantly low (ca. 20%). Intra-thyroidal lymphocytic infiltration was not observed. This model is also not suitable for detailed in vitro studies with splenocytes or sera because of non-specific adjuvant activity of this cell line; for example, injection of untransfected L cells itself induced a robust, antigen-nonspecific IFN-\gamma production form splenocytes.\textsuperscript{10} Of interest, L cells expressing the TSH receptor alone (without MHC class II expression) failed to induce disease.\textsuperscript{7}

The subsequent model reported by Prabhakar et al.\textsuperscript{9} used a similar approach, i.e., repetitive injections of a B lymphoma cell line (M12) permanently expressing the TSH receptor into syngeneic BALB/c mice. These cells, as professional antigen presenting cells, indeed express endogenous MHC class II and a costimulatory molecule B7-1. Surprisingly, disease reportedly developed virtually in all the mice.\textsuperscript{9} The reason for the greater efficacy of this model versus Shimojo model is at present unknown. Of interest, they also reported that injection of xenogeneic human embryonal kidney 293 cells stably expressing the extracellular domain of the TSH receptor alone or in combination with soluble TSH receptor extracellular domain protein and a Th2 adjuvant cholera toxin B (CTB) as well as immunization with TSH receptor extracellular domain protein and CTB was equally effective at inducing disease.\textsuperscript{9} However, histological findings in these models are not consistent with those of Graves' disease in humans ("thinning of the thyroid epithelium" versus "cuboidal thyroid epithelium"). Another negative aspect of this model is that it takes more than six months to induce Grave's disease, compared to about 2-3 months required for disease induction in other models. Furthermore, of most important, we could not replicate their data (our unpublished data).

The third model is DNA vaccination, i.e., intramuscular injections of the eukaryotic expression vector for the TSH receptor, reported by Vassart et al. In the original reports,\textsuperscript{13,14} this approach was only effective for outbred NMRI mice with low disease induction rate (ca. 15%), but not for inbred BALB/c mice, which are likely one of susceptible mouse strains to Graves' disease (refs. 8 and 15, also see below for a detail). Of interest, thyroid histology showed some intra-thyroidal lymphocytic infiltration in both NMRI and BALB/c mice. However, this model could not be reproduced by other groups including us.\textsuperscript{8,13,14} Later, TSH receptor-DNA vaccination was shown to induce Graves' hyperthyroidism in about 30% of HLA-DR3 transgenic mice on the non-obese diabetes (NOD) background.\textsuperscript{17}

The forth and fifth models were established by our group.\textsuperscript{15,16} The first one involved repeated intramuscular injections of recombinant, replication-defective adenovirus vector coding the full-length TSH receptor (AdTSHR).\textsuperscript{14} This model is reproducible in different groups using mice or hamsters\textsuperscript{18-20} and highly efficient with about 100% anti-TSH receptor antibody induction and about 50-60% disease incidence in BALB/c mice. Importantly, as determined using the chimeric TSH-lutropin receptors, TSAsbs in hyperthyroid Graves' sera recognized epitope(s) similar to those of TSAsbs in human Graves' sera. Another model used bone marrow-derived dendritic cells transduced with AdTSHR with the disease incidence of about 35%.\textsuperscript{15} Since dendritic cells are the most potent antigen presenting cells and are a prerequisite for the initiation of primary immune responses,\textsuperscript{25} this model may be physiologically highly relevant. These two models were later modified and optimized further by using adenovirus expressing the TSH receptor A subunit [AdTSHR289; truncated receptor corresponding to approximately two thirds of the N-terminal receptor ectodomain (289 amino acids)] instead of the full-length receptor (ref. 19 and our unpublished data; see below for a more detail). Thus disease incidence is now about 60-80% in BALB/c mice in both of our modified models. However, intra-thyroidal lymphocytic infiltration was not observed in these two models.

Taken together, our models, particularly intramuscular injection of AdTSHR289, appears to be the best Graves' model in terms of reproducibility, high disease incidence and the applicability to any mouse strain, despite lack of intra-thyroidal lymphocyte infiltrates.\textsuperscript{15,16}

What we can learn from establishment of these models?

Generations of these mouse models have provided some important implications for the pathogenesis of Graves' disease in humans. First, Shimojo model fits very well the hypothesis proposed by Bottazzo et al. more than two decades ago,\textsuperscript{1,2} i.e., thyroid cells in autoimmune thyroid diseases aberrantly express MHC class II antigen and, as non-professional antigen presenting cells, present thyroid-specific autoantigens to naïve T cells, leading to thyroid autoimmune reaction. However, thyroid cells reportedly do not express costimulatory molecules B7,\textsuperscript{22} suggesting induction of tolerance rather than immunity.\textsuperscript{25} More recently, thyroid-specific MHC class II transgenic mice are shown not to develop autoimmune thyroid disease spontaneously.\textsuperscript{24,25} Thus professional antigen presenting cells, i.e., dendritic cells, are most likely involved in the initiation of thyroid autoimmunity,\textsuperscript{24} as we have shown in our second model.\textsuperscript{17}

Second, all, but one,\textsuperscript{8,15} successful mouse models of Graves' hyperthyroidism utilized in vivo expression of the TSH receptor. In contrast, one report shows a failure of Graves' disease induction by conventional immunization with conformationally intact full-length TSH receptor protein and a strong adjuvant (complete Freund adjuvant).\textsuperscript{27} These discrepant data can possibly be best explained by the recent novel finding. It is now widely accepted that the TSH receptor is cleaved into two subunits, a TSH binding "A" subunit and a transmembrane/ cytoplasmic "B" subunit, on the cell surface, from which free A subunit is spontaneously released by shedding (ref. 1; Figure 1). Rapoport et al.\textsuperscript{28} clearly show that TSAbs in sera from Graves' patients preferentially recognize the free A subunit rather than the two-subunit receptor or the full-length single polypeptide receptor, indicating that the free A subunit may be the better autoantigen than other two forms of the receptor. In successful mouse models mentioned above, the free A subunit very likely sheds from the full-length receptor expressed in vivo. Contrary, it is unlikely that the full-length receptor used for the conventional immunization undergoes cleavage. This is why our models could be optimized with adenovirus expressing the TSH receptor A subunit (see above).
Th1 versus Th2 immune balance in development of Graves' hyperthyroidism

Graves' disease has long been thought to be a Th2-dominant autoimmune disease, because autoantibodies, not cytotoxic lymphocytes, are responsible for disease pathogenesis. In our model using intramuscular injection of AdTSHR, however, IgG subclasses of anti-TSH receptor antibodies induced were both of IgG1 and IgG2a, Th2 and Th1 subclasses, respectively, and splenocytes from AdTSHR-immunized mice produced a Th1 type cytokine interferon-γ (IFN-γ) and a Th2 type cytokine interleukin-10 (IL-10) in response to in vitro stimulation with TSH receptor antigen, indicating mixed Th1 and Th2 immune responses of TSH receptor-specific immune response. Furthermore, of surprising, co-injection of adenovirus expressing IL-4 deviated TSH receptor-specific immune response away from a Th1 phenotype, as demonstrated by increased IgG1 to IgG2a (Th2 to Th1) ratios and impaired secretion of IFN-γ from splenocytes of immunized mice, and almost completely suppressed disease induction. Thus TSH receptor-specific Th1 immune response appears to play an essential role in disease induction. The similar results were obtained by using prior infection with *Schistosoma mansoni* and simultaneous administration of α-galactosylceramide, a Th2-inducing infectious agent and a Th2-inducing glycolipid derived from marine sponge, respectively. It should be, however, noted here that these suppressive effects of adenovirus expressing IL-4, *Schistosoma mansoni* and α-galactosylceramide are all preventive, but not therapeutic, i.e., once anti-TSH receptor immune response is fully induced, Th2-immune deviation has no or if any little effect on disease development.

Consistent with the above-mentioned data for the importance of a Th1 response in AdTSHR-induced Graves' disease, adenovirus expressing IL-12 enhanced a Th1 immune response (enhancement of IFN-γ secretion from splenocytes) without changing disease incidence. The optimal Th1 immune response would have been already induced by AdTSHR immunization. However, unexpectedly, prior infection of *Mycobacterium bovis* BCG significantly prevented mice from AdTSHR induction of hyperthyroidism. In this study, anti-TSH receptor response was biased to a Th1 phenotype as shown by augmented IFN-γ secretion and impaired IL-10 secretion from splenocytes. Considering the effect of IL-12, this protective effect of *Mycobacterium bovis* BCG infection cannot solely be explained by Th1 immune deviation. The possible explanation of these unexpected data is discussed below.

The similar results were also obtained with our second model with dendritic cells transduced with AdTSHR. Thus Th2 adjuvants alum and pertussis toxin completely suppressed not only Graves' hyperthyroidism but also antibody production, whereas a Th1 adjuvant polyribosinonic polyribocytidylid acid augmented Th1 immune response (enhanced splenocyte production of IFN-γ) without affecting disease incidence.

Finally, studies with knockout mice provided somewhat complicated results. BALB/c mice deficient in IFN-γ or IL-4 by gene disruption are both resistant to AdTSHR induced hyperthyroidism. These data support the importance of a Th1 cytokine IFN-γ but are apparently inconsistent with the data obtained with IL-4 expressing adenovirus. However, these contradictory data may be explained by impairment of both Th1 and Th2 immune responses in IL-4 null mice, as demonstrated by decreased IgG1 to IgG2a ratios and loss of TSH receptor-specific IFN-γ production from splenocytes.

Altogether, Graves' hyperthyroidism is not simplistically a Th2-dominant autoimmune disease as previously considered. Our data clearly show that TSH receptor-specific Th1 immune response
appears critical for the pathogenesis of disease.

Genetic factors in Graves’ disease

The etiology of Graves’ disease in humans is thought to be multifactorial, i.e., both genetic and environmental factors appear to be involved in disease development. In genetic factors, associations have been observed between Graves’ disease and MHC genes [human leukocyte antigen (HLA) in humans] as well as non-MHC genes. As mentioned above, one of the advantages in our models is that they can be applied to any mouse strains, which prompted us to study the contribution of genetic factors on disease development using different mouse inbred strains. Our studies with intramuscular injection of AdTSHR clearly show that BALB/c and BALB.K mice which share the same non-MHC genes but have the different MHC genes (H-2d versus H-2k; MHC congenics) are both susceptible to Graves’ disease. In contrast, other mouse strains, for example DBA/2J and CBA/J mice with MHC haplotypes identical to those in BALB/c and BALB.K mice, respectively, are resistant strains. These data implicate non-MHC genes, rather than MHC genes, in disease susceptibility. A recent study show that F1 hybrids between BALB/c and C57BL/6 mice are susceptible to AdTSHR289-induced Graves’ hyperthyroidism, indicating the dominant role played by susceptibility genes rather than resistant genes in disease development. Furthermore, resistant strains can be divided into two subgroups, good and poor responders, in terms of non-stimulatory anti-TSH receptor antibody production. The former includes C57BL/6, SJL/J and DBA/2J mice, and the latter CBA/L and DBA/1J mice. Thus, there are at least two different types of genetic factors; one groups influencing production of thyroid stimulating antibodies, and another non-stimulatory antibody production. In addition, as mentioned above, recent studies show that DR3-transgenic NOD mice are more prone to develop Graves’ hyperthyroidism by AdTSHR than non-transgenic NOD mice, also suggesting involvement of MHC genes in disease occurrence. Of interest, the genetic susceptibility of thyroglobulin- or thyroid peroxidase-induced thyroiditis depends on certain MHC haplotypes.

Environmental factors in Graves’ disease

Environmental factors possibly contributing disease development include iodine, smoking, stressful life events, and infectious agents in humans. To clarify the effects of environmental microorganisms on disease induction, we first compared the responses to AdTSHR immunization in BALB/c mice housed conventionally versus in a specific pathogen-free condition. The disease incidence was not significantly different between two groups. Administration of bacterial and yeast adjuvants (LPS and zymosan A) also had little effect. However, as mentioned earlier, Schistosoma mansoni and Mycobacterium bovis BCG infections showed negative impact on disease development, indicating that some particular infectious pathogens may play a role in the etiology of Graves’ disease. These data cannot be explained by altered Th1 versus Th2 immune response. Instead, these results fit the “hygiene hypothesis” or “counter regulatory model.” These concepts proposes that reduced exposure to certain microorganisms, irrespective of their ability to induce a Th1 or a Th2-biased immune response, during childhood in developed countries impairs the development of an appropriately educated immune system, causing increased rates in development of not only Th1-type autoimmune diseases but also Th2-type allergic diseases in adults. Indeed the incidence of these diseases in humans is increasing in developed countries in general and Graves’ disease is relatively uncommon in developing countries. Overall, the development of Graves’ hyperthyroidism may be affected by certain infectious pathogens regardless of their ability to modify Th1 versus Th2 balance.

Iodide was used to elucidate the association between Graves’ disease and intrathyroidal lymphocytic infiltration that is frequently observed in Graves’ thyroid glands in humans. In this study, NOD, H-2b4 mice, which spontaneously develop autoimmune thyroiditis on high-iodide diet were immunized with AdTSHR289. However, AdTSHR immunization affected neither the degree of intrathyroidal lymphocytic infiltration nor anti-thyroglobulin antibody titers, and iodide administration did not influence anti-TSH receptor antibody titers. Although iodide did somewhat suppress the incidence of hyperthyroidism, this effect was considered non-immune mechanism. Thus the TSH receptor may not be the target to induce thyroiditis at least in this mouse strain. However, we have recently found that depletion of naturally occurring regulatory CD4+CD25+ T lymphocytes by anti-CD25 antibody renders resistant C57BL/6 mice susceptible to Graves’ hyperthyroidism to some extent, and the thyroid glands from hyperthyroid C57BL/6 mice show extensive lymphocytic infiltration (our unpublished data), indicating anti-TSH receptor autoimmunity can recruit lymphocytes into the thyroid glands in C57BL/6 genetic background. Further studies will be necessary to clarify the relationship of anti-TSH receptor autoimmunity and thyroiditis.

Conclusion

Significant progress in our understanding of the pathogenesis of Graves’ disease has been achieved following development of novel mouse models. Although we are still far away from the ultimate goal of clarifying the exact mechanisms for the initiation of anti-TSH receptor autoimmune response in Graves’ disease, further studies with these models will hopefully lead to the development of new approaches for treatment and ultimately prevention of Graves’ disease in the future.

References

Yuji Nagayama: Mouse Models of Graves' Disease

5. Shimoo N, Kohno Y, Yamaguchi K et al. Induction of Graves-like disease in mice by immunization with fibroblasts transfected with the thyrotropin receptor and a class II molecule. Proc Natl Acad Sci USA 93: 11074-11079, 1996