Current Concepts in the Molecular Pathogenesis of Thyroid-Associated Ophthalmopathy

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Graves' disease (GD) is a common autoimmune condition. At its core, stimulatory autoantibodies are directed at the thyroid-stimulating hormone receptor (TSHR), resulting in dysregulated thyroid gland activity and growth. Closely associated with GD is the ocular condition known as thyroid-associated ophthalmopathy (TAO). The pathogenesis of TAO remains enigmatic as do the connections between the thyroid and orbit. This review highlights the putative molecular mechanisms involved in TAO and suggests how these insights provide future directions for identifying therapeutic targets. Genetic, epigenetic, and environmental factors have been suggested as contributory to the development of GD and TAO. Thyroid-stimulating hormone receptor and insulin-like growth factor receptor (IGF-1R) are expressed at higher levels in the orbital connective tissue from individuals with TAO than in healthy tissues. Together, they form a functional complex and appear to promote signaling relevant to GD and TAO. Orbital fibroblasts display an array of cell surface receptors and generate a host of inflammatory molecules that may participate in T and B cell infiltration. Recently, a population of orbital fibroblasts has been putatively traced to bone marrow–derived progenitor cells, known as fibrocytes, as they express CD45, CD34, CXCR4, collagen I, functional TSHR, and thyroglobulin (Tg). Fibrocytes become more numerous in GD and we believe traffic to the orbit in TAO. Numerous attempts at developing complete animal models of GD have been largely unsuccessful, because they lack fidelity with the ocular manifestations seen in TAO. Better understanding of the pathogenesis of TAO and development of improved animal models should greatly accelerate the identification of medical therapy for this vexing medical problem.

Keywords: autoimmune, Graves' disease, inflammation

Graves' disease (GD) is a common autoimmune condition associated with dysregulated thyroid gland activity and growth.[1] Underlying glandular overactivity are stimulatory autoantibodies directed against the thyrotropin receptor (TSHR), known as thyroid-stimulating immunoglobulins (TSI).[1] Their actions result in the overproduction of thyroid hormones, which in turn causes numerous metabolic abnormalities in peripheral tissues.[1] Closely associated with GD is the manifestation localized in orbital connective tissues, known as thyroid-associated ophthalmopathy (TAO, also known as Graves' orbitopathy) that occurs in 25% to 50% of those with GD.[2,3] The clinical features of TAO include periorbital edema, eyelid retraction, proptosis, strabismus, exposure keratopathy, and compressive neuropathy (Figs. 1, 2). They are disfiguring and also may threaten sight.[4] The clinical course of TAO usually involves a self-limited active phase characterized by inflammation and tissue remodeling.[4] This process typically lasts between 18 and 36 months, and is followed by the stable phase. The exact pathogenesis of TAO appears to involve complex molecular and cellular processes that have yet to be understood fully.

The connection between the orbit and the pathologic events occurring within the thyroid in GD remains enigmatic, as does any relationship existing between thyroid hormone excess and TAO. The first signs of ocular involvement can precede, accompany, or follow development of thyroid dysfunction. The concept that orbital disease is not provoked directly by abnormal thyroid hormone levels associated with GD is now widely accepted. Rather, most experts believe that TAO occurs as the consequence of underlying autoimmune processes. Its complexity and diversity of presentation, combined with the absence of complete animal models, have delayed solving TAO. However, recent progress in identifying at least some of the pathologic elements involved in GD has begun to accelerate our understanding of the disease. This review attempts to crystallize these advances, and in so doing, identify weaknesses in the current concepts, and provide a roadmap for future studies that should facilitate basic discovery and translate to the clinic.

GENETIC, EPIGENETIC, AND ENVIRONMENTAL RISK FACTORS FOR TAO

Genetic Predisposition

Genetic and environmental factors contribute to the pathogenesis of GD.[5] However, clear-cut differences between genetic variations associated with GD and those peculiar to the subset of individuals developing TAO have not yet been identified.[6] Similar to other autoimmune conditions, GD and TAO are more prevalent among females.[7] However, men with GD appear to
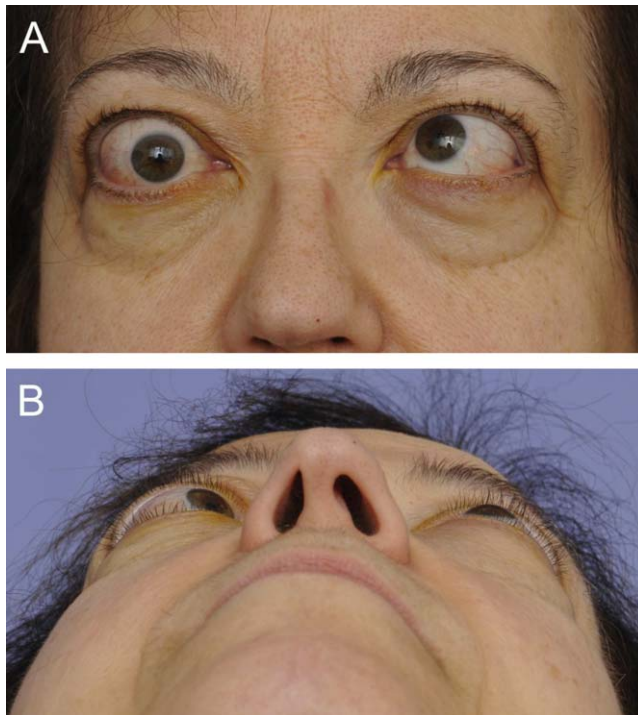
FIGURE 1. A 61-year-old woman with TAO. (A) Frontal view demonstrating bilateral upper lid retraction (right greater than left) and bilateral lower lid retraction with inferior scleral show. Bilateral proptosis, lateral flare, chemosis, injection, caruncular edema, and significant left esotropia also are evident. (B) Worms-eye view highlighting proptosis (right greater than left).
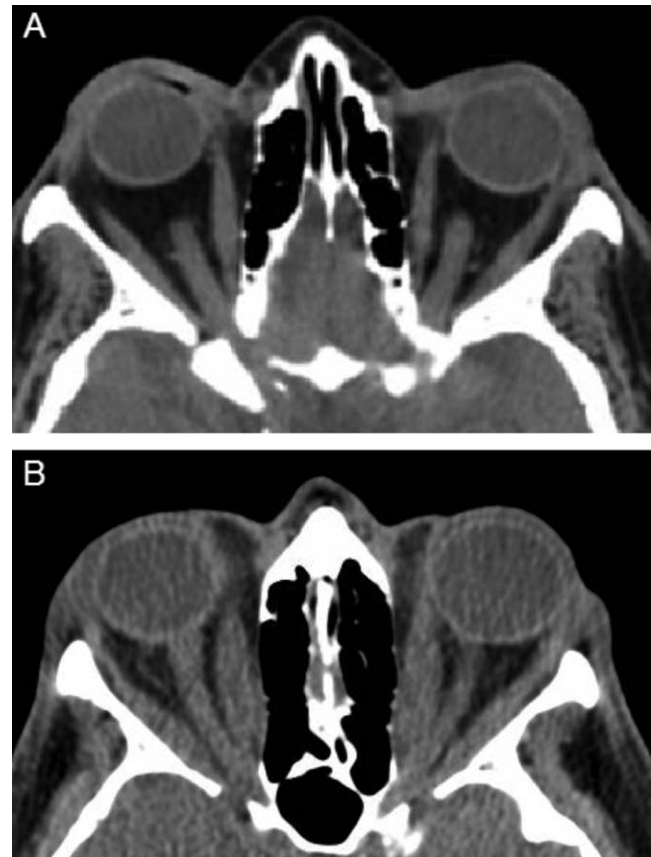

FIGURE 2. Orbital CT without contrast. (A) Uninvolved orbits. (B) Patient with TAO demonstrating bilateral exophthalmos. Bilateral marked enlargement of the extraocular muscles, especially the medial, superior, and inferior rectus muscles, causing apical crowding of the optic nerve. Stranding can be observed around both optic nerves.

be at greater risk of developing severe TAO.[8,9] Prevalence of TAO also diverges with respect to ethnicity. For instance, Asians are less likely to suffer TAO than are their European counterparts.[10] Increased incidence of GD among family members also indicates that genetic factors have a major role in susceptibility.[5,11] A recent study investigated the prevalence of ocular and eyelid signs in first and second-degree relatives from a single family harboring multiples cases of GD, TAO, and Hashimoto's thyroiditis.[12] The investigators reported that 33% of the euthyroid relatives had signs of TAO, such as upper lid retraction. These findings favor a genetic contribution to the development of TAO.[12]

Studies examining twins with GD were conducted by interrogating the Danish twin registry.[5,13] These demonstrated concordance rates as high as 30% for GD in monozygotic compared to 3% in dizygotic twins.[5,13] They indicated that approximately 79% of the risk for developing GD is attributable to genetics, while the remaining 21% derives from environmental factors.[13] In addition, several reports have appeared identifying multiple susceptibility genes associated with GD. Among these polymorphisms are variations in genes regulating immune function, such as HLA-DR3,[14,15] CTLA4,[16] PTPN22,[17] CD40,[18] IL-2RA,[19] FCRL3,[20] and IL-23R.[21] Others encode thyroid-specific proteins, such as TSHR[22] and thyroglobulin (Tg).[23]

Identification of novel single-nucleotide polymorphisms (SNPs) in disease susceptibility genes further contributes to our understanding of the genetic basis underlying GD. The Interleukin-21 and IL-21R polymorphisms have been associated with autoimmune conditions, such as type 1 diabetes mellitus,[24] juvenile idiopathic arthritis,[25] psoriasis,[26] celiac disease,[27,28] ulcerative colitis,[29] and multiple sclerosis.[30] The SNPs within the *IL-21* gene and those located within intron 1

of *TSHR*, such as rs2284720, also have been associated with GD and TAO.[31-34] The SNP rs6479778, identified within the *ARID5B* gene at 10q locus,[32,35] and SNP rs12147587, located within the *NRXN3* gene at 14q locus,[32] represent variations within genes that regulate adiposity and might predispose to GD.[36,37]

Because the vast majority of individuals with TAO have underlying GD, it would not be surprising that the two processes share disease susceptibility genes. One recent study examined polymorphisms of *HLA*, *CTLA4*, *IL23R*, and *TSHR* in a cohort with TAO and found no genetic differences compared to patients with GD without ocular involvement.[38] Most studies have concluded that the gene polymorphisms thus far identified contribute little to overall disease susceptibility. None identified appears to convey sufficient risk for developing TAO to warrant prophylactic treatment in individuals with GD. The relative contributions of specific genetic and environmental factors for developing TAO remain to be quantified. Moreover, the susceptibility conferred appears complex and varies with ethnicity.

Epigenetics

Besides genetic factors, epigenetic determinants, such as heritable alternations in gene function, also may have a role in GD. These could contribute through alterations in DNA methylation, histone modifications, genomic imprinting, RNA interference, and X chromosome inactivation.[39] As with genetic factors, those that emanate from the epigenenome

and provide unequivocal causality have yet to be identified. Yin et al.[39] found upward skewing of X chromosome inactivation (≥80% inactivation of one X chromosome in the same tissue) in GD when compared to healthy individuals. Yet, the mechanisms through which this inactivation leads to increased risk for GD are not yet known.[39] Nonetheless, this phenomenon could ultimately explain the higher incidence of GD and TAO in women.[40]

A recent study has identified a Tg promoter nucleotide substitution (−1623 A/G SNP, rs180195) that may predispose to autoimmune thyroid disease.[41] This G allele and G/G haplotype are more frequent in affected individuals, and interact epigenetically with IFNα following viral infections.[41] Subsequently, interferon regulatory factor-1 (IRF-1) binds the Tg promoter at rs180195, resulting in enhanced mono-methylation of the Lys-4 residue of H3.[41] Treatment with IFNα of thyroid cells transfected with a fragment of the *Tg* gene promoter fused to a reporter increases its activity only in the construct harboring the variant. Thus, it is possible that IFNα promotes IRF-1 binding to the variant *Tg* promoter, thereby directly modulating expression of gene(s) underlying thyroid autoimmunity.

Environmental Factors

Environmental factors, such as infectious agents, have been implicated in the initiation of immune responses to self-antigens.[7] These might underlie the development of GD and TAO. Bacteria can induce inflammatory responses leading to aberrant expression of co-stimulatory molecules, including MHC class II. This process often results in presentation of self-antigens and the activation of antigen-specific T cells.[7] Alternatively, infections can alter the expression of host proteins so that they become misrecognized as foreign.[42] Molecular mimicry, resulting from primary sequence identity or conformational similarities to antigens, also could have a pathogenic role in the development of GD, as has been proposed in other autoimmune conditions.[43-45]

An early study reported that DNA from human foamy viruses (HFV), otherwise known as spuma viruses, had been detected in peripheral DNA from a majority of those with GD, but was undetectable in healthy controls.[46] Subsequent studies have failed to confirm these findings.[47,48] However, another report detected HFV proteins in diseased thyroid tissue.[49] It remains unclear whether HFV infection might be associated with GD. A follow-up study utilizing more modern techniques could resolve this open question.

Yersinia enterocolitica was investigated initially for its participation in GD more than 40 years ago.[50,51] The large proportion of individuals with GD in whom antibodies against *Y. enterocolitica* can be detected suggests that these bacteria might express proteins resembling those of the host.[52,53] This concept is based in part on identification of high affinity TSH and TSI binding sites on *Y. enterocolitica*.[54-56] Furthermore, mice immunized with *Y. enterocolitica* envelope proteins have been shown to develop anti-TSHR antibodies.[50] A recent study demonstrated the outer membrane porin F protein of *Y. enterocolitica* cross-reacts immunologically with the leucine-rich domain of TSHR.[57] Furthermore, early precursor B cells can expand when exposed to *Y. enterocolitica* porin proteins and undergo somatic hypermutation to acquire cross-immunogeneticity with TSHR.[58] Although development of autoimmunity following certain infections has been suspected for many years, further study will be necessary before this mechanism can be linked causally to GD and TAO.

Cigarette smoking has been associated consistently with development and worsening of GD and TAO,[59-61] as well as other forms of human autoimmunity.[62,63] This connection was first described by Hagg and Asplund.[64] Subsequent studies have confirmed their findings, and smoking has emerged as an important risk factor for GD and TAO with odds ratios of 1.9 (95% confidence intervals [CI], 1.1–3.2) and 7.7 (95% CI, 4.3–13.7), respectively.[60] In individuals with GD who smoke more than 20 cigarettes per day, the relative risk for developing proptosis is 3.37 (1.50–7.58, $P = 0.003$) and as high as 7.04 (3.00–16.5, $P < 0.0001$) for developing diplopia.[65] Risk for developing TAO relates more to the number of cigarettes smoked following development of GD than the life-cumulative smoking burden.[65] In a matched case-control twin study, Brix et al.[66] found that the discordant monozygotic twin with GD was more likely to have smoked when compared to the healthy sibling. A meta-analysis of studies investigating the association between smoking and thyroid diseases confirmed the increased risk for developing or worsening of TAO beyond that associated with GD.[67] A retrospective analysis demonstrated that nonsmokers had a decreased risk of TAO progression, and better therapeutic response to orbital radiation and corticosteroids than did smokers.[68] While the mechanism underlying the deleterious effects of smoking on TAO remains uncertain, its cessation appears to improve treatment response and to lower the risk of developing TAO de novo.

THE PUTATIVE ROLE OF TSHR IN TAO

Thyroid-stimulating hormone receptor, a glycoprotein hormone receptor, is a member of the G protein coupled receptor family.[69] It contains a ligand-binding extracellular domain (ectodomain), a transmembrane domain, and an intracellular domain (endodomain).[69] Posttranslational intramolecular proteolytic cleavage of the extracellular domain results in the generation of the A-subunit, which exhibits immunoreactivity and is processed by antigen presenting cells.[70] Thyroid-stimulating immunoglobulins and TSH binding to TSHR results in receptor activation and unregulated thyroid hormone production. This appears to be the basis for hyperthyroidism and the development of goiter in GD.[1]

The frequently encountered close temporal relationship between the onset of thyroid dysfunction and development of TAO suggests that GD and TAO might share a common etiology, and perhaps share a common autoantigen.[71] In addition to thyroid epithelium, TSHR can be detected in several connective tissue/adipose depots, including those within the orbit.[72,73] Levels of TSHR mRNA are considerably lower in orbital fat than those found in thyroid.[74] They appear to be higher in orbital fibroblasts from patients with TAO compared to those from healthy donors.[74] While the role of TSI in TAO has not been established, these antibodies can activate TSHR displayed on orbital fibroblasts and lead to downstream signaling and production of IL-6.[75] While evidence suggesting that low-level TSHR expression on orbital fibroblasts is capable of transducing signals from TSI has been introduced, whether the receptor protein serves as an intraorbital antigen remains uncertain. To our knowledge, no compelling studies have demonstrated antigen-specific T cell infiltration of the orbit in TAO.

T AND B CELLS

In TAO, T and B cells infiltrate orbital fat (Fig. 3) and extraocular muscles.[76] This pattern of lymphocyte recruitment shares similarities with that occurring in the thyroid.[77,78] Both CD4+ and CD8+ T cells can be identified among the infiltrate, a process that apparently occurs early in TAO.[79] Th1 predominates early in the disease, whereas a bias toward the Th2 phenotype can be found later.[80] CD4+ Th17 T cells, which
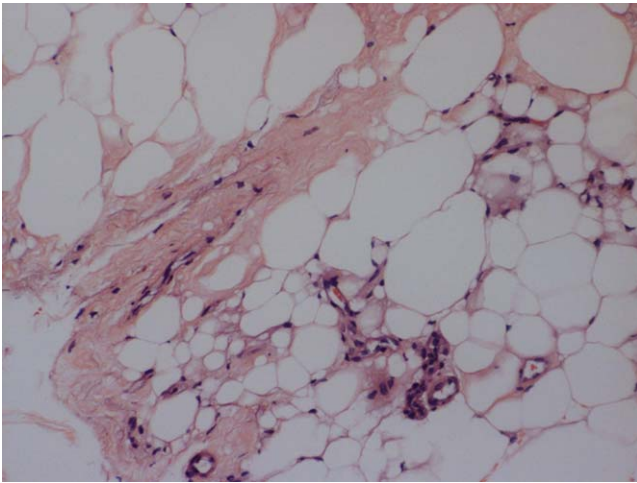
FIGURE 3. Histologic examination of orbital tissue of a patient with GD (hematoxylin and eosin, ×20). Mononuclear cell infiltrate is seen within the orbital fat compartment.

have been implicated in other autoimmune diseases, have yet to be identified in orbital infiltrates.[81] Despite the variants of IL-23R that have been associated with TAO,[21] and the increased frequency of circulating Th17 and Th22 cells in GD,[82,83] the possible involvement of the Th17 pathway in TAO has yet to be examined carefully.

CYTOKINES

Orbital tissue activation and remodeling associated with TAO appear to result from cytokine-dependent fibroblast activation.[84] This might be attributed, at least in part, to the unusual susceptibility of orbital fibroblasts to the actions of pro-inflammatory cytokines.[84] Evidence for involvement of specific cytokines derives from their detection in involved orbital fat. One study demonstrated immunoreactivity against IFNγ, TNFα, and IL-1α.[85] Messenger RNA encoding cytokines, including TNFα, IL-1β, IFNγ, IL-4, IL-6, and IL-10, was detected in extraocular muscle and fat from patients with TAO.[86] Prummel et al.[87] found elevated serum soluble IL-2R (sIL-2R) levels in approximately 45% of individuals with TAO. Although these levels failed to correlate with disease activity or severity, those individuals with the highest levels exhibited enlarged extraocular muscles.[87] Serum concentrations of IL-6 and soluble IL-6R were found to be elevated in GD compared to healthy individuals.[88] These were even higher in the subset with active TAO.[88] Serum IL-6 levels were higher in long-standing TAO.[89] Serum soluble IL-2R, IL-6, IL-6R, TNFαR I, II, and sCD30 were elevated in patients with moderately severe untreated TAO compared to healthy individuals matched for sex, age, and smoking habits.[90] Increased serum IL-17 levels were detected in TAO and were particularly elevated in those with active disease.[91]

ORBITAL FIBROBLASTS AND THE PUTATIVE ROLE OF BONE MARROW–DERIVED FIBROCYTES

A remaining central question concerns the identity of the primary autoimmune target in TAO. Extraocular muscle has been proposed by a few investigators,[92–97] but most have focused on orbital fibroblasts.[98] Supporting the latter point of view, infiltrating CD8+ T cells recognize orbital fibroblasts, and become activated through MHC class II and CD40-dependent

signaling,[7] suggesting that these cells represent autoimmune targets.

Orbital fibroblasts are a heterogeneous population of cells with complex structural and immunoregulatory functions.[99,100] They comprise spindle- and fusiform-shaped cells, projecting two or three dendritic processes.[101] Others are angular, with three or more dendritic processes. Thus, their shapes differ slightly from those of dermal fibroblasts. Their rate of cell division is predicated, at least in part, on whether they display the cell surface glycoprotein CD90, known as thymocyte antigen 1 (Thy-1).[102] For the first time, Koumas et al.[103,104] demonstrated that human orbital fibroblasts exhibited heterogeneous expression of Thy-1, and when separated into Thy-1+ and Thy-1− subsets, responded differently to extracellular stimuli, and showed distinct functionalities. When exposed to IL-1β or following CD40 ligation, Thy-1+ orbital fibroblasts produced considerably higher levels of PGE2 via upregulation of prostaglandin endoperoxide H synthase-2 (PGHS-2, also known as COX-2).[104] Further, Thy-1+ orbital fibroblasts differentiated into myofibroblasts when treated with TGF-β, as was evidenced by strong immunofluorescence activity to α-SMA,[105] whereas the Thy-1−subset underwent adipogenesis when treated with a PPARγ agonist.[103,104]

The cellular attributes of orbital fibroblasts currently are thought to predispose to the pathologic processes associated with TAO.[84] They display unique arrays of costimulatory molecules and cell surface receptors for various cytokines and growth factors.[84] It is the particular profile of inflammatory cues to which they respond that appears to set them apart from other fibroblasts. For instance, leukoregulin, IL-1β, and CD40 ligand (also known as CD40L or CD154) vigorously induce PGHS-2 in orbital fibroblasts when compared to dermal fibroblasts.[106–108] A major aspect of phenotypic divergence of orbital fibroblasts appears to relate to the disparities with which the IL-1 receptor antagonists (IL-1RA) isoforms are expressed.[109,110] Unlike those from the skin, orbital fibroblasts express vanishingly low levels of secreted IL-1RA (sIL-1RA), the antagonist molecule that has the dominant role in blocking IL-1–derived signaling. Instead, intracellular IL-1RA is far more highly expressed and inducible in these cells. The exaggerated induction of PGHS-2 resulting from cytokines, such as IL-1β, is mediated through enhanced PGHS-2 gene promoter activity and mRNA stability.[106,108] The upregulation of PGHS-2 was found to be accompanied by dramatically increased PGE2 production.[107] Orbital fibroblasts express PGE2 receptors and respond to this prostanoid by developing multiple long cytoplasmic processes[111] and generating cyclic adenosine monophosphate.[112] In addition, PGE2 influences B cell class-switching,[113] T cell differentiation,[114] and mast cell degranulation,[115] all of which might have roles in TAO. Hwang et al.[116] recognized that orbital fibroblasts from patients with TAO display higher levels of CD40 than do cells derived from healthy donors. These levels are further upregulated by IFNγ. When ligated with CD40L, they produce hyaluronan[117] as well as IL-6, IL-8, and MCP-1.[116] Interleukin-6 drives immunoglobulin production, development of plasma cells,[118] IL-4 synthesis, and biases T cells toward Th2 development.[119] Monocyte chemotactic factor-1, a powerful chemoattractant, may be involved in promoting mononuclear cell infiltration in TAO.[120] Interleukin-16 and RANTES[121] also are produced by orbital fibroblasts, once they are activated by cytokines, such as IL-1β[122] and IgGs,[123] from patients with GD through the IGF-1 receptor pathway.[124] Thus, fibroblasts may have important roles in T cell infiltration of the orbit and B cell differentiation.

The embryonic origins of orbital fibroblasts have been debated for many years. Recently, a potential explanation for the cellular heterogeneity found in TAO orbital connective tissue has been provided by the recognition that a subset of

these cells apparently derives from the bone marrow.[74] Progenitor cells, known as fibrocytes, have been found in these orbital tissues from individuals with TAO, but not in those from healthy donors. They derive from monocyte and B cell lineages and circulate as peripheral blood mononuclear cells (PMBCs).[125] Fibrocytes ordinarily comprise approximately 0.5% of circulating PBMCs and can infiltrate connective tissues at sites of injury.[126] They participate in inflammation, wound healing, and tissue remodeling, and also are involved in fibrotic lung and kidney diseases.[127,128] Fibrocytes synthesize collagen I (Col I), display CD34 and CXCR4, and traffic to tissues in response to multiple chemokines, including CXCL12.[129] They become more numerous in GD (Fig. 4), and can differentiate into myofibroblasts and adipocytes, and, thus, may account for the characteristic tissue remodeling associated with TAO.[130] The presence of fibrocytes in the TAO orbit may explain the divergent phenotypes observed in fibroblast populations.[74,131]

Fibrocytes unexpectedly express functional TSHR at levels comparable to those displayed on thyroid epithelial cells.[74] A greater proportion of fibrocytes from donors with TAO express TSHR than do those from healthy donors.[132] The levels of TSHR on fibrocytes are considerably higher than those on orbital fibroblasts, regardless of whether they derive from healthy tissues or those affected by TAO.[132] When TSHR on fibrocytes is ligated with bTSH or monoclonal TSI (M22), production of several cytokines, including IL-6, IL-8, RANTES, MCP-1, IL-1β, and TNF-α, is upregulated dramatically.[74,132] Further, fibrocytes are morphologically similar to orbital fibroblasts (Fig. 5A). The TAO orbital fat contains CD34$^+$TSHR$^+$CXCR4$^+$Col1$^+$ cells in situ, and the fibroblasts outgrowing these tissues display these markers[74] (Figs. 5B, C). CD34$^+$ orbital fibroblasts, like their circulating fibrocyte precursors,[133] differentiated into either adipocytes or myofibroblasts, depending on the culture conditions to which they were subjected.[103,104]

ADIPOGENESIS AND HYALURONAN PRODUCTION BY ORBITAL FIBROBLASTS: REFLECTIONS OF TISSUE REMODELING IN TAO

Thyroid-associated ophthalmopathy is characterized by the gross enlargement of extraocular muscles.[134] While this is due mostly to edema, the production of glycosaminoglycans (GAGs) by the orbital fibroblasts and hyperplasia of the adipose tissue also contribute to proptosis and can result in compression of the optic nerve.[134–136] Once lymphocytes infiltrate and activate the orbital fibroblasts, these cells produce GAGs and differentiate into myofibroblasts or adipocytes.[103,137–139]

The cardinal feature of remodeling seen in TAO is the disordered accumulation of hyaluronan, a nonsulfated GAG. The extraordinary hydrophilic nature of hyaluronan causes volume expansion within orbital tissues.[140] Orbital fibroblasts, as opposed to dermal fibroblasts, demonstrated a dramatic increase in hyaluronan production when exposed to leukoregulin, IFN-γ, and IL-1β through the induction of UDP-glucose dehydrogenase[141] and the hyaluronan synthases.[108,142] Further, when incubated with CD40L, they exhibited substantial coordinate increases in hyaluronan and PGE$_2$ synthesis, with the latter being mediated through PGHS-2 and IL-1α synthesis.[106] The robust response is due to low-level expression of sIL-1RA in orbital fibroblasts and subsequent poor inhibition of IL-1β.[106,109,110] Also, TGF-β has been shown to regulate hyaluronan production (Fig. 6). Recently, PPARγ activation was shown to suppress TGF-β–induced activation of fibrosis-
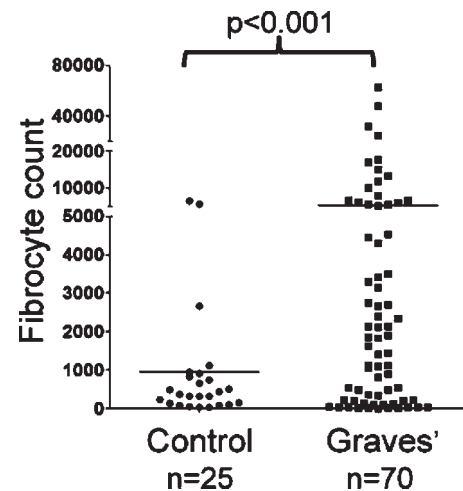

FIGURE 4. Increased generation of fibrocytes from PBMCs of patients with GD. There was approximately 5-fold more fibrocytes in individuals with GD compared to controls (5268 ± 1260 fibrocytes per 10^6 PBMCs, $n = 70$ versus control, 954 ± 329 fibrocytes per 10^6 PBMCs, $n = 25$, mean ± SD, $P < 0.001$). Reprinted with permission from Douglas RS, Afifiyan NF, Hwang CJ, et al. Increased generation of fibrocytes in thyroid-associated ophthalmopathy. *J Clin Endocrinol Metab.* 2010;95:430–438. Copyright 2010 The Endocrine Society.
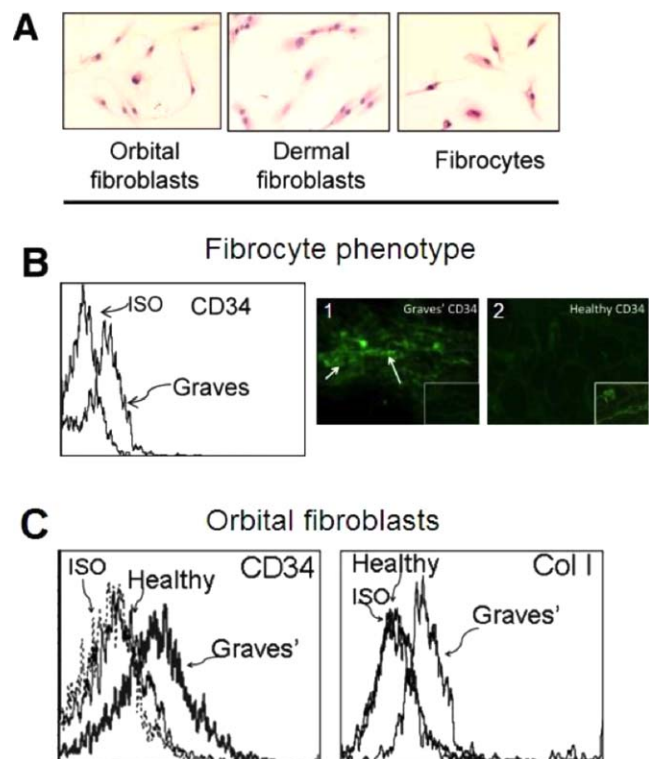

FIGURE 5. (A) Similar spindle-shaped phenotypes among orbital fibroblasts, dermal fibroblasts, and fibrocytes (hematoxylin and eosin, ×20). (B) Fibrocytes from individuals with GD display cell surface receptor CD34. 1, Immunofloresence staining of CD34 in TAO-derived tissue (*inset* as negative control). 2, Absence of CD34 expression in healthy orbital tissue (*inset* as positive control). (C) Orbital fibroblasts from individuals with and without TAO display similar receptors on fibrocytes, as shown by flow cytometric analysis with anti-CD34 and anti-Col I antibodies. Reprinted with permission from Douglas RS, Afifiyan NF, Hwang CJ, et al. Increased generation of fibrocytes in thyroid-associated ophthalmopathy. *J Clin Endocrinol Metab.* 2010;95:430–438. Copyright 2010 The Endocrine Society.
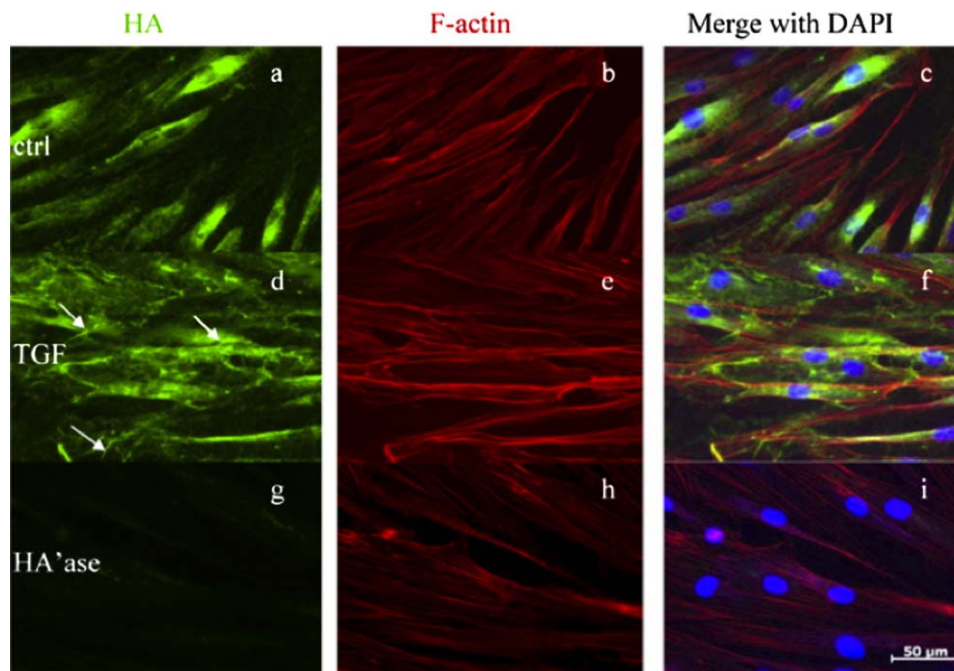
FIGURE 6. Immunofloresence of the induction of hyaluronan with TGF-β in human orbital fibroblasts. Cultures were treated with nothing (controls) or TGF-β1 for 24 hours. (**a, d, g**) Contain images of cells stained with biotinylated HABP and demonstrate hyaluronan. (**b, e, h**) Contain monolayers stained with phalloidin and demonstrate actin. (**c, f, i**) Show cultures stained with DAPI and disclose nuclei. (**a–c**) Untreated controls. Hyaluronan staining appears to be perinuclear. TGF-β1 induced hyaluronan staining and formation of microvillus-like projections. *Streptomyces* hyaluronidase-treated fibroblasts failed to exhibit hyaluronan staining, as in (**g–i**). Reprinted with permission from Guo N, Woeller CF, Feldon SE, Phipps RP. Peroxisome proliferator-activated receptor γ ligands inhibit transforming growth factor-β-induced, hyaluronan-dependent, T cell adhesion to orbital fibroblasts. *J Biol Chem*. 2011;286:18856–18867. Copyright 2011 The American Society for Biochemistry and Molecular Biology.

related processes.[143-145] Guo et al.[146] demonstrated that PPARγ ligands inhibited TGF-β–induced hyaluronan-dependent T cell adhesion to orbital fibroblasts. The same group reported that PGD$_2$, a major prostanoid produced by mast cells, regulates hyaluronan production in orbital fibroblasts, actions mediated through PD1.[147]

Crisp et al.[134] examined the role of TSHR in the adipogenesis of orbital tissues and found that the receptor is expressed differently at several stages of orbital and nonorbital fat differentiation. Further, levels of TSHR become elevated in orbital fibroblasts undergoing adipogenesis. Supraphysiologic TSH concentrations stimulated TSHR expression in TAO orbital preadipocyte fibroblasts.[134] In another study, PPARγ-expressing orbital fibroblasts underwent adipogenesis when co-cultured with activated T lymphocytes that produce PPARγ ligands. This activity could be attenuated by cyclooxygenase (COX) inhibitors.[148] When Zhang et al.[149] introduced TSHR harboring a gain-of-function mutation into orbital fibroblasts, cellular proliferation was slowed and the fibroblasts became refractory to PPARγ-induced adipogenesis. Other potentially important connections between TSHR and adipogenesis remain to be investigated thoroughly.

Individuals with TAO can be classified as manifesting either type I disease, which is characterized by expansion of adipose tissue, or type II, which is predominately extraocular muscle enlargement, or both.[86] While ample evidence suggests the phenotypic divergence of orbital fibroblasts, Kuiryan et al.[150] demonstrated that orbital fibroblasts from donors with type I TAO undergo adipogenesis more robustly than those from type II disease (Fig. 7). In contrast, type II fibroblasts exhibit a greater proliferative response to TGF-β. Therefore, it is possible that orbital fibroblast subtype determines clinical manifestation of TAO, as was suggested some time ago.[102,139] Further, inhibition of PGHS-1 and PGHS-2 by indomethacin can

attenuate 15-d-PGJ$_2$ (a PPARγ ligand)-induced adipogenesis only in fibroblasts from type II donors.[150] The mechanisms underlying this observation remain uncertain. Nonetheless, PGHS-2 inhibitors, such as celecoxib, may show promise in treating type II patients who prove unresponsive to corticosteroid treatment.[151]

THYROID PROTEINS IN THE ORBIT? A CONTINUING CONTROVERSY

Detection of "thyroid-specific" proteins in the orbit was first reported by Konishi et al.,[152] Kriss,[153] and McDougall et al.,[154] who detected Tg in tissues affected by TAO. This early report was followed by more recent work by Marino et al.,[155] who also identified Tg in orbit and in TAO orbital fibroblasts. The investigators assumed its origin to be the thyroid.[155] Fernando et al.[156] subsequently reported finding Tg expression by human CD34$^+$ fibrocytes and trace levels in TAO orbital fibroblasts. Their report suggested that fibrocytes express Tg as a consequence of substantial Tg gene promoter activity. This results in levels of Tg mRNA considerably below those found in thyroid tissue. Further, they found that the Tg was functional in that it could be iodinated in situ. Their studies suggest the potential for fibrocytes to generate iodothyronines, such as thyroid hormones. Further, they also raise the possibility that Tg might have some role as an orbital antigen.

Mature TSHR mRNA was detected initially using PCR by Fenzi et al.[72] in healthy orbital tissues and those affected by TAO. Their report soon was followed by that of Bahn et al.,[157] who detected TSHR mRNA in orbital fibroblasts (Fig. 8). Subsequently, these investigators found even higher levels in fibroblasts from individuals with TAO, especially when the cells were incubated under culture conditions favoring adipogenic
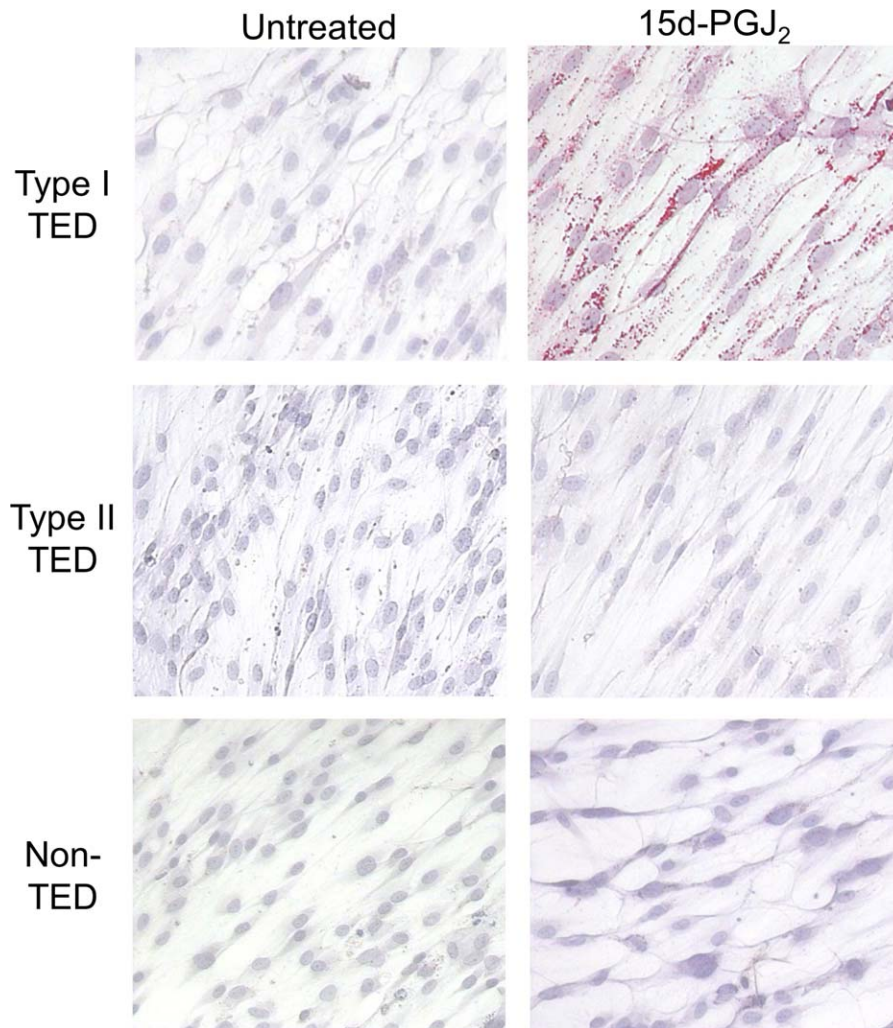
FIGURE 7. Treatment of orbital fibroblasts with 15d-PGJ$_2$ from different subtypes of TAO. Orbital fibroblasts were grown in the presence of 5 μM 15d-PGJ$_2$. Type I TAO orbital fibroblasts demonstrated more adipogenesis compared to type II or orbital fibroblasts from a healthy donor, as is evidenced by Oil Red O accumulation. TED, thyroid eye disease or TAO. Reprinted with permission from Kuriyan AE, Woeller CF, O'Loughlin CW, Phipps RP, Feldon SE. Orbital fibroblasts from thyroid eye disease patients differ in proliferative and adipogenic responses depending on disease subtype. *Invest Ophthalmol Vis Sci*. 2013;54:7370–7377. Copyright 2013 The Association for Research in Vision and Ophthalmology.

differentiation.[157] Thus, orbital tissues and their derivative fibroblasts express at least two proteins that were believed previously to be restricted to the thyroid epithelium. Furthermore, considerably higher levels of Tg and TSHR were found in fibrocytes.[156] Expression of these proteins in orbital fibroblasts localizes, albeit at considerably lower levels, to the CD34$^+$ orbital fibroblasts, which are derived putatively from fibrocytes.[156] This fibroblast subset is peculiar to cells derived from patients with TAO.[156] Orbital fibroblasts from healthy donors are uniformly CD34$^-$.[74] It would appear that expression of Tg and TSHR is dampened as fibrocytes infiltrate the orbit and cross-talk with CD34$^-$ fibroblasts.[156] The CD34$^-$ GD-orbital fibroblasts appear to downregulate Tg and TSHR expression.[156] Taken together, we can conclude that circulating fibrocytes become more numerous in patients with GD and can traffic to the orbit where they participate in the ocular manifestations of the disease (Fig. 9).

IGF-1R Pathway

Since Ingbar et al.[158] first described the functional relationship between TSH and IGF-1 pathways, much evidence has evolved to reinforce that proposed connectivity. They demonstrated that IGF-1 promoted rat thyroid epithelial cell proliferation and enhanced the effect of TSH on DNA synthesis.[158] Subsequently, substantial overlap between TSHR and insulin-like growth factor-1 receptor (IGF-1R) downstream signaling was reported. Both receptors extensively utilize the Akt/FRAP/mTOR/P70^{s6k} pathway.[159] Further, TSHR and IGF-1R form a functional and physical complex, suggesting a potential synergism that could promote abnormal signaling, such as that associated with GD.[160] Monoclonal antibodies used to block IGF-1R signaling also attenuate that downstream signaling from TSHR, suggesting that IGF-1R may participate in physiological TSHR signaling.[160]

Although TSHR has been established as the central autoantigen in GD, how it might participate in TAO remains less certain, as is the potential pathogenic involvement of other autoantigens. Insulin-like growth factor-1 influences several aspects of immunity, including thymic, B, and T cell development.[161] Overexpression of IGF-1R has been demonstrated in autoimmune processes, such as those occurring in GD.[98] The IGF-1 pathway was first implicated in GD when IgG
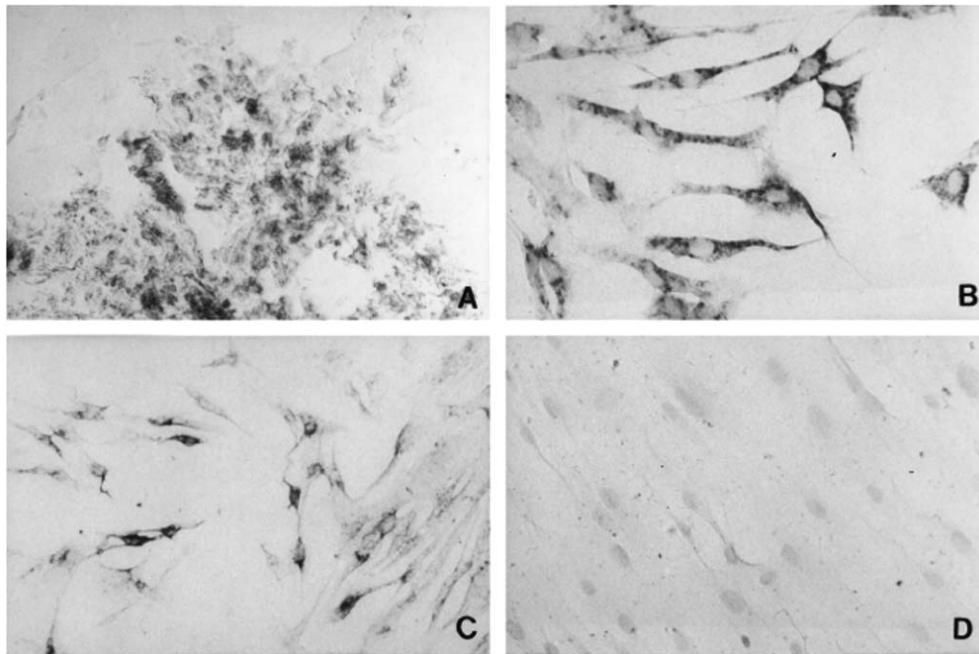
FIGURE 8. Immunohistochemical analysis of TSHR immunoreactivity on orbital connective tissue from a donor with TAO. The immunostaining was conducted with a monoclonal antibody directed against TSHR (amino acids 604-764). (**A**) Orbital connective tissue. (**B**) Passage one exhibits intense staining. (**C**) Passage three with reduced staining. (**D**) Passage 5 culture fails to show staining. Reprinted with permission from Bahn RS, Dutton CM, Natt N, Joba W, Spitzweg C, Heufelder AE. Thyrotropin receptor expression in Graves' orbital adipose/connective tissues: potential autoantigen in Graves' ophthalmopathy. *J Clin Endocrinol Metab*. 1998;83:998–1002. Copyright 1998 The Endocrine Society.

from patients was found to displace radiolabeled IGF-1 from the surface of orbital fibroblasts.[162]

Anti–IGF-1R antibodies have been detected in sera from many individuals with GD, whereas they are absent in the vast majority of sera from healthy controls.[123,124,163–167] At least a subset of these antibodies appear to activate IGF-1R and to initiate signaling that can be disrupted with a dominant negative IGF-1R, as well as with monoclonal anti–IGF-1R blocking antibodies.[124] Moreover, IGF-1R levels are increased on TAO orbital fibroblasts compared to those from healthy tissues.[124] When TAO orbital fibroblasts are treated with IGF-1 or IgG from patients, the cells produced hyaluronan[165] and

two powerful T-cell chemoattractants, namely IL-16 and RANTES.[123,124] These actions are mediated through the Akt/FRAP/mTOR/P70[s6k] pathway.[123] Furthermore, T cells and B cells from patients with GD also skew toward the IGF-1R^{+} phenotype.[168,169] Display of IGF-1R may protect against Fas-mediated apoptosis in B cells and is associated with the production of anti-TSHR antibodies by these cells.[169]

ANIMAL MODELS OF TAO

Among the first animal models attempting to recapitulate GD experimentally was that created by Shimojo et al.[170] These
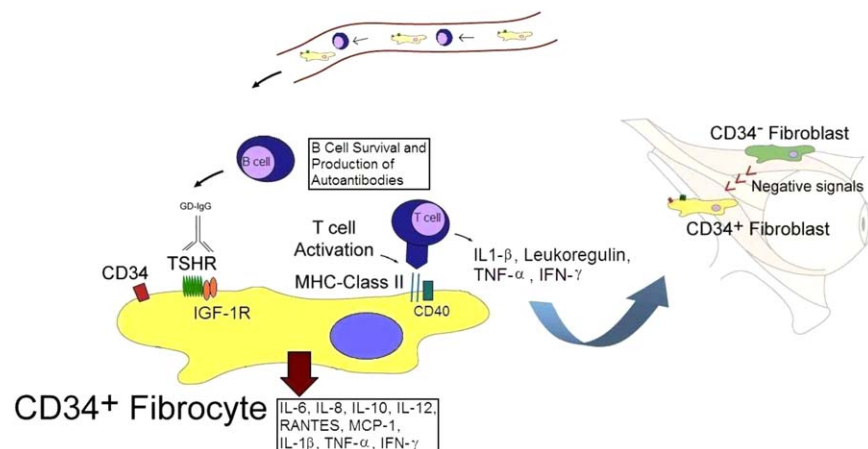

FIGURE 9. Schematic illustrating the putative role of fibrocytes in the pathogenesis of TAO. CD34^{+} fibrocytes derive from the bone marrow and appear to be trafficked specifically to the orbit in TAO where they transition into CD34^{+} fibroblasts. Fibrocytes express relatively high levels of functional TSHR. Further, they can differentiate into either adipocytes or myofibroblasts in vitro. CD34^{+} orbital fibroblasts interact with the native residential CD34^{−} orbital fibroblasts, resulting in dramatic reduction of expression of TSHR and other thyroid proteins. We postulate that the magnitude of this suppression may underlie susceptibility to TAO.

investigators immunized mice with human TSHR (hTSHR)-transfected fibroblasts also expressing MHC class II antigen.[170] Hyperthyroidism was detected in 20% of the animals. Later, Costagliola et al.[171] reported hyperthyroidism resulting from infection with an expression plasmid containing hTSHR cDNA. Nagayama et al.[172] injected an adenoviral vector expressing hTSHR into mice. This strategy resulted in a greater proportion (30%–50%) of animals developing hyperthyroidism.[172] When the free A-subunit of hTSHR was used for immunizations instead of the intact receptor, 65% to 80% of mice developed hyperthyroidism.[70] This model has proven replicable and is widely used as an animal model for GD.[173-176] More recent studies have combined TSHR plasmid injection with electroporation to enhance transfection efficacy.[177] However, these earlier attempts at creating a complete model of GD, including the ocular features of TAO, were not completely successful.

In 2011, Zhao et al.[178] attempted to induce hyperthyroidism and orbital pathology in mice by immunizing animals with plasmids encoding TSHR A and IGF-α1R. Deoxyribonucleic acid was delivered via skeletal muscle electroporation.[178] Many mice developed hyperthyroidism and generated TSI. Surprisingly, animals immunized with plasmid harboring TSHR also developed antibodies directed against IGF-1Rα. Histopathologic examination of the orbits revealed fibrosis. The IGF-1R–immunized mice also developed a strong anti–IGF-1R antibody response, but failed to exhibit a phenotype resembling GD. This study suggested an association between IGF-1R and TSHR,[160] although the basis of anti–IGF-1Rα antibody generation in TSHR A-immunized mice remains uncertain. Subsequently, Moshkelgosha et al.,[179] using the same plasmid electroporation strategy, demonstrated extensive tissue infiltration and remodeling within the orbit. The animals exhibited signs of marked orbital congestion, such as edema and chemosis. The majority of immunized animals developed blocking anti-TSHR antibodies and manifested hypothyroidism. A feature of the ocular pathology found by this group was the dramatic infiltration of optic nerves, which is strikingly uncharacteristic of TAO. Unfortunately, no details concerning the status of intraocular tissues or the central nervous system following immunization were included in the report. Further, an explanation for the dramatically different hypothyroid phenotype and predominately blocking anti-TSHR antibody profile from this group's earlier report[178] was not discussed in detail. Thus, greater definition of this model, including more careful and complete interrogation of the animals and their interesting phenotype, will be necessary before these findings can be evaluated critically. Nakahara et al.[180] described successful induction of TSI in wild type mice that received splenocytes from TSHR-immunized TSHR-knockout mice. Although this study suggests a role for anti-TSHR immune response in the development of GD, a low percentage of mice (22%) were hyperthyroid. Some of these mice later became hypothyroid. Furthermore, orbital tissue from two of the nine recipient mice demonstrated modest macrophage infiltration without the presence of striking extraocular muscle or fat enlargement, or lymphocytic infiltration. Thus, while encouraging reports of preclinical mouse models for GD have appeared recently, there have been inconsistent results and a potentially confounding deviation from the human disease. Consequently, further study is required before the implications of these reports can be fully assessed.

TREATMENT IMPLICATIONS AND FUTURE PERSPECTIVES

Current medical therapy for active moderate to severe TAO is limited to corticosteroids and external beam radiotherapy.[4] Surgical remediation usually awaits transition from active disease to the stable, chronic phase. This typically occurs over a course of a 36- to 48-month horizon.[4] Unfortunately, none of these therapeutic approaches appears to alter the natural course of TAO, making development of new therapies critical to addressing an important unmet need. Thyroid-associated ophthalmopathy is a complex autoimmune condition that only now is being clarified. Greater definition of the molecular and immunological underpinnings of this condition should facilitate the process of therapy development. In addition, better animal models should allow critical preclinical testing of candidate therapies. Potential immunotherapies based on our current understanding of GD and TAO include depleting T cells with anti-CD3 antibodies or targeting CTLA-4, a regulator of T lymphocyte activation.[181-184] Monoclonal antibodies against B cell surface antigen CD20, such as Rituximab, have demonstrated promising results in decreasing orbital inflammation in patients with TAO.[185-187] However, the preliminary findings from the two recently completed controlled prospective studies of Rituximab suggest that its effectiveness may not be uniform.[188,189] Alternative anti-B cell therapy might focus on anti-CD19, which would target plasmablasts and might provide a more complete response.[169] Anti-cytokine therapy, such as Etanercept and Infliximab, has been associated with anecdotal improvement in a very limited cohort of patients with TAO.[190-192] Controlled drug trials for these and related agents will be necessary before any conclusions can be drawn about their efficacy and safety in TAO. Anti-TSHR and anti-IGF-1R therapy also may prove to be effective. A trial of the latter strategy utilizing Teprotumumab as an IGF-1R blocking strategy currently is underway [available in the public domain at http://clinicaltrials.gov/show/NCT01868997].

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