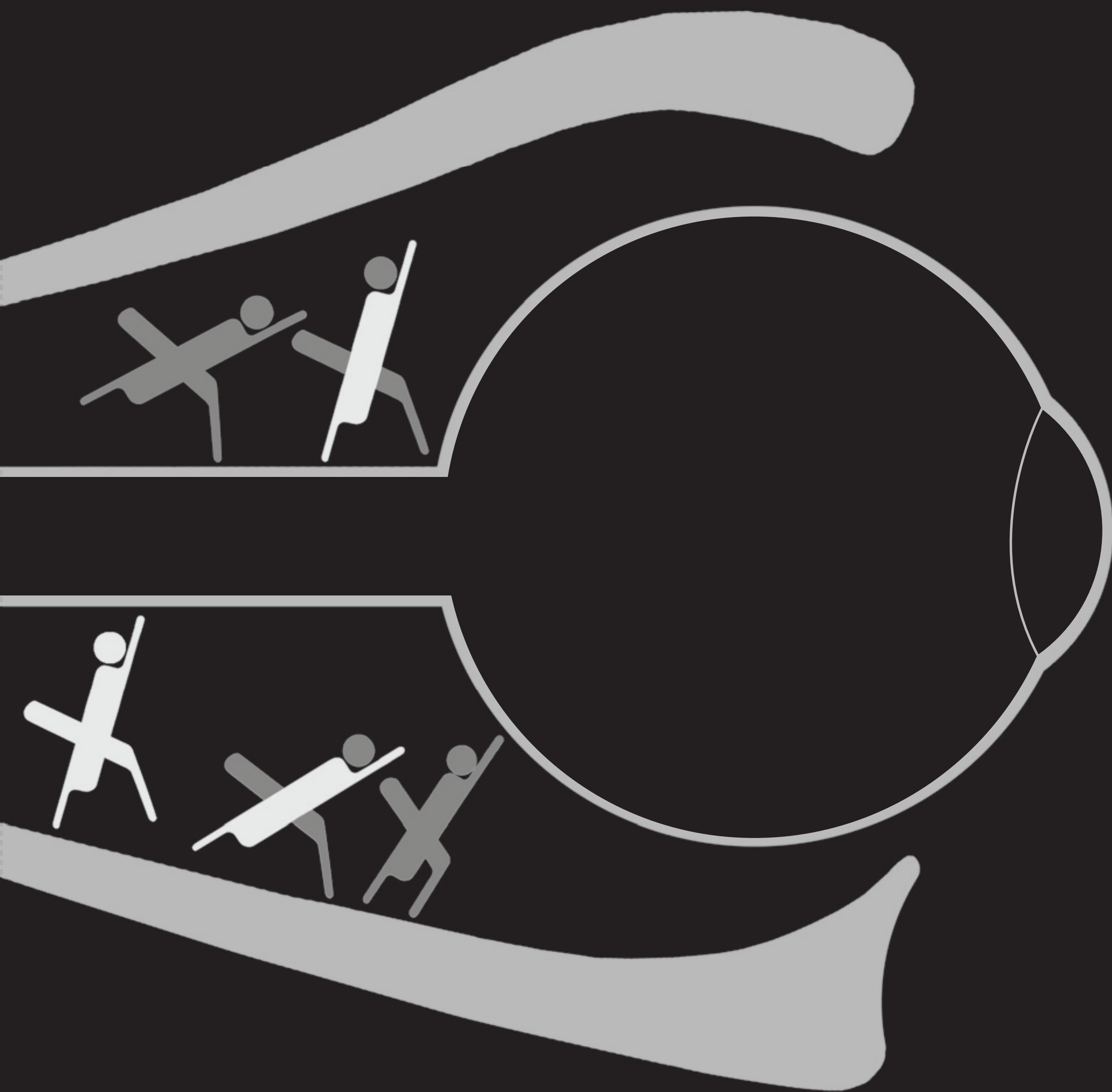
Graves' ophthalmopathy: a comprehensive role for platelet-derived growth factors



Leendert van Steensel

Graves' Ophthalmopathy:
A Comprehensive Role For Platelet-Derived Growth Factors

Leendert van Steensel

The printing of this thesis has financially been supported by:
Abbott B.V., Beckman Coulter Nederland B.V., Genzyme Nederland, J.E. Jurriaanse Stichting, Merck Sharpe & Dome B.V., MT-Diagnostics Netherlands B.V., Novartis Pharma B.V., Stichting Het Scholten-Cordes Fonds and Stichting Wetenschappelijk Onderzoek Oogziekenhuis.

ISBN: 978-90-7343-698-5
Layout and printing: Ridderprint B.V., Ridderkerk, The Netherlands
Cover: Jonatan Bevelander
Illustrations: Tar van Os, Sandra de Bruin

© L. van Steensel, Dordrecht, The Netherlands, 2012
No part of this thesis may be reproduced, stored in a retrieval system, or transmitted in any form or by any means without permission of the corresponding journals or the author.

Graves' Ophthalmopathy:
A Comprehensive Role For Platelet-Derived Growth Factors

Graves' ophthalmopathie:
een veelomvattende rol voor platelet-derived growth factors

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam

op gezag van de
rector magnificus
prof. dr. H.G. Schmidt

en volgens besluit van het College van Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 7 maart 2012 om 15.30 uur

door

Leendert van Steensel

geboren te Abakaliki,
Nigeria

PROMOTIECOMMISSIE

Promotoren:	prof.dr. P.M. van Hagen
	prof.dr. H. Hooijkaas
Overige leden:	prof.dr. T.J. Visser
	prof.dr. A. Kijlstra
	prof.dr. J.C. van Meurs
Copromotoren:	dr. W.A. Dik
	dr. A.D.A. Paridaens

CONTENTS

Chapter 1	Graves' disease	7
Chapter 2	Graves' ophthalmopathy	21
Chapter 3	Aims of the thesis	53
Chapter 4	Imatinib mesylate and AMN107 inhibit PDGF-signalling in orbital fibroblasts; a potential treatment for Graves' ophthalmopathy *Invest Ophthalmol Vis Sci. 2009 Jul;50(7):3091-8*	57
Chapter 5	Platelet-derived growth factor-BB: a stimulus for cytokine production by orbital fibroblasts in Graves' ophthalmopathy *Invest Ophthalmol Vis Sci. 2010 Feb;51(2):1002-7*	75
Chapter 6	Whole orbital tissue cultures identifies imatinib mesylate and adalimumab as potential therapeutics for Graves' ophthalmopathy *Br J Ophthalmol. 2011 May;95(5):735-8*	91
Chapter 7	Orbit-infiltrating mast cells, monocytes and macrophages produce PDGF isoforms that orchestrate orbital fibroblast activation in Graves' ophthalmopathy *J Clin Endocrinol Metab. 2012, in press*	101
Chapter 8	PDGF enhances orbital fibroblast responses to TSHR stimulating autoantibodies in Graves' ophthalmopathy patients *J Clin Endocrinol Metab. 2012, accepted for publication*	119
Chapter 9	General discussion	141
Chapter 10	Summary	161
	Populair-wetenschappelijke samenvatting	165
Chapter 11	Abbreviations	170
	Dankwoord	172
	Curriculum Vitae	174
	Publications	175
	PhD portfolio	176

Chapter 1

Graves' disease

Autoimmune thyroid disorders include Graves' disease (GD), which leads to hyperthyroidism, and Hashimoto's thyroiditis, which leads to hypothyroidism. The incidence of autoimmune hypothyroidism is around five times higher than the incidence of autoimmune hyperthyroidism[1], but together these autoimmune thyroid disorders are the most common autoimmune conditions in the Western population and affect around 2-5% of the population.[1-5]

Estimates for the incidence of autoimmune hyperthyroidism vary considerably, but a recent review estimates an incidence of 80/100.000/year for women and 8/100.000/year for men in Caucasian populations.[1, 5] Graves' disease (GD) is the most common cause of hyperthyroidism in Western and other iodine-sufficient populations, and accounts for ~80% of cases of hyperthyroidism. [6]

CLINICAL ASPECTS OF GRAVES' DISEASE

GD is caused by an autoimmune process that targets the thyroid gland and (in most cases) leads to increased thyroid hormone levels and often to thyroid gland enlargement (goiter). The clinical symptoms of GD are largely attributable to the increased thyroid hormone levels and include symptoms such as hyperactivity, heat intolerability, palpitations and weight loss. In addition to these signs of hyperthyroidism, more GD-specific symptoms can also be observed as patients with GD often also develop extra-thyroidal manifestations such as ophthalmopathy and localized dermopathy.[7-9]

Graves' ophthalmopathy (GO) is an orbital disorder which affects around 25-50% of the patients with GD, although subclinical changes can be detected in almost all GD patients. GO is characterized by orbital tissue expansion within the limited bony orbit and subsequent eye ball protrusion. When untreated, GO may cause blindness.[9] The pathophysiology and treatment of GO will be discussed in more detail in chapter 2.

Dermopathy is a rare complication of GD and occurs in ~4% of GD patients. Its localization is predominantly pretibial, although trauma or stress can initiate similar symptoms at other places of the body. Dermopathy is characterized by lymphocytic infiltration, accumulation of glycosaminoglycans and edema of the dermis.[10-12] Acropachy is the rarest complication of GD and occurs in less than 1% of GD patients. Acropachy is clinically characterized by clubbing of the fingers and toes, periosteal proliferation of the shafts of the phalanges and concomitant swelling of the overlaying soft tissues.[13-14] Dermopathy and acropachy may develop years after the onset of thyroid disease and their development is associated with severe GO.[13-14]

Diagnosis and treatment of Graves' disease

The diagnosis of GD is based on the combination of clinical symptoms and signs together with the biochemical determination of serum thyroid stimulating hormone (TSH) and free

thyroid hormone levels (fT4). Detection of serum levels of autoantibodies directed against the TSH receptor (TSHR), thyroid peroxidase (TPO) or thyroglobulin (TG) can be helpful in fine-tuning the treatment strategy.[7-9]

The major aim in the treatment of GD patients is to establish euthyroidism, in order to control the symptoms of hyperthyroidism as well as the clinical course of the ophthalmopathy and dermopathy. Euthyroidism can be reached through the administration of the thyrostatic thionamides, such as methimazole or propylthiouracil. These drugs block thyroid hormone synthesis, which is then followed by thyroid hormone supplementation. Euthyroidism can also be obtained by the use of a radioactive isotope of iodine (^{131}I). Thyroid hormone-producing cells specifically internalize the toxic isotope and are subsequently destroyed. Cases which bear contra-indications for both treatment possibilities are subjected to a subtotal thyroidectomy.[7-9]

GENETIC BASIS FOR GRAVES' DISEASE

Strong evidence for a genetic basis for autoimmune thyroid diseases comes from the finding that ~50% of patients have family members with GD and that siblings from GD patients have a risk of ~33% to develop an autoimmune thyroid disorder themselves.[4, 6, 15-16] Moreover, concordance rates for GD are ~35% in monozygotic twins, while this is only ~3% in dizygotic twins.[17-18] Statistical modelling estimates that ~79% of the predisposition to GD is determined by genetic factors, while environmental factors account for the other 21%.[17]

Several susceptibility genes for GD have been identified from the late 1970's onwards. The majority of these genes are involved in immune functions and especially in the interactions between immune cells.[19] A selection of genes associated with GD will be discussed in more detail hereafter and several of these are summarized in Table 1.

HLA

Early studies showed an association between major histocompatibility complex (MHC) regions and the development of GD. MHC regions encode for the human leukocyte antigen (HLA) proteins which are essential for peptide presentation to immune cells. Initially it was thought that the HLA class I molecule HLA-B8 accounted for the association between HLA and the development of GD in Caucasians[20-21], but later studies revealed that this association is merely due to the HLA class II molecule HLA-DR3.[22-24] In contrast, some HLA molecules, such as HLA-DR5, have a protective effect on the development of GD.[25] Further studies confirmed the previous associations and also showed positive associations between GD and HLA-DRB1, HLA-DQA1 and HLA-DQB1, but ethnic differences exist.[4, 6, 26-28]

CTLA4

The CTLA4 gene encodes the cytotoxic T-lymphocyte-associated serine esterase-4 (CTLA-4) protein that suppresses T-cell activation and subsequent T-cell driven immune responses. The CTLA-4 protein is not expressed by resting, naive T cells, but is expressed upon T-cell receptor - HLA interaction.[29] The CTLA4 gene is a highly polymorphic gene and specific polymorphisms have been associated with various autoimmune diseases, such as type 1 diabetes mellitus, autoimmune hypothyroidism, celiac disease, primary biliary sclerosis, systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis.[19, 30-31] Thus far, four different polymorphisms have been consistently linked to the development of GD across different ethnic groups.[4, 6, 26-28, 32-36] Together with the HLA genes, polymorphisms in CTLA4 have been predicted to account for ~50% of the genetic predisposition for GD.[26, 34]

CD40

The CD40 gene encodes a co-stimulatory molecule which is essentially involved in the activation of B cells. Ligation of CD40 on B cells provides essential signals that drive B-cell proliferation, maturation, antibody class-switching and secretion and the generation of long-lived memory cells. CD40 activation is also important in the activation of antigen presenting cells and various stromal cells.[37] A C/T polymorphism was detected in the Kozak sequence of the CD40 gene of which the CC genotype is strongly associated with GD in various ethnic groups.[38-41] The C-allele of this polymorphism is associated with increased CD40 expression on B cells and antigen presenting cells, which may result in higher activity of autoreactive B cells. In addition, increased CD40 expression on thyroid cells enhances thyroid pro-inflammatory functions, thereby perpetuating the inflammatory process.[40]

Other immune genes

Several other genes encoding proteins with immunological functions were also described to be associated with GD, including PTPN22, FCRL3, FOXP3 and IL2RA.[4, 6, 27-28, 32, 42-45] Also, polymorphisms in genes encoding cytokines such as interleukin 1 receptor antagonist (IL-1Ra), tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-4 as well as the vitamin D receptor gene have been associated with GD [46-51], but need to be validated in other and larger patient cohorts.

Thyroid-specific genes

Several thyroid-specific genes have been studied for associations with GD development, but most of these studies were disappointing. No association between the TPO gene and GD has been described [52], but associations between the TG gene and GD have been found in different ethnic groups. These associations were due to various single nucleotide polymorphisms which likely result in more immunogenic TG peptides.[53-55]

For a long time no consistent associations were found between GD and polymorphisms within the TSHR gene.[6] More recently however, associations between GD and (intronic) polymorphisms within the TSHR gene have been described in different ethnic groups.[28, 56-57] These studies still await confirmation in larger and ethnically distinct patient cohorts and therefore the contribution of genetic variants in the TSHR gene to GD is still largely debated.

Table 1. A selection of genes associated with Graves' disease

Gene	Protein function	Potential mechanism for contribution to GD	References
Immune response modulating genes			
HLA	Presentation of (foreign) peptides to immune cells	Alteration of peptide presentation to T cells	22-24
CTLA4	Negative regulator of T-cell and B-cell activation	Reduced suppression of T-cell activity	33-36
CD40	Co-stimulatory molecule expressed on B cells, antigen presenting cells and stromal cells	Increased CD40 expression leads to overactivation of B cells, antigen presenting cells and thyrocytes	39-41
PTPN22	powerful inhibitor of T-cell activation, plays a role in memory B-cell formation	Inhibition of T-cell activation, resulting in escape from thymic deletion(?), B-cell receptor activation defect	42-43, 58
Thyroid specific genes			
TSHR	Regulation of thyroid hormone production, target for autoantibodies in GD	Alterations in TSHR splicing which increases HLA-DR presentation of TSHR peptides?	56-57
TG	Precursor and store-house for thyroid hormone	Alterations in thyroglobulin peptide presentation by HLA-DR	53-55

ENVIRONMENTAL FACTORS PRE-DISPOSING FOR GRAVES' DISEASE

Besides genetic factors, environmental factors have also been implicated in the development of GD and are estimated to account for ~21% of the predisposition for GD.[17] Several of these environmental factors are discussed in more detail hereafter.

Smoking

Smoking is the best-recognized socio-environmental risk factor for GD development, especially for GO. Smoking causes oxidative stress and immune cell activation. A dose-dependent association exists between smoking and the risk to develop GD. Furthermore, smoking predisposes to a higher relapse chance and to a therapy-resistant and more severe clinical course of GD. In contrast, smoking protects against the development of autoimmune hypothyroidism.[32, 59-60]

Iodine

Iodine is required for the synthesis of thyroid hormones and a clear relationship exists between the amount of iodine intake and the occurrence of thyroid hormone disorders.[61] Hyperthyroidism is more common in geographical areas with sufficient iodine intake, in contrast to hypothyroidism which is more prevalent in iodine-deficient areas.[62-63] Increased iodine intake normally leads to a reduction of thyroid hormone production (the Wolff-Chaikoff effect). However, in patients having areas of autonomous thyroid hormone production in their thyroid, this may lead to increased thyroid hormone production (the Jod-Basedow effect). Usually these effects resolve within a few days, but when persisting, they may lead to thyroid damage and (auto)immune activation. Apart from this, iodine can also influence thyroid autoimmunity via direct activating effects on immune cells or by highly iodinated and therefore immunogenic TG.[59-61, 64] These data clearly demonstrates the importance of a well-balanced iodine intake.

Selenium

Selenium is an important trace mineral which is an essential nutrient for selenocysteine synthesis that influences thyroid hormone production.[65] Selenium deficiency has been associated with an increased thyroid gland volume and echogenicity. Oxidative stress plays a role in GD and selenium also functions as an anti-oxidant. Besides this, selenium exerts other immunomodulatory effects, such as the reduction of macrophage migration and a decreased T-cell proliferation.[65] Selenium supplementation results in a decrease of TPO antibody levels in hypothyroid patients and improves the clinical signs of moderate-to-severe GO.[59-60, 66-68]

Stress

Stressful life events have also been linked to GD.[32, 60] Activation of the hypothalamo-pituitary-adrenal axis by stress skews the immune systems more towards a T helper 2 (Th2) phenotype, which supports autoantibody-driven processes and may thus enhance the autoantibody driven pathology of GD. GD patients also encounter more stressful events (both positive and negative) in their life, both after and prior to the diagnosis.[69] Finally, a higher prevalence of GD was found in areas experiencing war.[70]

Infections

Differences in the occurrence of GD between seasons and geographic locations, together with serological evidence for recently encountered infections in GD patients, support the association between specific infections and GD.[32, 59-60, 71] For instance *Borrelia burgdorferi* and especially *Yersinia enterocolitica* infections have been associated with GD. The association between GD and *B. burgdorferi* is merely based on shared genetic homologies between *B. burgdorferi* and thyroid antigens, but experimental evidence is limited to a case study.[72-74]

The association between *Y. enterocolitica* infections and GD is supported by the increased frequency of *Y. enterocolitica*-specific antibodies in GD patients.[75] Molecular mimicry between certain *Y. enterocolitica* peptides and TSHR epitopes has been shown to play a role herein. Despite these data, recent prospective studies showed that *Y. enterocolitica* antibodies are independent from the occurrence of GD and that no causal or pathogenic role exists for *Y. enterocolitica* in GD.[12, 59-60, 76-78]

Viral infections (especially hepatitis C infections) have also been associated with GD. Increased frequencies of thyroid disorders or thyroid autoantibodies were found in patients infected with the hepatitis C virus, but conflicting data exist and the exact mechanism by which hepatitis C infections contribute to GD development is still unknown.[32, 79]

Drugs

Amiodarone and lithium aggravate thyroid dysfunction in patients with already subclinical thyroid disorders, but do not induce *de novo* thyroid autoimmunity. Highly active anti-retroviral therapy (HAART), which is used for the treatment of HIV-positive patients, and Campath-1H, a CD52 monoclonal antibody which is used for the treatment of multiple sclerosis, are drugs that have been associated with the development of GD.[32, 59-60, 80]

AUTOIMMUNE PROCESSES IN GRAVES' DISEASE

Genetic, environmental and endogenous factors contribute to a break-down of self tolerance and the formation of autoantibodies against the TSHR in GD. A complex interplay between dendritic cells (DC), T regulatory cells (Treg), T cells, B cells and thyrocytes plays a role in the development of GD.[81-83]

DC have been found elevated in the thyroid and peripheral blood from GD patients.[84-86] DC within the thyroid initially display an immature phenotype and are predominantly found in close contact with thyroid follicular cells.[85, 87] Following the uptake of thyroid antigens the DC mature and obtain a phenotype that is well-equipped for antigen presentation. Thyroid antigen presentation to lymphocytes may take place in the thyroid and the draining lymph nodes and leads to selective activation of T cells that express a T-cell receptor that recognizes thyroid antigens, such as the TSHR.[71, 85-87]

T cells that infiltrate the thyroid gland of GD patients are predominantly $CD4^+$ T cells and, together with B cells, they form germinal center-like structures.[82, 84] In contrast to Hashimoto's thyroiditis, GD is characterized by a mild lymphocytic infiltration and only little glandular destruction, indicating that destructive T-cell mediated reactions are hardly involved in GD.[82, 84] Therefore, although T cells in GD patients recognize multiple epitopes of the TSHR receptor and may target the TSHR directly[71, 82], it is more likely that the DC-

activated T cells are involved in providing the essential co-stimulatory signals to B cells for the initiation of efficient autoantibody responses (i.e. B-cell maturation, antibody class-switching) to thyroid antigens.

A special subpopulation of dendritic cells, plasmacytoid dendritic cells (pDC), respond to self-nucleic acids and viruses by producing type 1 interferons (e.g. IFN-α and IFN-β) and other pro-inflammatory cytokines. Plasmacytoid DC numbers are increased in GD patients and, together with so-called alternatively activated macrophages, produce type 1 interferons that lead to enhanced apoptosis of Treg.[81, 88] High expression of Fas and Fas ligand molecules on thyrocytes, DC and activated T cells may also contribute to Treg apoptosis.[89] Treg are potent suppressors of autoreactive T-cell proliferation, antibody production by B cells and DC maturation. The decreased numbers of Treg in GD patients may lead to high numbers of naive and autoreactive T cells in the thyroid gland.[81] The importance of Treg in the development of GD is further illustrated by studies showing that Treg infusion prevents the development of GD in mouse models.[90-91]

The loss of tolerance against thyroid antigens results in the production of autoantibodies directed against thyroid antigens. Well-known are the stimulatory autoantibodies directed against the TSHR which directly lead to increased production of thyroid hormone by the thyroid gland, thereby causing hyperthyroidism.[71] TSHR autoantibody levels do not necessarily correlate with the actual thyroid hormone concentrations, as also TSHR inhibitory antibodies can be formed. Autoantibodies directed against TG and TPO are often also found in GD, in many cases even before hyperthyroidism occurs. Autoantibodies against TG and TPO do not participate in the pathophysiological process of GD, but are considered to reflect thyroid damage.[71]

Activated T cells, B cells and DC produce various inflammatory cytokines, which amplify the (auto)inflammatory process. The thyroidal inflammatory environment of GD is dominated by Th2-related cytokines, including IL-4, IL-5, IL-10 and IL-13.[92] Thyrocytes play a central role in the maintenance and perpetuation of the autoimmune inflammatory process in the thyroid gland. This is reflected by their increased expression of adhesion molecules, such as ICAM-1, and production of cytokines/chemokines including IL-6, IL-8, CCL5, IL-16 and CXCL10.[71, 92]

Altogether, the autoimmune process in GO comprises aberrancies in the function and numbers of various immune cells. Together these lead to loss of cellular and humoral tolerance and subsequent development of GD.

CONCLUSION

GD is a thyroid disorder characterized by an autoimmune process that is associated with the formation of stimulatory autoantibodies against the TSHR. These TSHR autoantibodies induce hyperthyroidism and goiter formation.

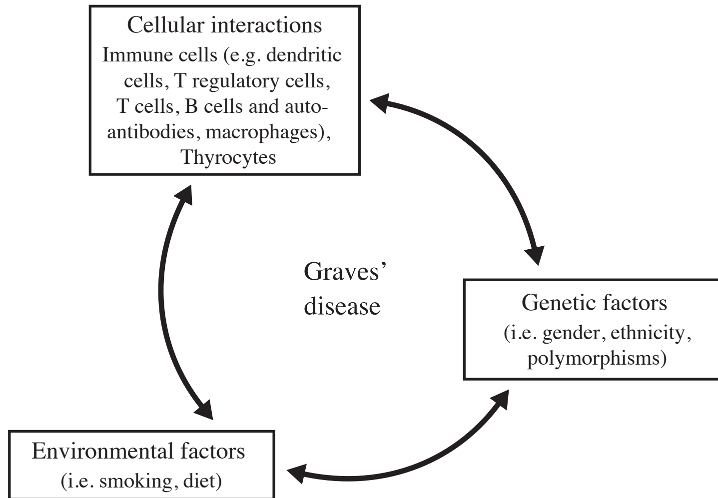


Figure 1 Graves' disease is a multifactorial disease in which environmental factors, genetic factors and cellular interactions between immune cells and thyrocytes lead to a loss of tolerance against thyroid antigens and a subsequent thyroid-directed autoimmune process.

The etiology of GD is complex and multifactorial and comprises genetic, environmental and immunological factors (Figure 1). The clinical signs and symptoms of GD that are associated with excessive thyroid hormone synthesis by the thyroid gland can be treated easily. Extra-thyroidal complications, and especially GO, however prove to be more difficult to control, as will be discussed in chapter 2.

REFERENCES

1. McGrogan A, Seaman HE, et al. *The incidence of autoimmune thyroid disease: a systematic review of the literature*. Clin Endocrinol (Oxf) 2008;69:687-696.
2. Tunbridge WM, Evered DC, et al. *The spectrum of thyroid disease in a community: the Whickham survey*. Clin Endocrinol (Oxf) 1977;7:481-493.
3. Jacobson DL, Gange SJ, et al. *Epidemiology and estimated population burden of selected autoimmune diseases in the United States*. Clin Immunol Immunopathol 1997;84:223-243.
4. Simmonds MJ, Gough SC. *Unravelling the genetic complexity of autoimmune thyroid disease: HLA, CTLA-4 and beyond*. Clin Exp Immunol 2004;136:1-10.
5. Wang C, Crapo LM. *The epidemiology of thyroid disease and implications for screening*. Endocrinol Metab Clin North Am 1997;26:189-218.
6. Tomer Y, Davies TF. *Searching for the autoimmune thyroid disease susceptibility genes: from gene mapping to gene function*. Endocr Rev 2003;24:694-717.
7. Weetman AP. *Graves' disease*. N Engl J Med 2000;343:1236-1248.
8. Brent GA. *Clinical practice. Graves' disease*. N Engl J Med 2008;358:2594-2605.
9. Bahn RS. *Graves' ophthalmopathy*. N Engl J Med 2010;362:726-738.
10. Fatourechi V. *Pretibial myxedema: pathophysiology and treatment options*. Am J Clin Dermatol 2005;6:295-309.
11. Schwartz KM, Fatourechi V, et al. *Dermopathy of Graves' disease (pretibial myxedema): long-term outcome*. J Clin Endocrinol Metab 2002;87:438-446.
12. Ai J, Leonhardt JM, et al. *Autoimmune thyroid diseases: etiology, pathogenesis, and dermatologic manifestations*. J Am Acad Dermatol 2003;48:641-659; quiz 660-642.
13. Anderson CK, Miller OF, 3rd. *Triad of exophthalmos, pretibial myxedema, and acropachy in a patient with Graves' disease*. J Am Acad Dermatol 2003;48:970-972.
14. Heymann WR. *Cutaneous manifestations of thyroid disease*. J Am Acad Dermatol 1992;26:885-902.
15. Martin L. *The hereditary and familial aspects of exophthalmic goitre and nodular goitre*. Q J Med 1945;14:207-219.
16. Hall R, Stanbury JB. *Familial studies of autoimmune thyroiditis*. Clin Exp Immunol 1967;2:Suppl:719-725.
17. Brix TH, Kyvik KO, et al. *Evidence for a major role of heredity in Graves' disease: a population-based study of two Danish twin cohorts*. J Clin Endocrinol Metab 2001;86:930-934.
18. Ringold DA, Nicoloff JT, et al. *Further evidence for a strong genetic influence on the development of autoimmune thyroid disease: the California twin study*. Thyroid 2002;12:647-653.
19. Cho JH, Gregersen PK. *Genomics and the multifactorial nature of human autoimmune disease*. N Engl J Med 2011;365:1612-1623.
20. Farid NR, Barnard JM, et al. *The association of HLA with autoimmune thyroid disease in Newfoundland. The influence of HLA homozygosity in Graves' disease*. Tissue Antigens 1976;8:181-189.
21. Bech K, Lumholtz B, et al. *HLA antigens in Graves' disease*. Acta Endocrinol (Copenh) 1977;86:510-516.
22. Farid NR, Sampson L, et al. *A study of human leukocyte D locus related antigens in Graves' disease*. J Clin Invest 1979;63:108-113.
23. Boehm BO, Kuhnl P, et al. *HLA-DRB3 gene alleles in Caucasian patients with Graves' disease*. Clin Investig 1992;70:956-960.
24. Heward JM, Allahabadia A, et al. *Linkage disequilibrium between the human leukocyte antigen class II region of the major histocompatibility complex and Graves' disease: replication using a population case control and family-based study*. J Clin Endocrinol Metab 1998;83:3394-3397.
25. Uno H, Sasazuki T, et al. *Two major genes, linked to HLA and Gm, control susceptibility to Graves' disease*. Nature 1981;292:768-770.
26. Chistyakov DA, Savost'anov KV, et al. *Genetic determinants of Graves disease*. Mol Genet Metab 2000;71:66-69.
27. Tomer Y. *Genetic susceptibility to autoimmune thyroid disease: past, present, and future*. Thyroid 2010;20:715-725.

28. Chu X, Pan CM, et al. *A genome-wide association study identifies two new risk loci for Graves' disease*. Nat Genet 2011;43:897-901.
29. Teft WA, Kirchhof MG, et al. *A molecular perspective of CTLA-4 function*. Annu Rev Immunol 2006;24:65-97.
30. Gough SC, Walker LS, et al. *CTLA4 gene polymorphism and autoimmunity*. Immunol Rev 2005;204:102-115.
31. Zhernakova A, Eerligh P, et al. *CTLA4 is differentially associated with autoimmune diseases in the Dutch population*. Hum Genet 2005;118:58-66.
32. Tomer Y, Huber A. *The etiology of autoimmune thyroid disease: a story of genes and environment*. J Autoimmun 2009;32:231-239.
33. Yanagawa T, Hidaka Y, et al. *CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population*. J Clin Endocrinol Metab 1995;80:41-45.
34. Vaidya B, Imrie H, et al. *The cytotoxic T lymphocyte antigen-4 is a major Graves' disease locus*. Hum Mol Genet 1999;8:1195-1199.
35. Allahabadia A, Heward JM, et al. *MHC class II region, CTLA4 gene, and ophthalmopathy in patients with Graves' disease*. Lancet 2001;358:984-985.
36. Kavvoura FK, Akamizu T, et al. *Cytotoxic T-lymphocyte associated antigen 4 gene polymorphisms and autoimmune thyroid disease: a meta-analysis*. J Clin Endocrinol Metab 2007;92:3162-3170.
37. Peters AL, Stunz LL, et al. *CD40 and autoimmunity: the dark side of a great activator*. Semin Immunol 2009;21:293-300.
38. Tomer Y, Concepcion E, et al. *A C/T single-nucleotide polymorphism in the region of the CD40 gene is associated with Graves' disease*. Thyroid 2002;12:1129-1135.
39. Kurylowicz A, Kula D, et al. *Association of CD40 gene polymorphism (C-1T) with susceptibility and phenotype of Graves' disease*. Thyroid 2005;15:1119-1124.
40. Jacobson EM, Huber AK, et al. *A CD40 Kozak sequence polymorphism and susceptibility to antibody-mediated autoimmune conditions: the role of CD40 tissue-specific expression*. Genes Immun 2007;8:205-214.
41. Ban Y, Tozaki T, et al. *Association of a C/T single-nucleotide polymorphism in the 5' untranslated region of the CD40 gene with Graves' disease in Japanese*. Thyroid 2006;16:443-446.
42. Gu LQ, Zhu W, et al. *Clinical associations of the genetic variants of CTLA-4, Tg, TSHR, PTPN22, PTPN12 and FCRL3 in patients with Graves' disease*. Clin Endocrinol (Oxf) 2010;72:248-255.
43. Velaga MR, Wilson V, et al. *The codon 620 tryptophan allele of the lymphoid tyrosine phosphatase (LYP) gene is a major determinant of Graves' disease*. J Clin Endocrinol Metab 2004;89:5862-5865.
44. Ban Y, Tozaki T, et al. *The regulatory T cell gene FOXP3 and genetic susceptibility to thyroid autoimmunity: an association analysis in Caucasian and Japanese cohorts*. J Autoimmun 2007;28:201-207.
45. Brand OJ, Lowe CE, et al. *Association of the interleukin-2 receptor alpha (IL-2Ralpha)/CD25 gene region with Graves' disease using a multilocus test and tag SNPs*. Clin Endocrinol (Oxf) 2007;66:508-512.
46. Blakemore AI, Watson PF, et al. *Association of Graves' disease with an allele of the interleukin-1 receptor antagonist gene*. J Clin Endocrinol Metab 1995;80:111-115.
47. Cuddihy RM, Bahn RS. *Lack of an association between alleles of interleukin-1 alpha and interleukin-1 receptor antagonist genes and Graves' disease in a North American Caucasian population*. J Clin Endocrinol Metab 1996;81:4476-4478.
48. Siegmund T, Usadel KH, et al. *Interferon-gamma gene microsatellite polymorphisms in patients with Graves' disease*. Thyroid 1998;8:1013-1017.
49. Ban Y, Taniyama M. *Vitamin D receptor gene polymorphism is associated with Graves' disease in the Japanese population*. J Clin Endocrinol Metab 2000;85:4639-4643.
50. Hunt PJ, Marshall SE, et al. *Cytokine gene polymorphisms in autoimmune thyroid disease*. J Clin Endocrinol Metab 2000;85:1984-1988.
51. Kamizono S, Hiromatsu Y, et al. *A polymorphism of the 5' flanking region of tumour necrosis factor alpha gene is associated with thyroid-associated ophthalmopathy in Japanese*. Clin Endocrinol (Oxf) 2000;52:759-764.
52. Pirro MT, De Filippis V, et al. *Thyroperoxidase microsatellite polymorphism in thyroid diseases*. Thyroid 1995;5:461-464.
53. Collins JE, Heward JM, et al. *Association of a rare thyroglobulin gene microsatellite variant with autoimmune thyroid disease*. J Clin Endocrinol Metab 2003;88:5039-5042.

54. Tomer Y, Greenberg DA, et al. *Thyroglobulin is a thyroid specific gene for the familial autoimmune thyroid diseases*. J Clin Endocrinol Metab 2002;87:404-407.
55. Ban Y, Greenberg DA, et al. *Amino acid substitutions in the thyroglobulin gene are associated with susceptibility to human and murine autoimmune thyroid disease*. Proc Natl Acad Sci U S A 2003;100:15119-15124.
56. Hiratani H, Bowden DW, et al. *Multiple SNPs in intron 7 of thyrotropin receptor are associated with Graves' disease*. J Clin Endocrinol Metab 2005;90:2898-2903.
57. Ploski R, Brand OJ, et al. *Thyroid stimulating hormone receptor (TSHR) intron 1 variants are major risk factors for Graves' disease in three European Caucasian cohorts*. PLoS One 2010;5:e15512.
58. Rieck M, Arechiga A, et al. *Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes*. J Immunol 2007;179:4704-4710.
59. Prummel MF, Strieder T, et al. *The environment and autoimmune thyroid diseases*. Eur J Endocrinol 2004;150:605-618.
60. Tanda ML, Piantanida E, et al. *Thyroid autoimmunity and environment*. Horm Metab Res 2009;41:436-442.
61. Brent GA. *Environmental exposures and autoimmune thyroid disease*. Thyroid 2010;20:755-761.
62. Laurberg P, Pedersen KM, et al. *Iodine intake and the pattern of thyroid disorders: a comparative epidemiological study of thyroid abnormalities in the elderly in Iceland and in Jutland, Denmark*. J Clin Endocrinol Metab 1998;83:765-769.
63. Laurberg P, Nohr SB, et al. *Thyroid disorders in mild iodine deficiency*. Thyroid 2000;10:951-963.
64. Papanastasiou L, Vatalas IA, et al. *Thyroid autoimmunity in the current iodine environment*. Thyroid 2007;17:729-739.
65. Duntas LH. *Selenium and the thyroid: a close-knit connection*. J Clin Endocrinol Metab 2010;95:5180-5188.
66. Marcocci C, Kahaly GJ, et al. *Selenium and the course of mild Graves' orbitopathy*. N Engl J Med 2011;364:1920-1931.
67. Weissel M. *Selenium and the course of mild Graves' orbitopathy*. N Engl J Med 2011;365:769-770; author reply 770-761.
68. Sheck L. *Selenium and the course of mild Graves' orbitopathy*. N Engl J Med 2011;365:770; author reply 770-771.
69. Matos-Santos A, Nobre EL, et al. *Relationship between the number and impact of stressful life events and the onset of Graves' disease and toxic nodular goitre*. Clin Endocrinol (Oxf) 2001;55:15-19.
70. Paunkovic N, Paunkovic J, et al. *The significant increase in incidence of Graves' disease in eastern Serbia during the civil war in the former Yugoslavia (1992 to 1995)*. Thyroid 1998;8:37-41.
71. Prabhakar BS, Bahn RS, et al. *Current perspective on the pathogenesis of Graves' disease and ophthalmopathy*. Endocr Rev 2003;24:802-835.
72. Benvenga S, Santarpia L, et al. *Human thyroid autoantigens and proteins of Yersinia and Borrelia share amino acid sequence homology that includes binding motifs to HLA-DR molecules and T-cell receptor*. Thyroid 2006;16:225-236.
73. Benvenga S, Guarneri F, et al. *Homologies between proteins of Borrelia burgdorferi and thyroid autoantigens*. Thyroid 2004;14:964-966.
74. Vaccaro M, Guarneri F, et al. *Association of lichen sclerosus and autoimmune thyroiditis: possible role of Borrelia burgdorferi?* Thyroid 2002;12:1147-1148.
75. Corapcioglu D, Tonyukuk V, et al. *Relationship between thyroid autoimmunity and Yersinia enterocolitica antibodies*. Thyroid 2002;12:613-617.
76. Effraimidis G, Tijssen JG, et al. *No causal relationship between Yersinia enterocolitica infection and autoimmune thyroid disease: evidence from a prospective study*. Clin Exp Immunol 2011;165:38-43.
77. Hansen PS, Wenzel BE, et al. *Yersinia enterocolitica infection does not confer an increased risk of thyroid antibodies: evidence from a Danish twin study*. Clin Exp Immunol 2006;146:32-38.
78. Brix TH, Hansen PS, et al. *Too early to dismiss Yersinia enterocolitica infection in the aetiology of Graves' disease: evidence from a twin case-control study*. Clin Endocrinol (Oxf) 2008;69:491-496.
79. Antonelli A, Ferri C, et al. *Thyroid disorders in chronic hepatitis C virus infection*. Thyroid 2006;16:563-572.

80. Jubault V, Penfornis A, et al. *Sequential occurrence of thyroid autoantibodies and Graves' disease after immune restoration in severely immunocompromised human immunodeficiency virus-1-infected patients*. J Clin Endocrinol Metab 2000;85:4254-4257.
81. Mao C, Wang S, et al. *Impairment of regulatory capacity of CD4+CD25+ regulatory T cells mediated by dendritic cell polarization and hyperthyroidism in Graves' disease*. J Immunol 2011;186:4734-4743.
82. Ben-Skowronek I, Sierocinska-Sawa J, et al. *Interaction of lymphocytes and thyrocytes in Graves' disease and nonautoimmune thyroid diseases in immunohistochemical and ultrastructural investigations*. Horm Res 2009;71:350-358.
83. Canning MO, Ruwhof C, et al. *Aberrancies in antigen-presenting cells and T cells in autoimmune thyroid disease. A role in faulty tolerance induction*. Autoimmunity 2003;36:429-442.
84. Kabel PJ, Voorbij HA, et al. *Intrathyroidal dendritic cells*. J Clin Endocrinol Metab 1988;66:199-207.
85. Quadbeck B, Eckstein AK, et al. *Maturation of thyroidal dendritic cells in Graves' disease*. Scand J Immunol 2002;55:612-620.
86. Quadbeck B, Stucke M, et al. *Dysregulation of TNF/TNFR superfamily members: a systemic link between intra- and extrathyroidal manifestations in Graves' disease*. Scand J Immunol 2006;64:523-530.
87. Molne J, Jansson S, et al. *Adherence of RFD-1 positive dendritic cells to the basal surface of thyroid follicular cells in Graves' disease*. Autoimmunity 1994;17:59-71.
88. Ruiz-Riol M, Barnils Mdel P, et al. *Analysis of the cumulative changes in Graves' disease thyroid glands points to IFN signature, plasmacytoid DCs and alternatively activated macrophages as chronicity determining factors*. J Autoimmun 2011;36:189-200.
89. Nakamura Y, Watanabe M, et al. *Intrathyroidal CD4+ T lymphocytes express high levels of Fas and CD4+ CD8+ macrophages/dendritic cells express Fas ligand in autoimmune thyroid disease*. Thyroid 2004;14:819-824.
90. McLachlan SM, Nagayama Y, et al. *The link between Graves' disease and Hashimoto's thyroiditis: a role for regulatory T cells*. Endocrinology 2007;148:5724-5733.
91. Nagayama Y, Horie I, et al. *CD4+CD25+ naturally occurring regulatory T cells and not lymphopenia play a role in the pathogenesis of iodide-induced autoimmune thyroiditis in NOD-H2h4 mice*. J Autoimmun 2007;29:195-202.
92. Gianoukakis AG, Khadavi N, et al. *Cytokines, Graves' disease, and thyroid-associated ophthalmopathy*. Thyroid 2008;18:953-958.

Chapter 2

Graves' ophthalmopathy

Graves' ophthalmopathy (GO) is the most common extra-thyroidal manifestation of Graves' disease (GD). GO is clinically manifest in around 25-50% of the patients with GD, although close examination reveals subclinical ocular symptoms in almost all patients with GD.[1-2] In the majority of patients the clinical course of GO is mild and can be treated with symptomatic treatment. However, 3-5% of the GO patients develop a severe and sometimes sight-threatening course of the disease due to severe corneal ulceration or compressive optic neuropathy.[2-5]

CLINICAL ASPECTS OF GRAVES' OPHTHALMOPATHY

Symptoms and clinical course of Graves' ophthalmopathy

Symptoms of GO are primarily of mechanical origin. An increase in orbital connective and/or muscle tissue within the space-limited bony orbit leads to protrusion of the eye and subsequent ocular symptoms such as upper eyelid retraction, edema, erythema of the peri-orbital tissues and conjunctivae, proptosis and strabismus (Figure 1).[2, 5]

The expansion of orbital connective and muscle tissue is the result of a pathophysiological process to which several cell types and mediators contribute. The pathophysiology of GO includes inflammation, excessive extracellular matrix (ECM) deposition (fibrosis), autoimmunity and adipogenesis. The orbital fibroblast is well-known for its central role in these processes, in which it interacts with B cells, T cells, monocytes, macrophages and mast cells.[2, 6] The pathophysiology of GO will be discussed in detail later.

Although most GO patients have a combination of adipose tissue and orbital muscle expansion, some patients exhibit a predominance for one of these processes. In case of the latter, patients younger than 40 years often show predominant involvement of adipose tissue whereas patients older than 70 years are more prone for orbital muscle enlargement. The orbital muscle enlargement is not associated with direct damage to the muscle cells, but is caused by infiltration of orbital fibroblasts and massive accumulation of ECM components between the muscle fibers.[2, 5]

The typical clinical course of GO in patients not receiving any treatment for their orbital complaints was first described and depicted by Rundle in 1957 (Figure 2).[7] In general, patients suffer an initial phase of progressive disease (the 'active' phase) which is characterized by active inflammation, orbital infiltration of immune cells, ECM production and edema. This may last several months and then the activity subsides and progresses to a phase of slow spontaneous recovery. This chronic 'inactive' phase may take months to years and is associated with fibrotic changes in the orbital tissue. The fibrosis is largely responsible for residual disease features such as adipose tissue expansion, proptosis and chronic eye movement dysfunction and determines the 'severity' of the disease.[2, 5, 8] Assessment of the

natural course of non-treated patients with mild-to-moderate GO revealed that ~22% of patients showed definite improvement, ~43% showed minor improvement, ~22% had no improvement and ~14% got worse.[9] Although GO is predominantly a mono-phasic disease; around 5% of GO patients experience a new flare up (Figure 2).[10]

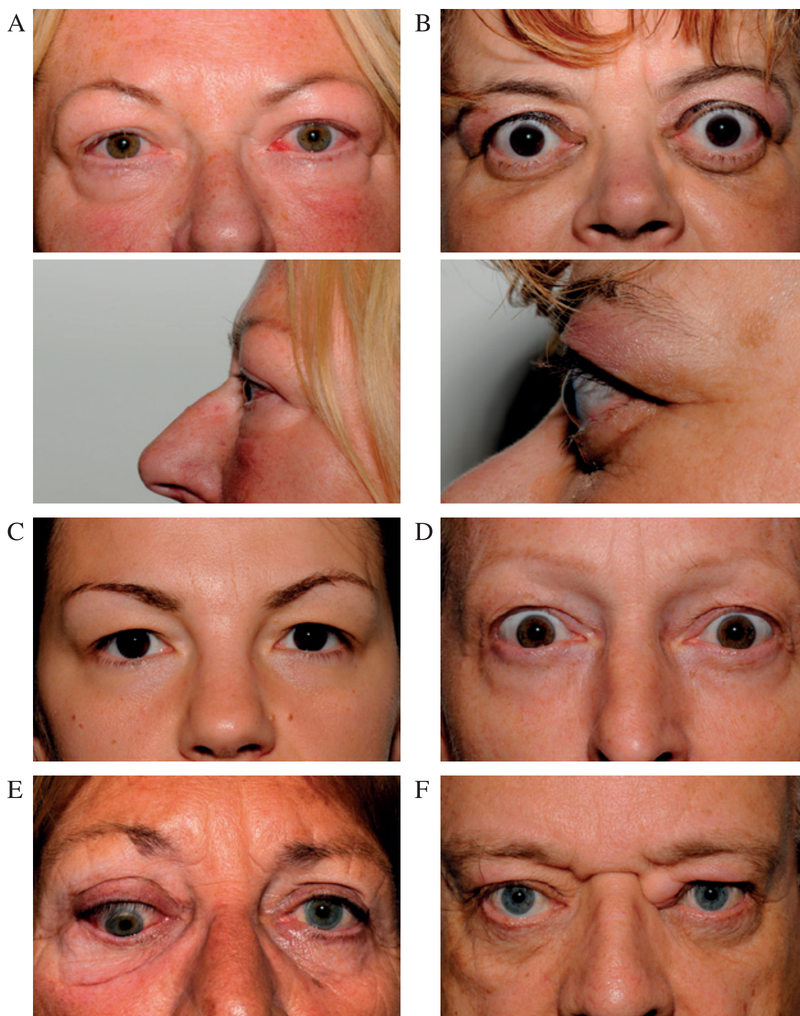


Figure 1 Clinical presentations of Graves' ophthalmopathy. A: moderate-to-severe, active GO showing asymmetrical peri-ocular swelling and (facial) redness, B: moderate-to-severe, inactive GO showing proptosis and eyelid retraction, C: young patient with mild-to-moderate, inactive GO showing eyelid retraction which is masked by dermatochalasis, D: older patient with mild-to-moderate, inactive GO showing upper eyelid retraction, E: mild-to-moderate, inactive GO showing asymmetrical strabismus of the right eye, and F: mild-to-moderate, inactive GO showing an asymmetrical periorbital adipose tissue prolaps. Courtesy of Dr. A.D.A. Paridaens

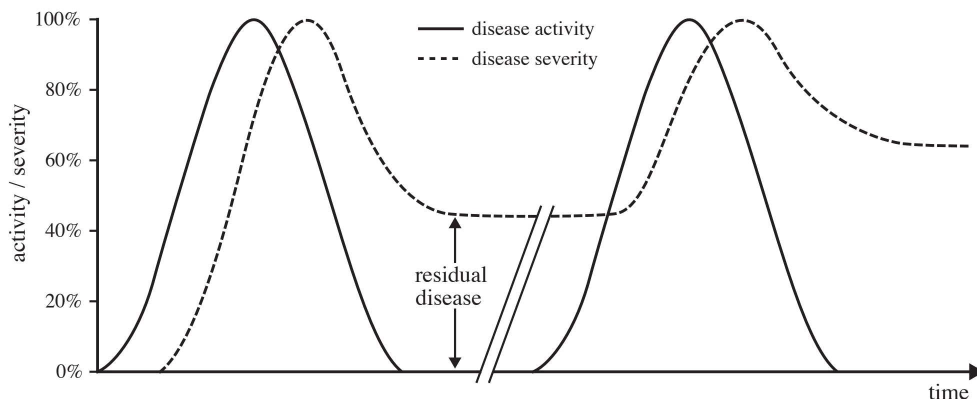


Figure 2 The natural course of GO generally involves a phase with active disease, which eventually subsides. The severity of GO follows the activity of GO and declines after resolvement of the activity of GO but residual disease features stay. Some patients suffer recurrent disease. Figure adapted from Cawood *et al*. 2004

Treatment of Graves' ophthalmopathy

GO patients are difficult to treat and treatment options are limited and did not change substantially in the last two decades. This is most likely related to the complex pathophysiology of GO and our limited understanding of this.

The management of hyperthyroidism and especially the prevention of thyroid hormone level fluctuations are essential in the treatment of GO. Anti-thyroid drugs such as the thionamides are a very efficient and rapid method to control hyperthyroidism. They restore euthyroidism and indirectly beneficially affect GO as well. However, relapses of hyperthyroidism (and worsening of GO) are frequently observed after drug withdrawal.[11-14] Radioactive iodine is a safe and widely used method to treat GD and is very efficient in establishing hypo- or euthyroidism. However, around 15-20% of patients (predominantly smokers) treated with radioactive iodine encounter worsening of their GO, which is probably caused by a rise in thyroid antigens due to thyroid damage that ameliorate the immune response. Concomitant treatment with low dose oral steroids efficiently prevents this side-effect.[13-15] Subtotal thyroidectomy is another effective method to treat hyperthyroidism, without having negative effects on the course of GO, but is not frequently used because of surgical complications.[13, 16]

The clinical activity and severity of GO is important in determining the appropriate treatment regimen. Mild-to-moderate GO is generally approached in a wait-and-see manner, and predominantly requires local measures, such as artificial tears, ointments and prisms.[12-13] Recently, 6-month supplementation of the trace-element selenium was reported to have positive effects on the course of GO and the related quality of life in patients with mild-to-moderate GO.[17] However, severe side-effects may occur upon chronic supplementation

of selenium.[18-19] Immunosuppressive drugs, such as oral corticosteroids, are usually not indicated for the treatment of mild-to-moderate GO.

It is more difficult to define a treatment regimen for patients with moderate-to-severe and sight-threatening GO. In patients with active disease, the use of corticosteroids is indicated to dampen the ongoing inflammation and especially a course of high-dose pulses of intravenous corticosteroids showed beneficial effects in ~80% of such patients.[13, 20-22] When the activity of GO is dampened, rehabilitative surgery may be indicated to correct residual disease features of the patients. Sight-threatening complications such as congestive optic neuropathy or corneal ulcerations need immediate treatment with high-dose intravenous corticosteroids and, in case of poor responsiveness, additional orbital decompressive surgery has to be performed.[13, 20, 23]

Other reported treatment options in Graves' ophthalmopathy

Immunomodulating agents are especially indicated for early intervention in the active inflammatory phase of GO to obtain the highest treatment effect and the lowest disease severity (Figure 3). In addition to the previously mentioned well-known en well-accepted therapeutic approaches, other treatments such as orbital radiotherapy and various immunomodulating drugs (e.g. somatostatin analogs, cyclosporine, intravenous immunoglobulins (IVIG) and biologicals) have also been tested in GO.[21-22]

Orbital radiotherapy has long been used for treatment of especially active GO patients, as lymphocytes are very sensitive to its cytotoxic effects. Improvement of diplopia was observed, but no improvement was observed in clinical activity score (CAS), proptosis or lid aperture and due to potential long-term side effects its use has gradually declined.[24-26]

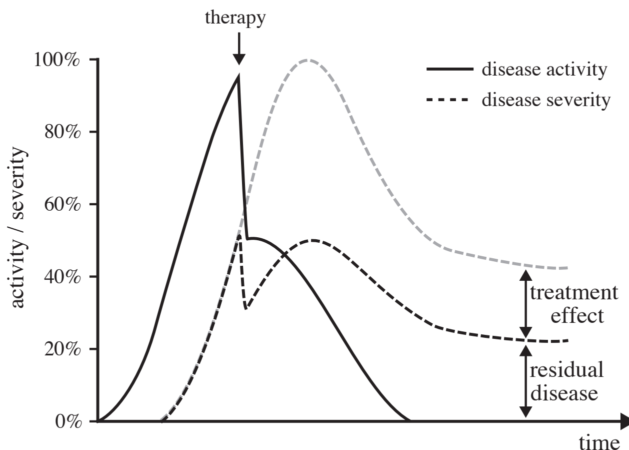


Figure 3 Immunomodulatory treatments are preferably administered in the early and active phase of GO to obtain the greatest treatment effect on the disease severity. Figure adapted from Cawood *et al*. 2004

Somatostatin is a peptide that inhibits the release of growth hormone by the pituitary. Somatostatin receptor subtypes (sst1-5) are broadly expressed on cells, amongst which lymphocytes (sst3) and fibroblasts (sst1).[27] Somatostatin and its analogs have shown therapeutic potential in various inflammatory disorders. Although initial studies suggested therapeutic value of somatostatin analogs for the treatment of GO[28-29], a few randomized controlled trials showed only marginal and non-significant differences compared to controls.[30-33] This could however be expected, as commercial available somatostatin analogs predominantly target sst2 and sst5, which are not the somatostatin receptor subtypes expressed in GO. These data, together with the high costs, limits the applicability of somatostatin for the treatment of GO. Still, novel long-acting analogs with a broader somatostatin receptor binding capacity have been developed, which may prove valuable for the treatment of GO in the future.[34]

Cyclosporine, a drug that especially targets T cells, was shown to reduce CAS and the relapse rate of GO in combination with corticosteroids. Treatment with cyclosporine alone was less effective than treatment with only oral corticosteroids.[35-36] IVIG treatment was as effective as corticosteroids in patients with active GO, but is not widely used due to the accompanying high costs.[37-38]

TNF-α-targeting therapies have also been tested in GO. Paridaens and co-workers demonstrated clinical improvement of especially soft tissue signs (e.g. peri-ocular chemosis and erythema) in 10 GO patients treated with etanercept, a TNF receptor – Fc fusion protein.[39] Case reports also reported beneficial effects of infliximab, a chimeric monoclonal anti-TNF-α antibody, in the treatment of GO.[40-41] However, no randomized controlled trials have been performed with these drugs yet.

Recently, various case studies and open-label pilot studies have shown beneficial effects of B-cell ablative therapy with rituximab (an anti-CD20 antibody) on GO.[42-45] The mechanism through which rituximab affects GO is still unclear. Peripheral and orbital $CD20^+$ B cells and possibly also orbital T cells were found depleted[46-48], but autoantibody levels and serum interleukin (IL)-6 and interleukin-6 receptor (IL-6R) levels do not change.[49] This indicates that the therapeutic potential of rituximab is most likely not mediated via attenuation of the humoral (autoantibody) reactions. Probably, rituximab affects GO via a local decrease in cytokine production and reduced antigen presentation by B cells (likely in lymph nodes) and indirectly through the increase of T regulatory cell numbers.[42, 50]

Severe adverse effects of rituximab treatment in GO patients are uncommon. However, El Fassi *et al*. recently showed serious articular and gastro-intestinal side effects in GD patients with or without GO.[51] In addition, rituximab was found ineffective in a corticosteroid-resistant GO patient.[52] Thus, although initial reports were promising, results from ongoing randomized clinical trials that investigate the effects of rituximab in larger cohorts of GO

patients are warranted before definite conclusions can be drawn regarding the efficacy, safety and usefulness of rituximab in the treatment of GO.[50]

Previous data illustrate that therapy for GO has not much improved in the last two decades and still heavily depends on corticosteroids and surgical decompression. The studies with rituximab and TNF-α inhibitors illustrate the potential of therapeutics that specifically target immune cells or their mediators in GO. However, more insight into the pathophysiology of GO and especially the cell types and mediators involved herein is essential to develop new treatment strategies.

DETERMINANTS IN THE DEVELOPMENT OF GRAVES' OPHTHALMOPATHY

Comparable to other autoimmune diseases, women have a higher risk to develop GO, which is around fourfold higher than for men.[2, 5] Male patients do however tend to develop a more severe phenotype. Furthermore, the prevalence increases from Asian to Caucasian to African populations.[53] Although this difference may be genetically determined, culture and life-style variations likely also contribute, as both endogenous (genetic factors, higher age, male sex) and exogenous (smoking, hypo/hyperthyroidism, iodine treatment) factors contribute to the development and the severity of GO.[53] The presence of such risk factors is important for the development of GO, as the presence of GD is necessary, but not required for the development of GO.[54]

Genetics

Genetic predisposition for GO development largely parallels that of GD (as discussed in chapter 1). The most important genes in which specific variations predispose for GO are those encoding CTLA4, PTPN22, CD40, TSHR and thyroglobulin.[53, 55-56] In addition, HLA-DR3 is also associated with pre-disposition for GO. Several studies using relatively small patient cohorts also revealed associations between polymorphisms in genes encoding Toll-like receptor-9[57], CD86[58], transforming growth factor (TGF)-β, IL-4, IL-10, IL-1α, interleukin-1 receptor antagonist (IL-1Ra), IL-12, interferon (IFN)-γ [59-61], tumour necrosis factor (TNF)-α[59, 62], intercellular adhesion molecule (ICAM)-1[63] and IL-23R[64], and GO. The reported associations however vary considerably between different populations and the majority of these studies lack numbers and power to detect associations with the occurrence and severity of GO.[56] Thus, although variations in genes, especially those encoding immunological factors, have been associated with GO, large and well-controlled studies are awaited before the exact contribution of specific gene variations can be determined. However, considering the clinical

heterogeneity of GO, the relatively high number of genetic candidates possibly involved, and especially the low relative risks of these genetic factors, environmental factors are likely more important determinants in GO development and severity than gene polymorphisms.[53, 65]

Smoking

Smoking is the best-known and strongest risk factor for the development and deterioration of GO. A dose-dependent relation exists between the number of pack years and the development of GO, with current smokers having a higher risk than past smokers.[66] Smoking also increases the likelihood of GO progression after radioiodine therapy and delays or decreases the responsiveness to treatment with steroids or orbital irradiation.[67] The detrimental effect of smoking on GO is considered to be due to induction of hypoxia. Hypoxia modulates cytokine networks and enhances HLA-DR expression on fibroblasts.[53, 66] Moreover, *in vitro* models have shown that cigarette smoke extract enhances adipogenesis and glycosaminoglycan production by orbital fibroblasts.[68]

Mechanical factors

The occurrence and the clinical course of GO is partly determined by mechanical factors related to the orbital anatomy. Expansion of the orbital tissue within the confined space of the orbit is a clear determinant of the clinical features of GO. Orbital tissue expansion impairs the venous and lymphatic outflow of the orbit, which subsequently leads to chemosis, peri-orbital edema and inflammation. The importance of mechanical factors in the course of GO is further illustrated by the rapid alleviation of clinical symptoms after decompressive surgery. Finally, only 6-14% of GO patients have unilateral disease, in which variations of the local orbital anatomy most likely play a role.[53, 69-70]

Thyroid hormone levels

Thyroid hormone level fluctuations, predominantly those associated with the occurrence of hypothyroidism after treatment of hyperthyroidism, is another important risk factor for the development of GO. Clearly, early and adequate stabilization of thyroid hormone levels decreases the risk and severity of GO in GD patients.[53, 71-72] In addition, TSHR stimulating antibody levels correlate positively with the clinical activity and severity of GO and also decrease and stabilize upon adequate treatment of the hyperthyroidism.[73-74] This may be related to the stimulatory effects that TSHR stimulating antibodies exert on orbital fibroblasts (as will be described later in this chapter).

Radioactive iodine

Treatment of GD hyperthyroidism with radioiodine is also associated with an increased risk for development and progression of GO. This is likely related to excessive release of thyroid antigens upon thyroid-toxic radioiodine treatment and the subsequent aggravation of the immune reaction, but the contribution of concurrent fluctuations in thyroid hormone levels cannot be ruled out. Prophylactic treatment with steroids very effectively prevents the adverse events of radioiodine treatment.[53, 75]

THE PATHOPHYSIOLOGY OF GRAVES' OPHTHALMOPATHY

The pathophysiology of GO comprises various cells that interact with each other and as such contribute to orbital inflammation and tissue expansion, either via direct cell-cell contact or via secreted factors. The immune cells involved in GO include at least T cells, B cells, monocytes, macrophages and mast cells[76-81], and will be discussed briefly hereafter. The orbital fibroblast is considered to fulfill a central role in GO and is a main focus in this thesis. Therefore, the role of orbital fibroblasts in GO will be discussed in more detail.

Leukocytes in Graves' ophthalmopathy

T cells

T cells that infiltrate the orbital tissue in GO predominantly express the adhesion molecules very late antigen (VLA)-4 and lymphocyte function associated protein (LFA)-1. Their migration is further facilitated by ICAM-1, vascular cell adhesion molecule (VCAM)-1 and CD44, which are expressed at elevated levels by orbital fibroblasts from GO patients.[82-86] T cells in GO orbital tissues are predominantly memory T cells[78] and mainly express the γδ T-cell receptor (TCR).[79] The TCR repertoire is restricted in early GO orbital tissue, suggesting orbit-antigen specific recruitment and expansion. T cells in orbital tissue from later stages of GO exhibit a much broader TCR repertoire.[87-88]

The infiltrated T cells communicate with target cells such as orbital fibroblasts via the secretion of soluble mediators (cytokines) or via direct cell-cell contact. In early GO, T cells predominantly produce T helper 1 (Th1) cytokines (e.g. IL-2, IFN-γ, TNF-α), which shifts towards the production of Th2 cytokines (e.g. IL-4, IL-5, IL-10) in late GO.[89-93] A predominance of Th2 cytokines is linked to tissue remodelling and fibrosis[94], which typically occurs during the later stages of GO.

T cells from GD patients specifically recognize autologous orbital fibroblasts [95-96], a process in which TCR interaction with certain TSHR epitopes expressed on orbital fibroblasts can

be involved.[97] In addition, cellular interaction between CD154 (also called CD40-Ligand) expressed by T cells and CD40 expressed by orbital fibroblasts also leads to production of various inflammatory mediators by orbital fibroblasts.[98-101] This data suggests that orbital fibroblasts are prime targets of the T-cell response in GO, which is further supported by *in vitro* studies showing that T cells stimulate proliferation, glycosaminoglycan production and adipogenic differentiation of orbital fibroblasts.[102-103]

Besides the effects of T cells on orbital fibroblasts, the CD40-CD154 co-stimulatory signal together with HLA class II – TCR interactions between B and T cells are essential to elicit a proper humoral immune response by B cells, which is evidently involved in GD and GO (see hereafter).[104] Conversely, GO patients have elevated levels of insulin-like growth factor-1 receptor (IGF-1R)-positive T cells which may become activated by IGF-1R autoantibodies that can be present in GO patients.[105] The exact role of this latter finding is so far unclear, but altogether it is clear that T cells contribute to the pathophysiology of GO.

B cells

Only few B cells are present in orbital tissue from GO patients.[77-78] Despite this, the concomitant occurrence of GO and Graves' hyperthyroidism suggests that TSHR stimulating antibodies play an important role in the development of GO. This is further supported by the positive correlation of the clinical activity of GO with TSHR autoantibody levels and the elevated expression of TSHR in GO orbital tissues.[81, 106] Besides TSHR autoantibodies, stimulating autoantibodies against the IGF-1R may also contribute to GO via activation of orbital fibroblasts.[107-109] Confirmation of these latter findings by other research groups is, however, eagerly awaited.

Autoantibodies directed against other proteins such as calsequestrin (a Ca^{2+} binding protein expressed in eye and skeletal muscle), G2S (the terminal fragment of the transcription factor FOXP1 that is expressed in eye muscle, thyroid and many other organs) and flavoprotein (a subunit of the mitochondrial enzyme succinate dehydrogenase that is expressed in various tissues such as liver, eye muscle and skeletal muscle) can also be detected in GO patients but are not specific.[110-113] Nevertheless, the prevalence of especially autoantibodies against calsequestrin and flavoprotein is increased amongst GO patients and they are sensitive markers of ocular muscle involvement.[111, 114] Recently, autoantibodies against collagen type-XIII were found to be increased in GO, especially in patients with active disease.[110-111] However, the clinical and pathological significance of these autoantibodies in GO still remains unclear.

The contribution of B cells to GO is nicely illustrated by the rapid clinical improvement that is achieved with B-cell-directed therapy, for instance with rituximab.[42, 45, 48, 50] Recent reports showed that autoantibody levels and serum cytokine levels remained unchanged in GO patients during rituximab treatment.[46-47, 50] Therefore, the rapid clinical improvement

achieved with rituximab treatment is very likely more related to the fact that B cells in GO also exert antigen presenting functions and produce many different pro-inflammatory and regulatory cytokines, rather than its effect on antibody levels and production.[50]

Monocytes and macrophages

Myeloid cells are well-known participants in inflamed tissues and macrophages participate in early as well as later pathophysiological processes of GO.[77-78, 115] GD patients without GO have increased monocyte numbers in their peripheral blood, while monocyte numbers are decreased in GD patients with GO, suggesting specific recruitment of monocytes into the orbital tissue.[79, 116] Serum levels of monocyte migration inhibitory factor (MIF), a factor which inhibits random tissue migration of monocytes under inflammatory conditions and facilitates localized inflammation, are increased in GO patients and may reflect orbit-localized monocyte infiltration.[117] Once infiltrated into the orbital tissue, the monocytes differentiate into tissue macrophages.[79, 116] The number of orbital tissue macrophages decreases slightly when active (early) GO progresses towards the inactive (late) phase of GO, but still remains elevated compared to control orbital tissue.[78-79, 115] The activity of tissue macrophages is also increased in GO, with resultant elevated production of cytokines that contribute to the inflammatory process in all stages of GO.[115, 118] Nevertheless, although monocytes and macrophages are prominently present in all stages of GO, limited insight exists on their exact participation in the pathophysiology of GO.

Mast cells

The presence of mast cells in orbital tissues from GO patients has already been noticed for a long time.[119] The importance of mast cells in GO is underscored by the improvement of tearing, itching and dryness of the eyes in a small cohort of GO patients treated with the mast cell stabilizing drugs montelukast and ceterizine.[120] Despite this, the exact role that mast cells fulfill in the pathophysiology of GO is largely unknown. In GO orbital tissue mast cells are mostly located in close proximity to adipocytes or fibroblasts and show features of degranulation.[81] This suggests that they might regulate orbital fibroblast and adipocyte activity. So far, however, only a limited number of *in vitro* studies explored the effect of mast cells on orbital fibroblast behaviour. These studies revealed that mast cells stimulate the production of hyaluronan and prostaglandin E2 (PGE_2) by orbital fibroblasts [121-122], processes in which mast cell-derived prostaglandin D2 (PGD_2) [121] and CD40 – CD154 ligation between orbital fibroblasts and mast cells are involved.[98, 101]

The reason of the increased mast cell numbers in GO orbital tissue is unknown, but stem cell factor (SCF, a growth factor for mast cells) is increased in serum from GD patients and may facilitate mast cell accumulation.[123] Serum levels of IgE can be increased in GD

patients and correlations between elevated serum IgE levels and the presence of GO have been described.[124-125] In addition to this, immunohistochemical studies revealed the presence of IgE in GO orbital tissue.[126] IgE binds FcRε on mast cells and upon interaction with an antigen, FcRε cross-linking causes degranulation of mast cells.[127] Some investigators found IgE molecules that specifically recognized TSHR in GO patients[128] which may possibly be involved in regulating mast cell recruitment and degranulation in GO. Although these data indicate an important role for mast cells in GO, further studies are required to delineate the exact contribution of mast cells to the pathophysiology of GO.

Orbital fibroblasts in Graves' ophthalmopathy

Fibroblasts represent the most abundant cell type in orbital tissue and have for a long time been seen as passive, inert cells with largely structural roles. However, orbital fibroblasts are now recognized as a heterogeneous cell population that fulfill complex functions in tissue homeostasis, both under normal and pathologic circumstances. Orbital fibroblasts are also potent regulators of immune cell recruitment and activation. Because of these features, orbital fibroblasts are nowadays considered as the central cell type in the pathophysiology of GO, as will be discussed hereafter and is depicted in Figure 4.

Orbital fibroblasts display a unique phenotype

Depending on the tissue they are located in, fibroblasts express distinct phenotypic features [129-130], indicating that the local tissue environment largely influences fibroblast behaviour. In addition to the anatomical location, orbital fibroblasts are from neuro-ectodermal origin, while most other tissue fibroblasts are from mesenchymal origin.[130]

Orbital fibroblasts have been shown to respond more vigorously to certain stimuli than fibroblasts from other anatomical locations. For instance, activation by IL-1β, TNF-α, leukoregulin or CD40 – CD154 ligation results in significantly higher cytokine, prostaglandin and plasminogen-activator inhibitor type-1 (PAI-1) production by orbital fibroblasts compared to other fibroblasts.[98-99, 131-134] Differences in the regulation of cell signalling pathways very likely underlie this phenomenon, but this hypothesis needs further studies.

Orbital fibroblasts from GO patients have also repeatedly been reported to exhibit different features compared to orbital fibroblasts from healthy controls. GO orbital fibroblasts have, for instance, a reduced basal production of IL-1Ra[135-136], exhibit increased expression of CD40[99], Thy1[137] and IGF-1R[109] and have a higher proliferation rate.[138] These data, however, are inconclusive, as conflicting data also exist.[139-140]

Orbital fibroblasts comprise a heterogeneous population that can be divided into two subpopulations based on the surface expression of the glycoprotein Thy1.[141] It has been reported that ~40% of the orbital fibroblasts are $Thy1^-$, and especially this population

differentiates into mature adipocytes upon stimulation with peroxisome proliferator-activated receptor gamma (PPAR-γ) agonists or cyclic adenosine monophosphate (cAMP)-enhancing agents.[141-143] In addition, Thy1$^-$ orbital fibroblasts produce higher levels of cytokines, amongst which IL-6, than Thy1$^+$ orbital fibroblasts. Around 60% of orbital fibroblasts express Thy1 and these fibroblasts have the potential to differentiate into myofibroblasts upon TGF-β treatment and produce higher levels of PGE_2 than their Thy1$^-$ counterparts.[137, 141, 144] From these data, it can be hypothesized that in GO the Thy1$^-$ orbital fibroblasts fulfill a role in orbital inflammation and adipogenesis, while Thy1$^+$ cells may be predominantly involved in the fibrotic component. This, however, needs further research.

Altogether, these data illustrate that orbital fibroblasts exhibit a unique phenotype which may explain the specific orbital involvement in patients with GD. This notion is supported by the fact that pretibial skin fibroblasts do share some very specific characteristics with orbital fibroblasts (e.g. TSHR expression) and that pretibial myxedema is another extra-thyroidal complication of GD.[131, 145-146]

Orbital fibroblasts and orbital inflammation

Orbital fibroblasts as a target for autoantibodies

The highest prevalence of GO is observed in GD patients with the highest TSHR autoantibodies, and TSHR autoantibody levels correlate positively with the clinical activity scores.[73-74, 147-148] This suggests a role for TSHR autoantibodies and orbital TSHR expression in the development of GO. TSHR is indeed expressed in orbital tissues, especially on orbital fibroblasts, and its expression is increased in orbital tissue and on orbital fibroblasts from GO patients.[149-152] In line with this, the level of TSHR expression in GO orbital tissues correlates positively with disease activity.[81, 93, 153]

Several lines of evidence suggest a role for TSHR activating autoantibodies in the pathogenesis of GO, but only few studies examined the effect of these autoantibodies on orbital fibroblasts. Activation of the TSHR on orbital fibroblasts by TSH or TSHR stimulating antibodies induce cAMP signalling, ICAM-1 expression, HLA-DR expression and IL-6 production.[154-156] Furthermore, TSHR-dependent activation of the phosphatidyl inositol 3 (PI3) kinase pathway is involved in adipogenic differentiation of orbital fibroblasts.[157-159] TSHR activation on orbital fibroblasts also results in increased hyaluronan production.[160-161] Despite the fact that these data point at an important role for TSHR activation on orbital fibroblasts in GO, limited knowledge exists on factors that regulate TSHR expression on orbital fibroblasts.

Few studies tried to identify factors that enhance TSHR expression by orbital fibroblasts. Stimulation of orbital fibroblasts with TSH was found to increase TSHR expression on orbital fibroblasts[162], but so far the differentiation of Thy1$^-$ orbital fibroblasts into mature

adipocytes with the concomitant increase in TSHR expression is regarded as the major cause of elevated orbital TSHR expression in GO.[144, 163-165] IL-6 and IL-1 further enhance adipogenic differentiation and TSHR expression by orbital fibroblasts, while IL-1α and IL-4 do not affect these processes.[140, 166] TNF-α and IFN-γ inhibit adipogenic differentiation and TSHR expression by orbital fibroblasts, while TGF-β reduces TSHR expression without influencing adipogenesis.[165] PPAR-γ agonists are also potent inducers of adipogenesis and TSHR expression by orbital fibroblasts.[143] Nevertheless, more insight into factors that regulate orbital TSHR expression is needed as this can contribute to improvement of treatment strategies.

IGF-1R is constitutively expressed at a high level on orbital fibroblasts and may also be a target for autoantibodies in GO.[106] Initially, IgG from GD patients was found to inhibit IGF-1 binding to IGF-1R.[167] Smith and co-workers subsequently identified that serum from GD patients contained IGF-1R stimulatory antibodies that induced the production of the T-cell chemoattractants IL-16 and CCL5, as well as hyaluronan.[108-109] More recently, physical and functional interactions between the TSHR and the IGF-1R were found in orbital fibroblasts, pointing at a functional interplay between TSHR autoantibodies, IGF-1R autoantibodies and their receptors in GO.[168] The effect of IGF-1R autoantibodies may not be limited to orbital fibroblasts in GO, but may also influence T cells and B cells, as fractions of IGF-1R-positive T cells and B cells are increased in GD patients.[169-171] The production of IGF-1R autoantibodies is most likely a consequence of thyroid autoimmunity rather than a cause, as illustrated by the formation of IGF-1R autoantibodies in mice immunized with TSHR.[172] Studies on the exact role and significance of IGF-1 receptor autoantibodies in GD/GO are therefore still required.

Orbital fibroblasts and inflammatory mediators

Orbital fibroblasts respond to inflammatory mediators but themselves also produce inflammatory factors and are thus involved in the shaping and maintenance of the orbital inflammatory environment.[173]

Recruitment of inflammatory cells into tissue requires the expression of adhesion molecules on inflammatory cells and tissue cells. GO patients have increased serum levels of soluble(s)-ICAM-1, sVCAM-1 and sELAM-1, which reflects (orbital) tissue infiltration by leukocytes.[174-177] Also, vascular endothelium within the orbital and perimysial connective tissue from GO patients expresses increased levels of ICAM-1, VCAM-1, ELAM-1 and CD44, with active GO patients showing the highest expression levels.[84-85, 178-179] Infiltration of leukocytes into orbital tissue especially depends on ICAM-1 and VCAM-1.[83, 175] ICAM-1 expression on orbital fibroblasts is markedly increased upon stimulation with inflammatory factors such as IL-1α, IFN-γ, TNF-α, but also by bovine TSH and immunoglobulins from GD patients (GD-IgG).[156, 175, 180]

CD40 expression is increased on orbital fibroblasts from GO patients[99] and CD40 – CD154 ligation is involved in the physical interaction between orbital fibroblasts and T cells and leads to increased orbital fibroblast proliferation.[103] CD40 ligation on orbital fibroblasts also induces expression of ICAM-1 and the production of cytokines (e.g. IL-6, IL-8, CCL2, IL-1α) and prostaglandins (e.g. PGE_2).[98-101]

The composition of inflammatory mediators within the orbital environment strongly influences orbital fibroblast behaviour. In early GO a Th1 cytokine-dominated environment exists, which shifts to a Th2-dominated cytokine pattern in late GO.[89, 181] Cytokines present in the early/active phase of GO include IL-1α, IL-1β, IL-2, IFN-γ and TNF-α[90-91, 93, 118, 181-182], while in the late phase IL-4, IL-5 and IL-10 dominate.[90-93] Cytokine measurements in serum from GO patients showed increased levels of IL-6, sIL-6R, sTNF-R1 and sTNF-R2[182-183], while levels of other factors such as sIL-2R, sCD30, IL-1α, IL-1Ra were variable.[182] CXCL10 is also increased in serum from GO patients, especially those with active disease.[184-185]

The effect of several cytokines on orbital fibroblast behaviour has been examined. The Th1 cytokine IFN-γ enhances HLA-DR, ICAM-1 and CD40 expression by orbital fibroblasts[82, 99-100, 156, 186], which is consistent with an inflammatory phenotype and which increases their potential to interact with leukocytes. IFN-γ also stimulates the production of the T cell chemoattractants CXCL9, CXCL10 and CXCL11 by orbital fibroblasts, which is synergistically enhanced by TNF-α.[184-185]

IL-1β is produced by many different cell-types and has strong activating capacities for orbital fibroblasts. IL-1β stimulates orbital fibroblasts to produce IL-6, IL-8, CCL2, CCL5, IL-16 and ICAM-1.[99, 109, 140, 187] However, the role of IL-1β and its interactions with other cytokines in the pathophysiology of GO is complex and incompletely understood, as illustrated by the divergent effects of IL-4 and IFN-γ on IL-1β-induced PGE_2 and hyaluronan production by orbital fibroblasts.[188]

PGE_2 stimulates B-cell maturation, activates mast cells and induces Th2 skewing.[189-191] Orbital fibroblasts produce increased levels of PGE_2 upon CD40-CD154 ligation, IL-1β stimulation[188, 192] and leukoregulin stimulation[193-195]. In return, PGE_2 induces the production of IL-6 by orbital fibroblasts via activation of the cAMP pathway.[196]

Orbital fibroblasts are thus important regulators of the orbital inflammatory process and, through the production of inflammatory mediators, they regulate leukocyte recruitment and activation in the orbit.

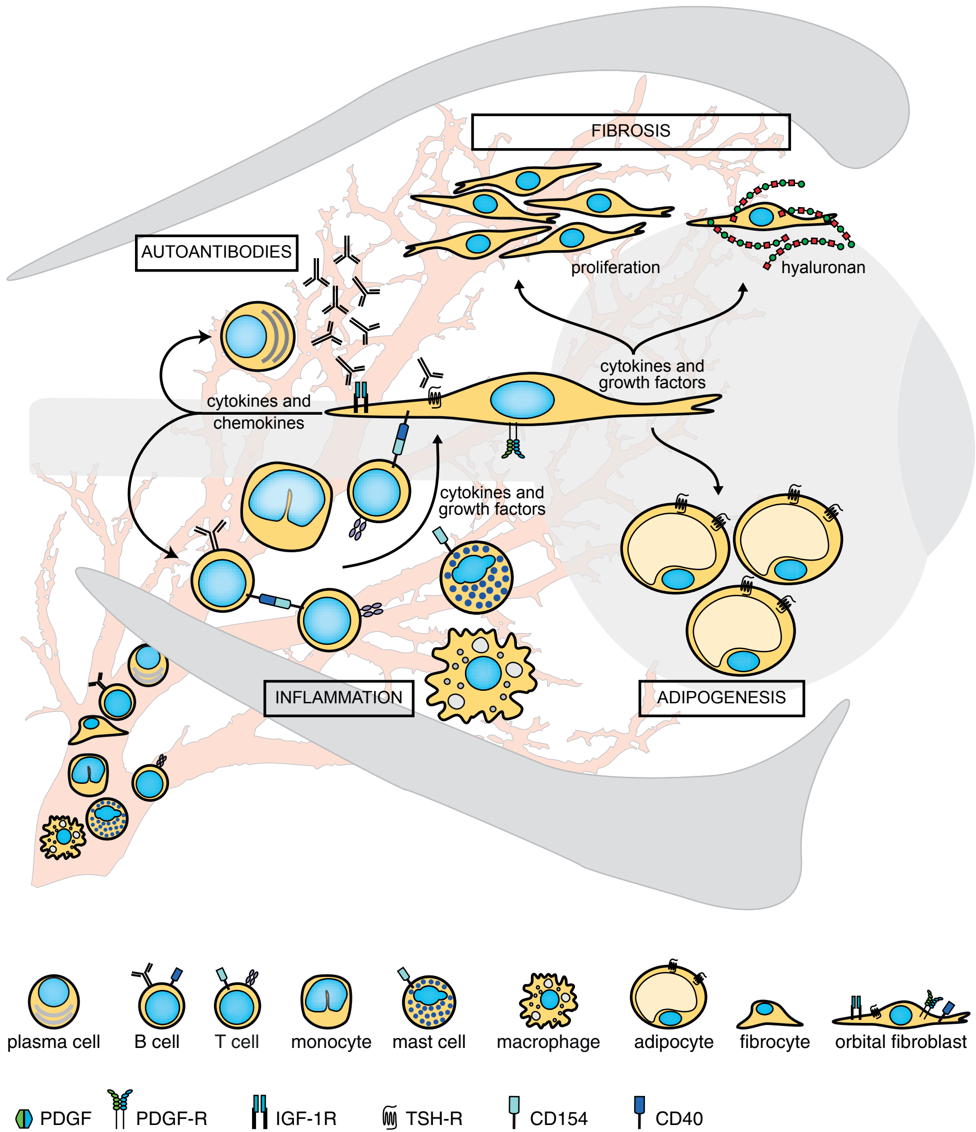


Figure 4 The pathophysiology of Graves' ophthalmopathy has four important components in which the orbital fibroblast is a central participant: inflammation, fibrosis, adipogenesis and the effect of autoantibodies on the orbital fibroblasts.

Orbital fibroblasts and orbital tissue expansion

Tissue expansion is the major contributor to proptosis in GO. Orbital fibroblasts are heavily involved herein, as they contribute to at least three pathophysiological mechanisms (orbital fibroblast proliferation, ECM production and adipogenesis) that are involved in tissue expansion (Figure 4).

Orbital fibroblast proliferation

Cellular proliferation can clearly contribute to tissue expansion and especially fibroblasts exhibit potent mitogenic responses upon activation.[197] The basal proliferative activity of GO orbital fibroblasts was found to be higher than that from orbital fibroblasts from healthy controls.[138] In addition, cellular interactions such as CD40 – CD154 ligation between T cells and orbital fibroblasts, but also various cytokines and growth factors, further increase orbital fibroblast proliferation.[103] Factors such as IL-4, IGF-1, TGF-β and PDGF-BB more strongly enhance the proliferation rate of GO orbital fibroblasts than that from control orbital fibroblasts.[138] Clearly, GO orbital fibroblasts emerge as extremely sensitive to mitogenic factors, which contributes to tissue expansion in GO.

Orbital fibroblasts and extra-cellular matrix deposition

ECM components are important in regulating tissue homeostasis and define the microenvironment in the orbit in which cellular communication, adhesion and migration take place.[198] (Immuno)histological and biochemical analysis of GO orbital tissues reveal predominant deposition of non-sulfated glycosaminoglycans (e.g. hyaluronan, chondroitin sulphate and dermatan sulphate), which are produced by fibroblasts.[198-200] Hyaluronan is regarded as the most important glycosaminoglycan contributing to orbital tissue expansion in GO and is estimated to occupy ~75000 times the volume of collagen. This latter is mainly related to the massive water binding potential of hyaluronan.[198] Synthesis of hyaluronan is controlled by the combined action of three hyaluronan synthases (HAS1-3) that are expressed at the cell surface membrane. In orbital fibroblasts, HAS2 is the constitutively highest expressed hyaluronan synthase.[132]

Stimulatory signals generally lead to higher production of glycosaminoglycans/ hyaluronan in orbital fibroblasts than in fibroblasts from other anatomical locations.[133, 187, 194] Not only soluble factors such as leukoregulin, IL-1, TNF-α, IFN-γ, TGF-β, IGF-1, PDGF and prostaglandins increase hyaluronan production[132-133, 139-140, 201-203], but also cellular interactions between orbital fibroblasts and immune cells can do so.[121-122]

IL-1β stimulates hyaluronan production by orbital fibroblasts by enhancing HAS2 expression in a Ca^{2+} - PKC pathway-dependent manner. [132, 204] IL-1Ra inhibits the stimulatory effect of IL-1β on hyaluronan production by orbital fibroblasts[205], but GO orbital fibroblasts produce significantly lower levels of IL-1Ra than control orbital fibroblasts.[135-136] IL-4 and

IFN-γ further enhance the effect of IL-1β on hyaluronan production by orbital fibroblasts.[188]

Although early studies found no effect of TSH on hyaluronan production by orbital fibroblasts[139, 206], later studies did find stimulatory effects of TSHR or cAMP-dependent signalling on the production of hyaluronan by orbital fibroblasts.[158, 201] GD-IgG were also found to induce hyaluronan production by orbital fibroblasts, which was, dependent on the adipogenic differentiation of the orbital fibroblasts, either caused by TSHR or IGF-1R-directed autoantibodies.[161, 206]

Deposition of ECM is the net effect of production and degradation of these components by various enzymes, such as hyaluronidases or metalloproteinases. Fibrinolysis is also an important process in ECM remodelling and the conversion of inactive plasminogen into active plasmin is essential herein. This conversion is established by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), which are controlled by their natural inhibitor PAI-1.[207] In contrast to fibroblasts from other anatomical locations, orbital fibroblasts do not express hyaluronidases[208] and very low levels of PAI-1. PAI-1 expression by orbital fibroblasts is however highly inducible by factors such as TGF-β or leukoregulin.[209] Non-inflammatory conditions in the orbit are therefore characterized by low PAI-1 activity and a relative high level of ECM degradation. Inflammatory conditions (such as in GO) do however lead to high PAI-1 levels that decreased ECM degradation which subsequently contributes to excessive ECM deposition.

Limited data is available on the contribution of matrix metalloproteinases (MMP) and their natural inhibitors, the tissue inhibitor of metalloproteinases (TIMP), in GO. Expression of MMP-3, which degrades various collagens and proteoglycans, was found increased in orbital tissues from patients with active GO.[210] In addition, IL-1β stimulation was found to increase the production of TIMP-1 by orbital fibroblasts.[211-212] The role and significance of the MMP family and their inhibitors in GO however still needs to be elucidated.

Adipogenesis

Increased levels of adipogenic markers such as leptin, adiponectin, PPAR-γ and Thy1 are found in GO orbital tissue, which indicates the accumulation of adipose tissue in GO.[137, 163, 210, 213] Orbital fibroblasts have the capacity to differentiate into adipocytes, and increased adipogenesis of orbital fibroblasts is a characteristic of GO. Orbital fibroblasts that undergo adipogenic differentiation accumulate lipid vacuoles, and increase their TSHR expression and cytokine production.[141-142] Especially $Thy1^-$ orbital fibroblasts differentiate into mature adipocytes when cultured under serum-free conditions in the presence of insulin, triiodothyronine, carbaprostacyclin, dexamethasone and isobutylmethylxanthine.[141-142, 164] Possibly, differences in the size of the $Thy1^-$ orbital fibroblast pool may be related to the observed differences in adipose tissue expansion between GO patients.[106]

Various factors stimulate adipogenic differentiation of orbital fibroblasts, amongst which IL-1β[68, 140], IL-6[166], PGD_2[121], TSH[157] and TSHR stimulating antibodies.[155] Also the physical interaction between orbital fibroblasts and autologous T cells[102] and cigarette smoke constituents[68, 140] promote adipogenic differentiation by orbital fibroblasts. Remarkably, Th1 cytokines such as TNF-α and IFNγ inhibit adipogenic differentiation by orbital fibroblasts.[140, 165, 214] This fits their role in early inflammation rather than in the later tissue expanding processes, although the Th2 related factor TGF-β also inhibits the adipogenic differentiation by orbital fibroblasts.[165]

2

PPAR-γ is a nuclear transcription factor that is involved in cellular metabolism. Activation of PPAR-γ with thiazolidinediones such as pioglitazone or rosiglitazone is used as a treatment for type 2 diabetes as it increases lipid metabolism.[104] GO patients who were treated with these drugs for type 2 diabetes sometimes encounter orbital deterioration due to PPAR-γ activation.[143, 215-216] Besides their stimulating effect on adipogenesis, PPAR-γ agonists may also inhibit orbital inflammation and remodelling.[104, 217] Therefore, PPAR-γ may be an important regulatory factor in GO and a well-balanced PPAR-γ activity may be beneficial in GO.[104]

Table 1. The influence of some mediators on orbital fibroblast functions

	Involved in:					
factor	inflammatory mediators	adipogenesis	proliferation	hyaluronan production	prostaglandin production	references
IL-1β	IL-6, IL-8, ICAM-1	↑		↑	↑	139-140, 187-188, 201, 218
IFN-γ	ICAM-1, CD40, CXCL9,10,11	↓		↑		133, 184-186
TNF-α		↓		↑	↑	133, 140, 165
Leukoregulin	IL-1β			↑	↑	193-194, 219
CD40-CD154 interaction	IL-6, IL-8, CCL2 IL-1α, ICAM-1		↑	↑	↑	98-99, 101, 103
TSH	ICAM-1	↑		↑		139, 160-161
IGF-1	CCL5, IL-16		↑	↑		108, 202
TGF-β			↑	↑		139, 165, 201, 217

Fibrocytes: a source of orbital fibroblasts?

Besides through proliferation, the orbital fibroblast pool possibly also expands as the result of the recruitment and differentiation of so-called fibrocytes. Fibrocytes are bone-marrow derived cells that express CD45, CD34, CXCR4 and collagen type-1. Fibrocytes rapidly

infiltrate wounds and contribute to the healing process, but are also involved in pathological tissue remodelling (fibrosis). For instance in lung fibrosis, increased numbers of fibrocytes have been found in the fibrotic tissue and in the blood.[220-221]

Smith and co-workers reported increased fibrocyte counts in orbital tissue and peripheral blood from GO patients.[222] The fibrocytes within the orbital tissue expressed the IGF-1R as well as the TSHR and stimulation with TSH increased IL-6 and TNF-α production by these cells. This implies that fibrocytes that infiltrate into the orbital tissue of GO patients may act as targets for especially TSHR autoantibodies.[222-223] However, the mechanism by which orbital fibrocyte recruitment is regulated and whether fibrocyte recruitment correlates with clinical parameters remains to be investigated in GO.

GRAVES' OPHTHALMOPATHY AS A FIBRO-PROLIFERATIVE DISORDER

The central role of orbital fibroblasts in GO with regard to excessive proliferation, ECM production, regulation of inflammatory mediators but also fibrocyte recruitment parallels fibrotic processes in other organ systems, including the lungs, the liver and the skin. GO can therefore be regarded as an inflammatory, fibro-proliferative disorder of the orbit. The pathogenesis of fibro-proliferative/fibrotic disorders, most typically those occurring in the lungs, has been studied extensively. Various mediators have been identified to contribute to these fibrotic reactions, of which platelet-derived growth factor (PDGF) can be regarded as one of the most critical ones.[6, 197, 224-226]

Platelet-derived growth factor

PDGF is a family of growth stimulating polypeptides that exerts broad functions in organogenesis and organ/tissue homeostasis but also under pathological conditions.[227-228] The PDGF family comprises four genes that encode the peptide chains PDGF-A, PDGF-B, PDGF-C and PDGF-D. Disulfide bridging between these PDGF-chains results in the formation of the active homodimers PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD and the heterodimer PDGF-AB (Figure 5).[229-234] The pro-peptide chains of PDGF-A and PDGF-B form dimers intracellularly which have to be activated before secretion by the removal of their N-terminal ends.[228, 234] PDGF-CC and PDGF-DD are secreted as latent molecules with CUB domains attached to their N-terminal ends and are activated by removal of these CUB domains by proteases.[228, 235] Secreted PDGF isoforms can bind to extracellular proteins (e.g α_2-macroglobulin) and to ECM components (e.g. SPARC, heparin sulfate) which thereby fulfill a reservoir function.[228, 234-236]

PDGF dimers act via two receptor chains: PDGF-Rα and PDGF-Rβ. These receptor chains comprise five extracellular immunoglobulin loops and a split intracellular tyrosine

kinase domain. Upon ligand binding, the receptor chains dimerize, leading to PDGF-Rαα, PDGF-Rαβ or PDGF-Rββ dimers. Subsequently, autophosphorylation of the tyrosine residues in the intracellular receptor chains occurs. The PDGF-A and PDGF-C chains are ligands for PDGF-Rα, PDGF-D for PDGF-Rβ and PDGF-B can bind both PDGF-Rα and PDGF-Rβ (Figure 5).[228, 234-235] The PDGF receptor-ligand complex is eventually internalized by endosomes and degraded in lysosomes or the receptor chains are recycled and re-expressed on the cell-membrane. Finally, PDGF-receptor activity can be opposed by the activity of tyrosine phosphatases.[228, 234-235]

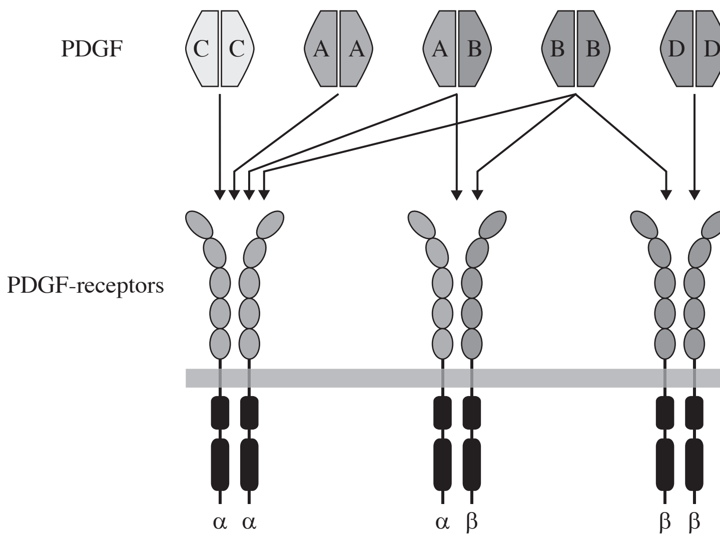


Figure 5 The PDGF family comprises 4 chains that can lead to 5 dimeric isoforms (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD) and 2 receptor chains that can lead to 3 dimeric PDGF receptors (PDGF-Rαα, PDGF-Rαβ and PDGF-Rββ).

PDGF is a major pro-fibrotic growth factor which has been associated with fibro-proliferative disorders in the lungs, kidneys, liver, heart and skin.[226, 228, 237-239] PDGF promotes fibroblast proliferation and chemotaxis, increases the production of ECM and inflammatory cytokines by fibroblasts and facilitates cell adhesion.[237] The expression of the different PDGF isoforms may differ between organs, indicating an organ-specific expression pattern of PDGF.[228, 237, 239] Also, differences in stimulatory capacity can exist between the different PDGF isoforms for certain cell types.[228] This underscores the importance to determine organ-specific PDGF expression and involvement in pathological conditions associated with tissue remodeling and fibrosis. However, no such information is available for PDGF in orbital tissues from GO patients.

REFERENCES

1. Wiersinga WM, Bartalena L. *Epidemiology and prevention of Graves' ophthalmopathy*. Thyroid 2002;12:855-860.
2. Bahn RS. *Graves' ophthalmopathy*. N Engl J Med 2010;362:726-738.
3. Bartalena L, Tanda ML. *Clinical practice*. *Graves' ophthalmopathy*. N Engl J Med 2009;360:994-1001.
4. Weetman AP. *Graves' disease*. N Engl J Med 2000;343:1236-1248.
5. Wiersinga WM, Kahaly GJ (eds). *Graves' Orbitopathy: a multidisciplinary approach*. Basel: Karger; 2007.
6. van Steensel L, Dik WA. *The orbital fibroblast: a key player and target for therapy in graves' ophthalmopathy*. Orbit 2010;29:202-206.
7. Rundle FF. *Management of exophthalmos and related ocular changes in Graves' disease*. Metabolism 1957;6:36-48.
8. Cawood T, Moriarty P, et al. *Recent developments in thyroid eye disease*. BMJ 2004;329:385-390.
9. Perros P, Crombie AL, et al. *Natural history of thyroid associated ophthalmopathy*. Clin Endocrinol (Oxf) 1995;42:45-50.
10. Selva D, Chen C, et al. *Late reactivation of thyroid orbitopathy*. Clin Experiment Ophthalmol 2004;32:46-50.
11. Abraham P, Avenell A, et al. *A systematic review of drug therapy for Graves' hyperthyroidism*. Eur J Endocrinol 2005;153:489-498.
12. Bartalena L. *The dilemma of how to manage Graves' hyperthyroidism in patients with associated orbitopathy*. J Clin Endocrinol Metab 2011;96:592-599.
13. Bartalena L, Baldeschi L, et al. *Consensus statement of the European Group on Graves' orbitopathy (EUGOGO) on management of GO*. Eur J Endocrinol 2008;158:273-285.
14. Hegedus L. *Treatment of Graves' hyperthyroidism: evidence-based and emerging modalities*. Endocrinol Metab Clin North Am 2009;38:355-371, ix.
15. Acharya SH, Avenell A, et al. *Radioiodine therapy (RAI) for Graves' disease (GD) and the effect on ophthalmopathy: a systematic review*. Clin Endocrinol (Oxf) 2008;69:943-950.
16. Stalberg P, Svensson A, et al. *Surgical treatment of Graves' disease: evidence-based approach*. World J Surg 2008;32:1269-1277.
17. Marcocci C, Kahaly GJ, et al. *Selenium and the course of mild Graves' orbitopathy*. N Engl J Med 2011;364:1920-1931.
18. Weissel M. *Selenium and the course of mild Graves' orbitopathy*. N Engl J Med 2011;365:769-770; author reply 770-761.
19. Sheck L. *Selenium and the course of mild Graves' orbitopathy*. N Engl J Med 2011;365:770; author reply 770-771.
20. Zang S, Ponto KA, et al. *Clinical review: Intravenous glucocorticoids for Graves' orbitopathy: efficacy and morbidity*. J Clin Endocrinol Metab 2011;96:320-332.
21. Stiebel-Kalish H, Robenshtok E, et al. *Treatment modalities for Graves' ophthalmopathy: systematic review and metaanalysis*. J Clin Endocrinol Metab 2009;94:2708-2716.
22. Krassas GE, Boboridis K. *Recent developments in the medical treatment of thyroid eye disease*. Orbit 2006;25:117-122.
23. Wakelkamp IM, Baldeschi L, et al. *Surgical or medical decompression as a first-line treatment of optic neuropathy in Graves' ophthalmopathy? A randomized controlled trial*. Clin Endocrinol (Oxf) 2005;63:323-328.
24. Mourits MP, van Kempen-Harteveld ML, et al. *Radiotherapy for Graves' orbitopathy: randomised placebo-controlled study*. Lancet 2000;355:1505-1509.
25. Prummel MF, Terwee CB, et al. *A randomized controlled trial of orbital radiotherapy versus sham irradiation in patients with mild Graves' ophthalmopathy*. J Clin Endocrinol Metab 2004;89:15-20.
26. Gorman CA, Garrity JA, et al. *A prospective, randomized, double-blind, placebo-controlled study of orbital radiotherapy for Graves' ophthalmopathy*. Ophthalmology 2001;108:1523-1534.
27. Ferone D, van Hagen PM, et al. *Somatostatin receptor distribution and function in immune system*. Dig Liver Dis 2004;36 Suppl 1:S68-77.

28. Pasquali D, Notaro A, et al. *Somatostatin receptor genes are expressed in lymphocytes from retroorbital tissues in Graves' disease*. J Clin Endocrinol Metab 2002;87:5125-5129.
29. Pasquali D, Vassallo P, et al. *Somatostatin receptor gene expression and inhibitory effects of octreotide on primary cultures of orbital fibroblasts from Graves' ophthalmopathy*. J Mol Endocrinol 2000;25:63-71.
30. Vaidya B, Shenton BK, et al. *Analysis of peripheral blood T-cell subsets in active thyroid-associated ophthalmopathy: absence of effect of octreotide-LAR on T-cell subsets in patients with thyroid-associated ophthalmopathy*. Thyroid 2005;15:1073-1078.
31. Dickinson AJ, Vaidya B, et al. *Double-blind, placebo-controlled trial of octreotide long-acting repeatable (LAR) in thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 2004;89:5910-5915.
32. Wemeau JL, Caron P, et al. *Octreotide (long-acting release formulation) treatment in patients with graves' orbitopathy: clinical results of a four-month, randomized, placebo-controlled, double-blind study*. J Clin Endocrinol Metab 2005;90:841-848.
33. Stan MN, Garrity JA, et al. *Randomized, double-blind, placebo-controlled trial of long-acting release octreotide for treatment of Graves' ophthalmopathy*. J Clin Endocrinol Metab 2006;91:4817-4824.
34. Bartalena L, Lai A, et al. *Novel immunomodulating agents for Graves orbitopathy*. Ophthal Plast Reconstr Surg 2008;24:251-256.
35. Kahaly G, Schrezenmeir J, et al. *Ciclosporin and prednisone v. prednisone in treatment of Graves' ophthalmopathy: a controlled, randomized and prospective study*. Eur J Clin Invest 1986;16:415-422.
36. Prummel MF, Mourits MP, et al. *Prednisone and cyclosporine in the treatment of severe Graves' ophthalmopathy*. N Engl J Med 1989;321:1353-1359.
37. Baschieri L, Antonelli A, et al. *Intravenous immunoglobulin versus corticosteroid in treatment of Graves' ophthalmopathy*. Thyroid 1997;7:579-585.
38. Kahaly G, Pitz S, et al. *Randomized trial of intravenous immunoglobulins versus prednisolone in Graves' ophthalmopathy*. Clin Exp Immunol 1996;106:197-202.
39. Paridaens D, van den Bosch WA, et al. *The effect of etanercept on Graves' ophthalmopathy: a pilot study*. Eye (Lond) 2005;19:1286-1289.
40. Durrani OM, Reuser TQ, et al. *Infliximab: a novel treatment for sight-threatening thyroid associated ophthalmopathy*. Orbit 2005;24:117-119.
41. Komorowski J, Jankiewicz-Wika J, et al. *Monoclonal anti-TNFalpha antibody (infliximab) in the treatment of patient with thyroid associated ophthalmopathy*. Klin Oczna 2007;109:457-460.
42. Khanna D, Chong KK, et al. *Rituximab treatment of patients with severe, corticosteroid-resistant thyroid-associated ophthalmopathy*. Ophthalmology 2010;117:133-139 e132.
43. Silkiss RZ, Reier A, et al. *Rituximab for thyroid eye disease*. Ophthal Plast Reconstr Surg 2010;26:310-314.
44. Salvi M, Vannucchi G, et al. *Treatment of Graves' disease and associated ophthalmopathy with the anti-CD20 monoclonal antibody rituximab: an open study*. Eur J Endocrinol 2007;156:33-40.
45. El Fassi D, Nielsen CH, et al. *Treatment-resistant severe, active Graves' ophthalmopathy successfully treated with B lymphocyte depletion*. Thyroid 2006;16:709-710.
46. Nielsen JF, El Fassi D, et al. *Evidence of orbital B and T cell depletion after rituximab therapy in Graves' ophthalmopathy*. Acta Ophthalmol 2009;87:927-929.
47. Salvi M, Vannucchi G, et al. *Rituximab treatment in a patient with severe thyroid-associated ophthalmopathy: effects on orbital lymphocytic infiltrates*. Clin Immunol 2009;131:360-365.
48. Salvi M, Vannucchi G, et al. *Efficacy of rituximab treatment for thyroid-associated ophthalmopathy as a result of intraorbital B-cell depletion in one patient unresponsive to steroid immunosuppression*. Eur J Endocrinol 2006;154:511-517.
49. Vannucchi G, Campi I, et al. *Rituximab treatment in patients with active Graves' orbitopathy: effects on proinflammatory and humoral immune reactions*. Clin Exp Immunol 2010;161:436-443.
50. Hegedus L, Smith TJ, et al. *Targeted biological therapies for Graves' disease and thyroid-associated ophthalmopathy. Focus on B-cell depletion with Rituximab*. Clin Endocrinol (Oxf) 2011;74:1-8.
51. El Fassi D, Nielsen CH, et al. *Systemic adverse events following rituximab therapy in patients with Graves' disease*. J Endocrinol Invest 2010.
52. Krassas GE, Stafilidou A, et al. *Failure of rituximab treatment in a case of severe thyroid ophthalmopathy unresponsive to steroids*. Clin Endocrinol (Oxf) 2010;72:853-855.

53. Stan MN, Bahn RS. *Risk factors for development or deterioration of Graves' ophthalmopathy*. Thyroid 2010;20:777-783.
54. Salvi M, Zhang ZG, et al. *Patients with endocrine ophthalmopathy not associated with overt thyroid disease have multiple thyroid immunological abnormalities*. J Clin Endocrinol Metab 1990;70:89-94.
55. Eckstein AK, Johnson KT, et al. *Current insights into the pathogenesis of Graves' orbitopathy*. Horm Metab Res 2009;41:456-464.
56. Bednarczuk T, Gopinath B, et al. *Susceptibility genes in Graves' ophthalmopathy: searching for a needle in a haystack?* Clin Endocrinol (Oxf) 2007;67:3-19.
57. Liao WL, Chen RH, et al. *Toll-like receptor gene polymorphisms are associated with susceptibility to Graves' ophthalmopathy in Taiwan males*. BMC Med Genet 2010;11:154.
58. Liao WL, Chen RH, et al. *The association between polymorphisms of B7 molecules (CD80 and CD86) and Graves' ophthalmopathy in a Taiwanese population*. Ophthalmology 2011;118:553-557.
59. Anvari M, Khalilzadeh O, et al. *Genetic susceptibility to Graves' ophthalmopathy: the role of polymorphisms in proinflammatory cytokine genes*. Eye (Lond) 2010;24:1058-1063.
60. Khalilzadeh O, Anvari M, et al. *Graves' ophthalmopathy and gene polymorphisms in interleukin-1alpha, interleukin-1beta, interleukin-1 receptor and interleukin-1 receptor antagonist*. Clin Experiment Ophthalmol 2009;37:614-619.
61. Khalilzadeh O, Anvari M, et al. *Genetic susceptibility to Graves' ophthalmopathy: The role of polymorphisms in anti-inflammatory cytokine genes*. Ophthalmic Genet 2010;31:215-220.
62. Kamizono S, Hiromatsu Y, et al. *A polymorphism of the 5' flanking region of tumour necrosis factor alpha gene is associated with thyroid-associated ophthalmopathy in Japanese*. Clin Endocrinol (Oxf) 2000;52:759-764.
63. Kretowski A, Wawrusiewicz N, et al. *Intercellular adhesion molecule 1 gene polymorphisms in Graves' disease*. J Clin Endocrinol Metab 2003;88:4945-4949.
64. Huber AK, Jacobson EM, et al. *Interleukin (IL)-23 receptor is a major susceptibility gene for Graves' ophthalmopathy: the IL-23/T-helper 17 axis extends to thyroid autoimmunity*. J Clin Endocrinol Metab 2008;93:1077-1081.
65. Villanueva R, Inzerillo AM, et al. *Limited genetic susceptibility to severe Graves' ophthalmopathy: no role for CTLA-4 but evidence for an environmental etiology*. Thyroid 2000;10:791-798.
66. Hegedius L, Brix TH, et al. *Relationship between cigarette smoking and Graves' ophthalmopathy*. J Endocrinol Invest 2004;27:265-271.
67. Eckstein A, Quadbeck B, et al. *Impact of smoking on the response to treatment of thyroid associated ophthalmopathy*. Br J Ophthalmol 2003;87:773-776.
68. Cawood TJ, Moriarty P, et al. *Smoking and thyroid-associated ophthalmopathy: A novel explanation of the biological link*. J Clin Endocrinol Metab 2007;92:59-64.
69. Bahn RS. *Clinical review 157: Pathophysiology of Graves' ophthalmopathy: the cycle of disease*. J Clin Endocrinol Metab 2003;88:1939-1946.
70. Perros P, Krassas GE. *Graves orbitopathy: a perspective*. Nat Rev Endocrinol 2009;5:312-318.
71. Kung AW, Yau CC, et al. *The incidence of ophthalmopathy after radioiodine therapy for Graves' disease: prognostic factors and the role of methimazole*. J Clin Endocrinol Metab 1994;79:542-546.
72. Tallstedt L, Lundell G, et al. *Does early administration of thyroxine reduce the development of Graves' ophthalmopathy after radioiodine treatment?* Eur J Endocrinol 1994;130:494-497.
73. Gerding MN, van der Meer JW, et al. *Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2000;52:267-271.
74. Ponto KA, Kanitz M, et al. *Clinical Relevance of Thyroid-Stimulating Immunoglobulins in Graves' Ophthalmopathy*. Ophthalmology 2011.
75. Ponto KA, Zang S, et al. *The tale of radioiodine and Graves' orbitopathy*. Thyroid 2010;20:785-793.
76. Weetman AP, Cohen S, et al. *Immunohistochemical analysis of the retrobulbar tissues in Graves' ophthalmopathy*. Clin Exp Immunol 1989;75:222-227.
77. Kahaly G, Hansen C, et al. *Immunohistochemical staining of retrobulbar adipose tissue in Graves' ophthalmopathy*. Clin Immunol Immunopathol 1994;73:53-62.
78. Pappa A, Lawson JM, et al. *T cells and fibroblasts in affected extraocular muscles in early and late thyroid associated ophthalmopathy*. Br J Ophthalmol 2000;84:517-522.

79. Eckstein AK, Quadbeck B, et al. *Thyroid associated ophthalmopathy: evidence for CD4(+) gammadelta T cells; de novo differentiation of RFD7(+) macrophages, but not of RFD1(+) dendritic cells; and loss of gammadelta and alphabeta T cell receptor expression*. Br J Ophthalmol 2004;88:803-808.
80. Pappa A, Calder V, et al. *Analysis of extraocular muscle-infiltrating T cells in thyroid-associated ophthalmopathy (TAO)*. Clin Exp Immunol 1997;109:362-369.
81. Boschi A, Daumerie C, et al. *Quantification of cells expressing the thyrotropin receptor in extraocular muscles in thyroid associated orbitopathy*. Br J Ophthalmol 2005;89:724-729.
82. Heufelder AE, Gorman CA, et al. *Modulation of HLA-DR expression on retroocular fibroblasts from patients with active thyroid-related ophthalmopathy: in vitro effects of agents used in the management of hyperthyroidism and ophthalmopathy*. Exp Clin Endocrinol 1991;97:206-211.
83. Heufelder AE, Bahn RS. *Graves' immunoglobulins and cytokines stimulate the expression of intercellular adhesion molecule-1 (ICAM-1) in cultured Graves' orbital fibroblasts*. Eur J Clin Invest 1992;22:529-537.
84. Heufelder AE, Bahn RS. *Elevated expression in situ of selectin and immunoglobulin superfamily type adhesion molecules in retroocular connective tissues from patients with Graves' ophthalmopathy*. Clin Exp Immunol 1993;91:381-389.
85. Heufelder AE, Bahn RS, et al. *[Detection, localization and modulation of hyaluronic acid/CD44 receptor expression in patients with endocrine orbitopathy] Nachweis, Lokalisation und Modulation der Hyaluronsaure/ CD44-Rezeptor-Expression bei Patienten mit endokriner Orbitopathie*. Med Klin (Munich) 1993;88:181-184, 277.
86. Heufelder AE. *Pathogenesis of ophthalmopathy in autoimmune thyroid disease*. Rev Endocr Metab Disord 2000;1:87-95.
87. Heufelder AE, Herterich S, et al. *Analysis of retroorbital T cell antigen receptor variable region gene usage in patients with Graves' ophthalmopathy*. Eur J Endocrinol 1995;132:266-277.
88. Heufelder AE, Schworm HD, et al. *Molecular analysis of antigen receptor variable region repertoires in T lymphocytes infiltrating the intrathyroidal and extrathyroidal manifestations in patients with Graves' disease*. Exp Clin Endocrinol Diabetes 1996;104 Suppl 4:84-87.
89. Aniszewski JP, Valyasevi RW, et al. *Relationship between disease duration and predominant orbital T cell subset in Graves' ophthalmopathy*. J Clin Endocrinol Metab 2000;85:776-780.
90. Forster G, Otto E, et al. *Analysis of orbital T cells in thyroid-associated ophthalmopathy*. Clin Exp Immunol 1998;112:427-434.
91. Hiromatsu Y, Yang D, et al. *Cytokine profiles in eye muscle tissue and orbital fat tissue from patients with thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 2000;85:1194-1199.
92. McLachlan SM, Prummel MF, et al. *Cell-mediated or humoral immunity in Graves' ophthalmopathy? Profiles of T-cell cytokines amplified by polymerase chain reaction from orbital tissue*. J Clin Endocrinol Metab 1994;78:1070-1074.
93. Wakelkamp IM, Bakker O, et al. *TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients*. Clin Endocrinol (Oxf) 2003;58:280-287.
94. Barron L, Wynn TA. *Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages*. Am J Physiol Gastrointest Liver Physiol 2011;300:G723-728.
95. Grubeck-Loebenstein B, Trieb K, et al. *Retrobulbar T cells from patients with Graves' ophthalmopathy are CD8+ and specifically recognize autologous fibroblasts*. J Clin Invest 1994;93:2738-2743.
96. Otto EA, Ochs K, et al. *Orbital tissue-derived T lymphocytes from patients with Graves' ophthalmopathy recognize autologous orbital antigens*. J Clin Endocrinol Metab 1996;81:3045-3050.
97. Arnold K, Tandon N, et al. *T cell responses to orbital antigens in thyroid-associated ophthalmopathy*. Clin Exp Immunol 1994;96:329-334.
98. Cao HJ, Wang HS, et al. *Activation of human orbital fibroblasts through CD40 engagement results in a dramatic induction of hyaluronan synthesis and prostaglandin endoperoxide H synthase-2 expression. Insights into potential pathogenic mechanisms of thyroid-associated ophthalmopathy*. J Biol Chem 1998;273:29615-29625.
99. Hwang CJ, Afifiyan N, et al. *Orbital fibroblasts from patients with thyroid-associated ophthalmopathy overexpress CD40: CD154 hyperinduces IL-6, IL-8, and MCP-1*. Invest Ophthalmol Vis Sci 2009;50:2262-2268.

100. Sempowski GD, Rozenblit J, et al. *Human orbital fibroblasts are activated through CD40 to induce proinflammatory cytokine production*. Am J Physiol 1998;274:C707-714.
101. Zhao LQ, Wei RL, et al. *The expression of intercellular adhesion molecule-1 induced by CD40-CD40L ligand signaling in orbital fibroblasts in patients with Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2010;51:4652-4660.
102. Feldon SE, O'Loughlin C W, et al. *Activated human T lymphocytes express cyclooxygenase-2 and produce proadipogenic prostaglandins that drive human orbital fibroblast differentiation to adipocytes*. Am J Pathol 2006;169:1183-1193.
103. Feldon SE, Park DJ, et al. *Autologous T-lymphocytes stimulate proliferation of orbital fibroblasts derived from patients with Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2005;46:3913-3921.
104. Lehmann GM, Feldon SE, et al. *Immune mechanisms in thyroid eye disease*. Thyroid 2008;18:959-965.
105. Douglas RS, Brix TH, et al. *Divergent frequencies of IGF-I receptor-expressing blood lymphocytes in monozygotic twin pairs discordant for Graves' disease: evidence for a phenotypic signature ascribable to nongenetic factors*. J Clin Endocrinol Metab 2009;94:1797-1802.
106. Khoo TK, Bahn RS. *Pathogenesis of Graves' ophthalmopathy: the role of autoantibodies*. Thyroid 2007;17:1013-1018.
107. Pang SJ, Liu F, et al. *Tracking the algal origin of the Ulva bloom in the Yellow Sea by a combination of molecular, morphological and physiological analyses*. Mar Environ Res 2010;69:207-215.
108. Pritchard J, Han R, et al. *Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway*. J Immunol 2003;170:6348-6354.
109. Pritchard J, Horst N, et al. *Igs from patients with Graves' disease induce the expression of T cell chemoattractants in their fibroblasts*. J Immunol 2002;168:942-950.
110. De Bellis A, Sansone D, et al. *Serum antibodies to collagen XIII: a further good marker of active Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2005;62:24-29.
111. Gopinath B, Musselman R, et al. *Study of serum antibodies against three eye muscle antigens and the connective tissue antigen collagen XIII in patients with Graves' disease with and without ophthalmopathy: correlation with clinical features*. Thyroid 2006;16:967-974.
112. Lahooti H, Parmar KR, et al. *Pathogenesis of thyroid-associated ophthalmopathy: does autoimmunity against calsequestrin and collagen XIII play a role?* Clin Ophthalmol 2010;4:417-425.
113. Mizokami T, Salvi M, et al. *Eye muscle antibodies in Graves' ophthalmopathy: pathogenic or secondary epiphenomenon?* J Endocrinol Invest 2004;27:221-229.
114. Wall JR, Lahooti H. *Pathogenesis of thyroid eye disease--does autoimmunity against the TSH receptor explain all cases?* Endokrynol Pol 2010;61:222-227.
115. Chen MH, Liao SL, et al. *Role of macrophage infiltration in the orbital fat of patients with Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2008;69:332-337.
116. Quadbeck B, Stucke M, et al. *Dysregulation of TNF/TNFR superfamily members: a systemic link between intra- and extrathyroidal manifestations in Graves' disease*. Scand J Immunol 2006;64:523-530.
117. van der Gaag R, Broersma L, et al. *Circulating monocyte migration inhibitory factor in serum of Graves' ophthalmopathy patients: a parameter for disease activity?* Clin Exp Immunol 1989;75:275-279.
118. Kumar S, Bahn RS. *Relative overexpression of macrophage-derived cytokines in orbital adipose tissue from patients with graves' ophthalmopathy*. J Clin Endocrinol Metab 2003;88:4246-4250.
119. Wegelius O, Asboe-Hansen G, et al. *Retrobulbar connective tissue changes in malignant exophthalmos*. Acta Endocrinol (Copenh) 1957;25:452-456.
120. Lauer SA, Silkiss RZ, et al. *Oral montelukast and cetirizine for thyroid eye disease*. Ophthal Plast Reconstr Surg 2008;24:257-261.
121. Guo N, Baglole CJ, et al. *Mast cell-derived prostaglandin D2 controls hyaluronan synthesis in human orbital fibroblasts via DP1 activation: implications for thyroid eye disease*. J Biol Chem 2010;285:15794-15804.
122. Smith TJ, Parikh SJ. *HMC-1 mast cells activate human orbital fibroblasts in coculture: evidence for up-regulation of prostaglandin E2 and hyaluronan synthesis*. Endocrinology 1999;140:3518-3525.
123. Yamada T, Sato A, et al. *An elevation of stem cell factor in patients with hyperthyroid Graves' disease*. Thyroid 1998;8:499-504.

124. Molnar I, Horvath S, et al. *Detectable serum IgE levels in Graves' ophthalmopathy*. Eur J Med Res 1996;1:543-546.
125. Sato A, Takemura Y, et al. *A possible role of immunoglobulin E in patients with hyperthyroid Graves' disease*. J Clin Endocrinol Metab 1999;84:3602-3605.
126. Raikow RB, Dalbow MH, et al. *Immunohistochemical evidence for IgE involvement in Graves' orbitopathy*. Ophthalmology 1990;97:629-635.
127. Theoharides TC, Kempuraj D, et al. *Differential release of mast cell mediators and the pathogenesis of inflammation*. Immunol Rev 2007;217:65-78.
128. Metcalfe R, Jordan N, et al. *Demonstration of immunoglobulin G, A, and E autoantibodies to the human thyrotropin receptor using flow cytometry*. J Clin Endocrinol Metab 2002;87:1754-1761.
129. Chang HY, Chi JT, et al. *Diversity, topographic differentiation, and positional memory in human fibroblasts*. Proc Natl Acad Sci U S A 2002;99:12877-12882.
130. Kazim M, Goldberg RA, et al. *Insights into the pathogenesis of thyroid-associated orbitopathy: evolving rationale for therapy*. Arch Ophthalmol 2002;120:380-386.
131. Young DA, Evans CH, et al. *Leukoregulin induction of protein expression in human orbital fibroblasts: evidence for anatomical site-restricted cytokine-target cell interactions*. Proc Natl Acad Sci U S A 1998;95:8904-8909.
132. Kaback LA, Smith TJ. *Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 1999;84:4079-4084.
133. Metcalfe RA, Weetman AP. *Stimulation of extraocular muscle fibroblasts by cytokines and hypoxia: possible role in thyroid-associated ophthalmopathy*. Clin Endocrinol (Oxf) 1994;40:67-72.
134. Han R, Tsui S, et al. *Up-regulation of prostaglandin E2 synthesis by interleukin-1beta in human orbital fibroblasts involves coordinate induction of prostaglandin-endoperoxide H synthase-2 and glutathione-dependent prostaglandin E2 synthase expression*. J Biol Chem 2002;277:16355-16364.
135. Muhlberg T, Heberling HJ, et al. *Detection and modulation of interleukin-1 receptor antagonist messenger ribonucleic acid and immunoreactivity in Graves' orbital fibroblasts*. Invest Ophthalmol Vis Sci 1997;38:1018-1028.
136. Muhlberg T, Joba W, et al. *Interleukin-1 receptor antagonist ribonucleic acid and protein expression by cultured Graves' and normal orbital fibroblasts is differentially modulated by dexamethasone and irradiation*. J Clin Endocrinol Metab 2000;85:734-742.
137. Khoo TK, Coenen MJ, et al. *Evidence for enhanced Thy-1 (CD90) expression in orbital fibroblasts of patients with Graves' ophthalmopathy*. Thyroid 2008;18:1291-1296.
138. Heufelder AE, Bahn RS. *Modulation of Graves' orbital fibroblast proliferation by cytokines and glucocorticoid receptor agonists*. Invest Ophthalmol Vis Sci 1994;35:120-127.
139. Korducki JM, Loftus SJ, et al. *Stimulation of glycosaminoglycan production in cultured human retroocular fibroblasts*. Invest Ophthalmol Vis Sci 1992;33:2037-2042.
140. Cawood TJ, Moriarty P, et al. *The effects of tumour necrosis factor-alpha and interleukin1 on an in vitro model of thyroid-associated ophthalmopathy; contrasting effects on adipogenesis*. Eur J Endocrinol 2006;155:395-403.
141. Koumas L, Smith TJ, et al. *Fibroblast subsets in the human orbit: Thy-1+ and Thy-1- subpopulations exhibit distinct phenotypes*. Eur J Immunol 2002;32:477-485.
142. Valyasevi RW, Erickson DZ, et al. *Differentiation of human orbital preadipocyte fibroblasts induces expression of functional thyrotropin receptor*. J Clin Endocrinol Metab 1999;84:2557-2562.
143. Valyasevi RW, Harteneck DA, et al. *Stimulation of adipogenesis, peroxisome proliferator-activated receptor-gamma (PPARgamma), and thyrotropin receptor by PPARgamma agonist in human orbital preadipocyte fibroblasts*. J Clin Endocrinol Metab 2002;87:2352-2358.
144. Koumas L, Smith TJ, et al. *Thy-1 expression in human fibroblast subsets defines myofibroblastic or lipofibroblastic phenotypes*. Am J Pathol 2003;163:1291-1300.
145. Rapoport B, Alsabeh R, et al. *Elephantiasic pretibial myxedema: insight into and a hypothesis regarding the pathogenesis of the extrathyroidal manifestations of Graves' disease*. Thyroid 2000;10:685-692.
146. Stadlmayr W, Spitzweg C, et al. *TSH receptor transcripts and TSH receptor-like immunoreactivity in orbital and pretibial fibroblasts of patients with Graves' ophthalmopathy and pretibial myxedema*. Thyroid 1997;7:3-12.

147. Eckstein AK, Plicht M, et al. *Thyrotropin receptor autoantibodies are independent risk factors for Graves' ophthalmopathy and help to predict severity and outcome of the disease*. J Clin Endocrinol Metab 2006;91:3464-3470.

148. Khoo DH, Ho SC, et al. *The combination of absent thyroid peroxidase antibodies and high thyroid-stimulating immunoglobulin levels in Graves' disease identifies a group at markedly increased risk of ophthalmopathy*. Thyroid 1999;9:1175-1180.

149. Feliciello A, Porcellini A, et al. *Expression of thyrotropin-receptor mRNA in healthy and Graves' disease retro-orbital tissue*. Lancet 1993;342:337-338.

150. Heufelder AE, Bahn RS. *Evidence for the presence of a functional TSH-receptor in retroocular fibroblasts from patients with Graves' ophthalmopathy*. Exp Clin Endocrinol 1992;100:62-67.

151. Starkey KJ, Janezic A, et al. *Adipose thyrotrophin receptor expression is elevated in Graves' and thyroid eye diseases ex vivo and indicates adipogenesis in progress in vivo*. J Mol Endocrinol 2003;30:369-380.

152. Wiersinga WM. *Autoimmunity in Graves' Ophthalmopathy: The Result of an Unfortunate Marriage Between TSH Receptors and IGF-1 Receptors?* J Clin Endocrinol Metab 2011.

153. Bahn RS, Dutton CM, et al. *Thyrotropin receptor expression in Graves' orbital adipose/connective tissues: potential autoantigen in Graves' ophthalmopathy*. J Clin Endocrinol Metab 1998;83:998-1002.

154. Bell A, Gagnon A, et al. *Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts*. Am J Physiol Cell Physiol 2000;279:C335-340.

155. Kumar S, Schiefer R, et al. *A stimulatory thyrotropin receptor antibody (M22) and thyrotropin increase interleukin-6 expression and secretion in Graves' orbital preadipocyte fibroblasts*. Thyroid 2010;20:59-65.

156. Li Y, Chen L, et al. *Effect of immunoglobulin G from patients with Graves' ophthalmopathy and interferon gamma in intercellular adhesion molecule-1 and human leucocyte antigen-DR expression in human retroocular fibroblasts*. Chin Med J (Engl) 2000;113:752-755.

157. Kumar S, Nadeem S, et al. *A stimulatory TSH receptor antibody enhances adipogenesis via phosphoinositide 3-kinase activation in orbital preadipocytes from patients with Graves' ophthalmopathy*. J Mol Endocrinol 2011;46:155-163.

158. Zhang L, Baker G, et al. *Biological effects of thyrotropin receptor activation on human orbital preadipocytes*. Invest Ophthalmol Vis Sci 2006;47:5197-5203.

159. Zhang L, Paddon C, et al. *Gsalpha signalling suppresses PPARgamma2 generation and inhibits 3T3L1 adipogenesis*. J Endocrinol 2009;202:207-215.

160. van Zeijl CJ, Fliers E, et al. *Thyrotropin receptor-stimulating Graves' disease immunoglobulins induce hyaluronan synthesis by differentiated orbital fibroblasts from patients with Graves' ophthalmopathy not only via cyclic adenosine monophosphate signaling pathways*. Thyroid 2011;21:169-176.

161. Zhang L, Bowen T, et al. *Thyrotropin receptor activation increases hyaluronan production in preadipocyte fibroblasts: contributory role in hyaluronan accumulation in thyroid dysfunction*. J Biol Chem 2009;284:26447-26455.

162. Bahn RS, Dutton CM, et al. *Thyrotropin receptor expression in cultured Graves' orbital preadipocyte fibroblasts is stimulated by thyrotropin*. Thyroid 1998;8:193-196.

163. Kumar S, Coenen MJ, et al. *Evidence for enhanced adipogenesis in the orbits of patients with Graves' ophthalmopathy*. J Clin Endocrinol Metab 2004;89:930-935.

164. Sorisky A, Pardasani D, et al. *Evidence of adipocyte differentiation in human orbital fibroblasts in primary culture*. J Clin Endocrinol Metab 1996;81:3428-3431.

165. Valyasevi RW, Jyonouchi SC, et al. *Effect of tumor necrosis factor-alpha, interferon-gamma, and transforming growth factor-beta on adipogenesis and expression of thyrotropin receptor in human orbital preadipocyte fibroblasts*. J Clin Endocrinol Metab 2001;86:903-908.

166. Jyonouchi SC, Valyasevi RW, et al. *Interleukin-6 stimulates thyrotropin receptor expression in human orbital preadipocyte fibroblasts from patients with Graves' ophthalmopathy*. Thyroid 2001;11:929-934.

167. Weightman DR, Perros P, et al. *Autoantibodies to IGF-1 binding sites in thyroid associated ophthalmopathy*. Autoimmunity 1993;16:251-257.

168. Tsui S, Naik V, et al. *Evidence for an association between thyroid-stimulating hormone and insulin-like growth factor 1 receptors: a tale of two antigens implicated in Graves' disease*. J Immunol 2008;181:4397-4405.

169. Douglas RS, Gianoukakis AG, et al. *Circulating mononuclear cells from euthyroid patients with thyroid-associated ophthalmopathy exhibit characteristic phenotypes*. Clin Exp Immunol 2007;148:64-71.
170. Douglas RS, Gianoukakis AG, et al. *Aberrant expression of the insulin-like growth factor-1 receptor by T cells from patients with Graves' disease may carry functional consequences for disease pathogenesis*. J Immunol 2007;178:3281-3287.
171. Douglas RS, Naik V, et al. *B cells from patients with Graves' disease aberrantly express the IGF-1 receptor: implications for disease pathogenesis*. J Immunol 2008;181:5768-5774.
172. Zhao SX, Tsui S, et al. *Orbital fibrosis in a mouse model of Graves' disease induced by genetic immunization of thyrotropin receptor cDNA*. J Endocrinol 2011.
173. Smith TJ. *Orbital fibroblasts exhibit a novel pattern of responses to proinflammatory cytokines: potential basis for the pathogenesis of thyroid-associated ophthalmopathy*. Thyroid 2002;12:197-203.
174. Wakelkamp IM, Gerding MN, et al. *Smoking and disease severity are independent determinants of serum adhesion molecule levels in Graves' ophthalmopathy*. Clin Exp Immunol 2002;127:316-320.
175. Heufelder AE, Bahn RS. *Soluble intercellular adhesion molecule-1 (sICAM-1) in sera of patients with Graves' ophthalmopathy and thyroid diseases*. Clin Exp Immunol 1993;92:296-302.
176. De Bellis A, Bizzarro A, et al. *Behavior of soluble intercellular adhesion molecule-1 and endothelial-leukocyte adhesion molecule-1 concentrations in patients with Graves' disease with or without ophthalmopathy and in patients with toxic adenoma*. J Clin Endocrinol Metab 1995;80:2118-2121.
177. Mysliwiec J, Kretowski A, et al. *Serum L-selectin and ICAM-1 in patients with Graves' ophthalmopathy during treatment with corticosteroids*. Immunol Lett 2001;78:123-126.
178. Heufelder AE, Scriba PC. *Characterization of adhesion receptors on cultured microvascular endothelial cells derived from the retroorbital connective tissue of patients with Grave's ophthalmopathy*. Eur J Endocrinol 1996;134:51-60.
179. Pappa A, Calder V, et al. *Adhesion molecule expression in vivo on extraocular muscles (EOM) in thyroid-associated ophthalmopathy (TAO)*. Clin Exp Immunol 1997;108:309-313.
180. Heufelder AE, Bahn RS. *Detection and localization of cytokine immunoreactivity in retro-ocular connective tissue in Graves' ophthalmopathy*. Eur J Clin Invest 1993;23:10-17.
181. de Carli M, D'Elios MM, et al. *Cytolytic T cells with Th1-like cytokine profile predominate in retroorbital lymphocytic infiltrates of Graves' ophthalmopathy*. J Clin Endocrinol Metab 1993;77:1120-1124.
182. Wakelkamp IM, Gerding MN, et al. *Both Th1- and Th2-derived cytokines in serum are elevated in Graves' ophthalmopathy*. Clin Exp Immunol 2000;121:453-457.
183. Salvi M, Girasole G, et al. *Increased serum concentrations of interleukin-6 (IL-6) and soluble IL-6 receptor in patients with Graves' disease*. J Clin Endocrinol Metab 1996;81:2976-2979.
184. Antonelli A, Ferrari SM, et al. *Monokine induced by interferon gamma (IFNgamma) (CXCL9) and IFNgamma inducible T-cell alpha-chemoattractant (CXCL11) involvement in Graves' disease and ophthalmopathy: modulation by peroxisome proliferator-activated receptor-gamma agonists*. J Clin Endocrinol Metab 2009;94:1803-1809.
185. Antonelli A, Rotondi M, et al. *Interferon-gamma-inducible alpha-chemokine CXCL10 involvement in Graves' ophthalmopathy: modulation by peroxisome proliferator-activated receptor-gamma agonists*. J Clin Endocrinol Metab 2006;91:614-620.
186. Yan SX, Wang Y. *Inhibitory effects of Triptolide on interferon-gamma-induced human leucocyte antigen-DR, intercellular adhesion molecule-1, CD40 expression on retro-ocular fibroblasts derived from patients with Graves' ophthalmopathy*. Clin Experiment Ophthalmol 2006;34:265-271.
187. Chen B, Tsui S, et al. *IL-1 beta induces IL-6 expression in human orbital fibroblasts: identification of an anatomic-site specific phenotypic attribute relevant to thyroid-associated ophthalmopathy*. J Immunol 2005;175:1310-1319.
188. Han R, Smith TJ. *T helper type 1 and type 2 cytokines exert divergent influence on the induction of prostaglandin E2 and hyaluronan synthesis by interleukin-1beta in orbital fibroblasts: implications for the pathogenesis of thyroid-associated ophthalmopathy*. Endocrinology 2006;147:13-19.
189. Roper RL, Brown DM, et al. *Prostaglandin E2 promotes B lymphocyte Ig isotype switching to IgE*. J Immunol 1995;154:162-170.
190. Betz M, Fox BS. *Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines*. J Immunol 1991;146:108-113.

191. Kuehn HS, Jung MY, et al. *Prostaglandin E2 activates and utilizes mTORC2 as a central signaling locus for the regulation of mast cell chemotaxis and mediator release*. J Biol Chem 2011;286:391-402.
192. Cao HJ, Han R, et al. *Robust induction of PGHS-2 by IL-1 in orbital fibroblasts results from low levels of IL-1 receptor antagonist expression*. Am J Physiol Cell Physiol 2003;284:C1429-1437.
193. Hogg MG, Evans CH, et al. *Leukoregulin induces plasminogen activator inhibitor type 1 in human orbital fibroblasts*. Am J Physiol 1995;269:C359-366.
194. Smith TJ, Wang HS, et al. *Leukoregulin is a potent inducer of hyaluronan synthesis in cultured human orbital fibroblasts*. Am J Physiol 1995;268:C382-388.
195. Wang HS, Cao HJ, et al. *Leukoregulin induction of prostaglandin-endoperoxide H synthase-2 in human orbital fibroblasts. An in vitro model for connective tissue inflammation*. J Biol Chem 1996;271:22718-22728.
196. Raychaudhuri N, Douglas RS, et al. *PGE2 induces IL-6 in orbital fibroblasts through EP2 receptors and increased gene promoter activity: implications to thyroid-associated ophthalmopathy*. PLoS One 2010;5:e15296.
197. Wynn TA. *Cellular and molecular mechanisms of fibrosis*. J Pathol 2008;214:199-210.
198. Smith TJ, Bahn RS, et al. *Connective tissue, glycosaminoglycans, and diseases of the thyroid*. Endocr Rev 1989;10:366-391.
199. Hufnagel TJ, Hickey WF, et al. *Immunohistochemical and ultrastructural studies on the exenterated orbital tissues of a patient with Graves' disease*. Ophthalmology 1984;91:1411-1419.
200. Hansen C, Rouhi R, et al. *Increased sulfatation of orbital glycosaminoglycans in Graves' ophthalmopathy*. J Clin Endocrinol Metab 1999;84:1409-1413.
201. Imai Y, Ibaraki K, et al. *Analysis of proteoglycan synthesis by retro-ocular tissue fibroblasts under the influence of interleukin 1 beta and transforming growth factor-beta*. Eur J Endocrinol 1994;131:630-638.
202. Imai Y, Odajima R, et al. *Effect of growth factors on hyaluronan and proteoglycan synthesis by retroocular tissue fibroblasts of Graves' ophthalmopathy in culture*. Acta Endocrinol (Copenh) 1992;126:541-552.
203. Wang HS, Tung WH, et al. *TGF-beta induced hyaluronan synthesis in orbital fibroblasts involves protein kinase C betaII activation in vitro*. J Cell Biochem 2005;95:256-267.
204. Wong YK, Tang KT, et al. *Stimulation of hyaluronan synthesis by interleukin-1beta involves activation of protein kinase C betaII in fibroblasts from patients with Graves' ophthalmopathy*. J Cell Biochem 2001;82:58-67.
205. Tan GH, Dutton CM, et al. *Interleukin-1 (IL-1) receptor antagonist and soluble IL-1 receptor inhibit IL-1-induced glycosaminoglycan production in cultured human orbital fibroblasts from patients with Graves' ophthalmopathy*. J Clin Endocrinol Metab 1996;81:449-452.
206. Smith TJ, Hoa N. *Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, insulin-like growth factor-I receptor*. J Clin Endocrinol Metab 2004;89:5076-5080.
207. Kucharewicz I, Kowal K, et al. *The plasmin system in airway remodeling*. Thromb Res 2003;112:1-7.
208. Weigel PH, Hascall VC, et al. *Hyaluronan synthases*. J Biol Chem 1997;272:13997-14000.
209. Cao HJ, Hogg MG, et al. *Transforming growth factor-beta induces plasminogen activator inhibitor type-1 in cultured human orbital fibroblasts*. Invest Ophthalmol Vis Sci 1995;36:1411-1419.
210. Lantz M, Vondrichova T, et al. *Overexpression of immediate early genes in active Graves' ophthalmopathy*. J Clin Endocrinol Metab 2005;90:4784-4791.
211. Han R, Smith TJ. *Induction by IL-1 beta of tissue inhibitor of metalloproteinase-1 in human orbital fibroblasts: modulation of gene promoter activity by IL-4 and IFN-gamma*. J Immunol 2005;174:3072-3079.
212. Kim H, Choi YH, et al. *Antifibrotic effect of Pirfenidone on orbital fibroblasts of patients with thyroid-associated ophthalmopathy by decreasing TIMP-1 and collagen levels*. Invest Ophthalmol Vis Sci 2010;51:3061-3066.
213. Kumar S, Leontovich A, et al. *Gene expression profiling of orbital adipose tissue from patients with Graves' ophthalmopathy: a potential role for secreted frizzled-related protein-1 in orbital adipogenesis*. J Clin Endocrinol Metab 2005;90:4730-4735.
214. Weetman AP. *Determinants of autoimmune thyroid disease*. Nat Immunol 2001;2:769-770.
215. Starkey K, Heufelder A, et al. *Peroxisome proliferator-activated receptor-gamma in thyroid eye disease: contraindication for thiazolidinedione use?* J Clin Endocrinol Metab 2003;88:55-59.

216. Smith TJ, Koumas L, et al. *Orbital fibroblast heterogeneity may determine the clinical presentation of thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 2002;87:385-392.
217. Guo N, Woeller CF, et al. *Peroxisome proliferator-activated receptor gamma ligands inhibit transforming growth factor-beta-induced, hyaluronan-dependent, T cell adhesion to orbital fibroblasts*. J Biol Chem 2011;286:18856-18867.
218. Yoon JS, Lee HJ, et al. *Quercetin Inhibits IL-1beta-Induced Inflammation, Hyaluronan Production and Adipogenesis in Orbital Fibroblasts from Graves' Orbitopathy*. PLoS One 2011;6:e26261.
219. Cao HJ, Smith TJ. *Leukoregulin upregulation of prostaglandin endoperoxide H synthase-2 expression in human orbital fibroblasts*. Am J Physiol 1999;277:C1075-1085.
220. Keeley EC, Mehrad B, et al. *Fibrocytes: bringing new insights into mechanisms of inflammation and fibrosis*. Int J Biochem Cell Biol 2010;42:535-542.
221. Reilkoff RA, Bucala R, et al. *Fibrocytes: emerging effector cells in chronic inflammation*. Nat Rev Immunol 2011;11:427-435.
222. Douglas RS, Afifiyan NF, et al. *Increased generation of fibrocytes in thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 2010;95:430-438.
223. Smith TJ. *Potential role for bone marrow-derived fibrocytes in the orbital fibroblast heterogeneity associated with thyroid-associated ophthalmopathy*. Clin Exp Immunol 2010;162:24-31.
224. Mutsaers SE, Bishop JE, et al. *Mechanisms of tissue repair: from wound healing to fibrosis*. Int J Biochem Cell Biol 1997;29:5-17.
225. Barrientos S, Stojadinovic O, et al. *Growth factors and cytokines in wound healing*. Wound Repair Regen 2008;16:585-601.
226. Boor P, Ostendorf T, et al. *Renal fibrosis: novel insights into mechanisms and therapeutic targets*. Nat Rev Nephrol 2010;6:643-656.
227. Betsholtz C. *Insight into the physiological functions of PDGF through genetic studies in mice*. Cytokine Growth Factor Rev 2004;15:215-228.
228. Andrae J, Gallini R, et al. *Role of platelet-derived growth factors in physiology and medicine*. Genes Dev 2008;22:1276-1312.
229. Johnsson A, Heldin CH, et al. *Platelet-derived growth factor: identification of constituent polypeptide chains*. Biochem Biophys Res Commun 1982;104:66-74.
230. Li X, Ponten A, et al. *PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor*. Nat Cell Biol 2000;2:302-309.
231. LaRochelle WJ, Jeffers M, et al. *PDGF-D, a new protease-activated growth factor*. Nat Cell Biol 2001;3:517-521.
232. Bergsten E, Uutela M, et al. *PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor*. Nat Cell Biol 2001;3:512-516.
233. Betsholtz C, Johnsson A, et al. *cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines*. Nature 1986;320:695-699.
234. Heldin CH, Westermark B. *Mechanism of action and in vivo role of platelet-derived growth factor*. Physiol Rev 1999;79:1283-1316.
235. Reigstad LJ, Varhaug JE, et al. *Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family*. FEBS J 2005;272:5723-5741.
236. Bonner JC, Badgett A, et al. *Inhibition of platelet-derived growth factor-BB-induced fibroblast proliferation by plasmin-activated alpha 2-macroglobulin is mediated via an alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein-dependent mechanism*. J Biol Chem 1995;270:6389-6395.
237. Bonner JC. *Regulation of PDGF and its receptors in fibrotic diseases*. Cytokine Growth Factor Rev 2004;15:255-273.
238. Trojanowska M. *Role of PDGF in fibrotic diseases and systemic sclerosis*. Rheumatology (Oxford) 2008;47 Suppl 5:v2-4.
239. Ostendorf T, Eitner F, et al. *The PDGF family in renal fibrosis*. Pediatr Nephrol 2011; DOI 10.1007/s00467-011-1892-z.

Chapter 3

Aims of the thesis

Graves' ophthalmopathy (GO) leads to a considerable physical and mental burden for patients and medical treatment has not significantly improved in the last two decades. The central role of orbital fibroblast activation in GO shares many similarities with fibro-proliferative disorders in other organs systems, in which PDGF is considered as a central stimulator of fibroblast activity. Therefore, the aim of this thesis was to delineate the involvement of PDGF in the pathophysiology of GO and to investigate whether PDGF may be a new therapeutic target for the treatment of GO patients.

Chapter 4

Imatinib mesylate and AMN107 inhibit PDGF-signalling in orbital fibroblasts: a potential treatment for Graves' ophthalmopathy

Leendert van Steensel [1], Dion Paridaens [2], Benjamin Schrijver [1], Gemma M. Dingjan [1], Paul L.A. van Daele [1], P. Martin van Hagen [1,2], Willem A. van den Bosch [2], Hemmo A. Drexhage [1], Herbert Hooijkaas [1] and Willem A. Dik [1]

[1] *Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands*
[2] *Rotterdam Eye Hospital, Rotterdam, The Netherlands*

Invest Ophthalmol Vis Sci. 2009 jul;50(7):3091-8

ABSTRACT

Purpose

Excessive orbital fibroblast proliferation and hyaluronan production are characteristic for Graves' ophthalmopathy (GO) and are driven by local mediators. Imatinib mesylate and AMN107 are tyrosine kinase inhibitors that inhibit fibroblast proliferation and collagen production in lungs and skin. Here we determined whether imatinib mesylate and AMN107 inhibit orbital fibroblast proliferation and hyaluronan production induced by PDGF-BB and TGF-β_1 and whether gene expressions for PDGF-B and TGF-β_1 (growth factors suggested to play a role in GO) were indeed increased in GO orbital tissues.

Methods

PDGF-B and TGF-β_1 mRNA levels were determined in orbital tissues from thirteen GO and five controls patients. Orbital fibroblasts were cultured from eight GO and three control patients and the effect of imatinib mesylate and AMN107 on PDGF-BB and TGF-β_1-induced orbital fibroblast proliferation, signaling cascades, hyaluronan synthase (HAS) gene expression and hyaluronan production were determined.

Results

PDGF-B and TGF-β_1 mRNA levels were significantly increased in GO orbital tissues. Imatinib mesylate and AMN107 inhibited PDGF-BB-induced orbital fibroblast proliferation, *HAS* induction and hyaluronan production by blocking PDGF-receptor phosphorylation. TGF-β_1 induced HAS expression and hyaluronan production. This was not inhibited by imatinib mesylate or AMN107, due to the inability of TGF-β_1 to activate c-Abl kinase activity in orbital fibroblasts.

Conclusions

Imatinib mesylate and AMN107 inhibit orbital fibroblast proliferation and hyaluronan production induced by PDGF-BB; a factor highly expressed in orbital tissue from GO patients. The drugs, however, had no effect on TGF-β_1-induced HAS expression and hyaluronan production. Nevertheless, imatinib mesylate and AMN107 should be considered as treatment candidates for GO.

INTRODUCTION

Graves' ophthalmopathy (GO) is an autoimmune inflammatory disease of the orbit. The active stage of GO is characterized by an orbital infiltrate, consisting of mainly T cells, macrophages, mast cells, some B cells and plasma cells.[1-3] These inflammatory cells produce cytokines, growth factors and immunoglobulins that subsequently stimulate orbital fibroblasts to proliferate and to produce an excess of extracellular matrix (ECM) components, especially hyaluronan. Orbital fibroblast proliferation and ECM production are considered key events in the pathophysiology of GO and contribute to clinical manifestations such as proptosis and extraocular motility dysfunction.[1, 3-5] The treatment of GO is still limited. Despite treatment with steroids to reduce inflammation, a considerable number of patients need (recurrent) decompressive surgery.[1, 4-5] Therefore, novel therapies for GO are required.

Excessive proliferation and ECM synthesis by fibroblasts are central to fibrotic diseases in general and are assumed to be driven by mediators produced by inflammatory cells and resident cells. Recently, it was demonstrated that TGF-β_1 induced c-Abl kinase activity in a Smad-independent way and that imatinib mesylate prevented bleomycin-induced lung and renal fibrosis through inhibition of TGF-β_1-induced c-Abl activation and subsequent collagen production.[6-7] Imatinib mesylate is a tyrosine kinase inhibitor that blocks c-Abl kinase activity and is used to target the product of the BCR-ABL fusion gene in chronic myeloid leukemia.[8] In addition, imatinib mesylate inhibits PDGF receptor (PDGF-R) tyrosine kinase activity, thus preventing PDGF-R autophosphorylation upon PDGF binding.[8-9] We and others found that imatinib mesylate efficiently inhibited PDGF-BB-induced proliferation and TGF-β_1-induced collagen synthesis by lung and dermal fibroblasts obtained from systemic sclerosis patients.[10-11] Nilotinib (AMN107) and dasatinib, two novel inhibitors of c-Abl and PDGF-R tyrosine kinase activity, were recently reported to inhibit collagen production by dermal fibroblasts as well as bleomycin-induced dermal fibrosis in mice.[12] These data suggest that these tyrosine kinase inhibitors are promising drugs for the treatment of fibrotic diseases and therefore possibly also for GO.

Mediators implicated in the pathogenesis of orbital fibrosis should fit three basic criteria: (1) they should stimulate fibroblast proliferation and ECM production, (2) their expression must be increased in the affected tissue and (3) inhibitors of the mediators function should attenuate the development of fibrosis.[13] Mediators which fit these criteria are the earlier mentioned PDGF-BB and TGF-β_1[14] which have been suggested as potential pro-fibrotic mediators in GO based on *in vitro* experiments.[15-17] However, so far no evidence has been generated that increased levels of these mediators do indeed exist in orbital tissue from GO patients.

In this study we demonstrate increased PDGF-B and TGF-β_1 mRNA levels in orbital tissues from GO patients. In addition, we show that imatinib mesylate and AMN107 are potent inhibitors of PDGF-BB-induced orbital fibroblast proliferation as well as hyaluronan synthesis through inhibition of PDGF-R phosphorylation. TGF-β_1 induced HAS expression

and hyaluronan production was not inhibited by imatinib mesylate or AMN107. This was due to the inability of TGF-β_1 to activate c-Abl kinase activity in orbital fibroblasts. Smad signaling was activated by TGF-β_1. This study provides evidence that imatinib mesylate and AMN107 should be considered as candidates for the treatment of therapy resistant GO patients, especially those suffering of severe peri-ocular edema and ocular motility dysfunction and not responding to regular therapies.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin and trypsin/EDTA were purchased from Cambrex BioWhittaker (Verviers, Belgium). The human fetal lung fibroblast cell line (HFL-1) was obtained from ATCC (USA). Recombinant human PDGF-BB, recombinant human TGF-β_1 and a hyaluronan ELISA were obtained from R&D Systems (Abingdon, UK). Anti-PDGF-Rβ antibody (SC-339) was purchased from Santa Cruz Biotechnologies (Heidelberg, Germany). Anti-phospho-tyrosine (#9411), anti-c-Abl (#2862), anti-Smad3 (#9513) and anti-phospho-Smad3 (#9520S) antibodies were purchased from Cell Signaling (Danvers, USA). Anti-β-actin (AB6276) was purchased from Abcam (Cambridge, UK). Anti-c-Abl antibody (8E9) was kindly provided by Dynomics (Rotterdam, the Netherlands). Imatinib mesylate and AMN107 were kindly provided by Novartis Pharma (Basel, Switzerland). SB431542 was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). GST-CRK was a kind gift from E.B. Leof (Minnesota, USA).

Patients and controls

Patient details are given in Table 1A and 1B and clinical scores were determined as described before.[18] Orbital tissue was obtained from fifteen GO patients undergoing orbital decompression surgery. Six of the orbital tissues were used for mRNA detection and yielded orbital fibroblast strains, seven of the tissues were only used for mRNA detection and from two tissues only fibroblast strains were established (see methods below). Of the patients three had active and twelve inactive disease. All patients undergoing orbital surgery were euthyroid. Furthermore, orbital tissue was obtained from seven control patients without thyroid or inflammatory disease and undergoing orbital surgery for other reasons. One control orbital tissue was used for mRNA detection and yielded an orbital fibroblast strain, four control tissues were only used for mRNA detection and from two control tissues only fibroblast strains were established. All tissues were obtained in the Rotterdam Eye Hospital (Rotterdam, The Netherlands) after informed consent and in accordance with the principles

of the Declaration of Helsinki and after approval by the institutional review board at the Erasmus MC, University Medical Center (Rotterdam, The Netherlands).

Table 1. Patients and controls

A. Patients and controls from whom orbital tissues were used for mRNA determination		
	GO patients (n=13)	Controls (n=5)
Age (range)	51 (27-78)	53 (37-80)
Sex (m/v)	1 / 12	0 / 5
Smoking	7 / 13	0 / 5
Graves' Disease	13 / 13	0/5
- RAI	12 / 13	-
- Surgery	1 / 13	-
- Strumazol	8 / 13	-
Treatment GO	13/13	0/5
- Surgery	13 / 13	-
- Prednison	13 / 13	-
- Radiation	4 / 13	-
Euthyroid	13 / 13	5/5
TSH-Receptor antibodies	13 / 13	0 / 5
TPO antibodies	6 / 13	0 / 5
Clinical Activity Score (CAS) (range)	2 (1 - 6)	-
NO-SPECS (range) #	3 (1 – 7)	-
B. Patients and controls from whom orbital fibroblasts strains were obtained		
	GO patients (n=8)	Controls (n=3)
Age (range)	54 (42-78)	59 (49-70)
Sex (m/v)	1 / 7	1 / 2
Smoking	5 / 8	0 / 0
Graves' Disease	8 / 8	0 / 0
- RAI	7 / 8	-
- Surgery	0 / 8	-
- Strumazol	8 / 8	-
Treatment GO	8 / 8	0 / 0
- Surgery	8 / 8	-
- Prednison	8 / 8	-
- Radiation	3 / 8	-
Euthyroid	8 / 8	3 / 3
TSH-Receptor antibodies	8 / 8	0 / 3
TPO antibodies	7 / 8	0 / 3
Clinical Activity Score (CAS) (range)	4 (1 - 7)	-
NO-SPECS (range) #	4 (1 - 7)	-

NO-SPECS score: as described before [18]

Detection of PDGF-B and TGF-β_1 mRNA levels in orbital tissue

RNA was isolated using Rneasy columns (Qiagen, Hilden, Germany) and reverse transcribed into cDNA.[19] PDGF-B and TGF-β_1 transcript levels were determined by real-time quantitative PCR (RQ-PCR) using an Applied Biosystems 7700 PCR machine (Foster City, USA). Transcript levels were normalized to the control gene Abelson.[19] Primer - probe combinations used are listed in Table 2.

Orbital fibroblast culture

Fibroblast strains were established from orbital tissues as described previously.[20] Once fibroblast monolayers were obtained, cultures were serially passaged after gentle treatment with trypsin/EDTA. Eight GO fibroblast strains were obtained, of which three from patients with active GO and five with inactive GO. Three control fibroblast strains were obtained. Fibroblast strains used for experiments were between the 6th and 13th passage.

Orbital fibroblast proliferation assay

Initial cytotoxicity studies based on total cell number determination and lactate dehydrogenase release revealed that imatinib mesylate and AMN107 were non-toxic to orbital fibroblasts at concentrations up to 2.5 μg/ml. In our subsequent studies imatinib mesylate and AMN107 were used at a concentration of 2.5 μg/ml. The effect of imatinib mesylate and AMN107 on PDGF-BB-induced fibroblast proliferation was determined as described previously for the PDGF receptor specific tyrosine kinase inhibitor tyrphostin AG1296.[21] Briefly, fibroblasts were seeded at 6 $x10^3$ cells/well into 96-well plates in DMEM containing 10% FCS and antibiotics (DMEM 10% FCS) and allowed to adhere for 24 hours. Hereafter, medium was changed to DMEM containing 1.0% FCS and antibiotics (DMEM 1.0% FCS) with or without imatinib mesylate or AMN107 for 16 hours. Subsequently, cells were cultured in DMEM 1.0% FCS with or without 50 ng/ml PDGF-BB at six replicates per condition. Proliferation was assessed after 24 hours using a colorimetric assay based on the uptake and subsequent release of methylene blue dye as described before.[21] Proliferation was expressed as percentage change in mean absorbance from that of cells exposed to DMEM 1.0% FCS alone.

Hyaluronan synthase *(*HAS*)* mRNA expression in orbital fibroblasts

Fibroblasts were seeded at 4 $x10^5$ cells/well into 6-well plates in DMEM 10% FCS and allowed to adhere. Hereafter, the fibroblasts were incubated in DMEM 1.0% FCS in the presence or absence of imatinib mesylate or AMN107 for 16 hours. Subsequently, cells were cultured in DMEM 1.0% FCS with or without TGF-β_1 (10 ng/ml) or PDGF-BB (50 ng/ml) for 6 hours. RNA was isolated and reverse transcribed into cDNA.[19] HAS1, HAS2 and HAS3 expression was determined by RQ-PCR and normalized to the control gene Abelson. Because of variability in basal HAS mRNA, results were expressed as fold induction relative to basal HAS expression. Primer - probe combinations used are listed in Table 2.

Hyaluronan production by orbital fibroblasts

Fibroblasts were seeded at 1.5 $x10^5$ cells/well into 24-well plates in DMEM 10% FCS and allowed to adhere. Hereafter, the fibroblasts were incubated in DMEM 1.0% FCS in the presence or absence of imatinib mesylate or AMN107 for 16 hours. Subsequently, cells were cultured in DMEM 1.0% FCS with or without TGF-β_1 (10 ng/ml), PDGF-BB (50 ng/ml) or a combination of TGF-β_1 and PDGF-BB for 24 hours. Supernatants were harvested and

hyaluronan levels were determined by ELISA. Because of variability in basal production levels, results were expressed as fold induction relative to the basal hyaluronan production.

Table 2. RQ-PCR primer – probe combinations

Gene	Forward primer	Reverse primer	Probe
PDGF-B	Fw_Hu_PDGFB_EMC: TCCCGAGGAGCTTTATGA-GATG	Rv_Hu_PDGFB_EMC: CGGGTCATGTTCAGGTCCAAC	T_Hu_PDGFB_EMC: AGTGACCACTGATCGCTC-CTTTG
TGF-β_1	Fw_Hu_TGFB1_EMC: CGCGTGCTAATGGTGGAA	Rv_Hu_TGFB1_EMC: AGAGCAACACGGGTT-CAGGT	T_Hu_TGFB1_EMC: CCACAACGAAATCTAT-GACAAGTTCAAGCAGA
HAS1	Fw_Hu_HAS1_EMC: GCAAGCGCGAGGTCATGT	Rv_Hu_HAS1_EMC: CGGGGGTCCTCGTCCA	T_Hu_HAS1_EMC: ACTACGTGCAGGTCTGT-GACTCGGACAC
HAS2	Fw_Hu_HAS2_EMC: AATGGGGTGGAAAAAGA-GAAGTC	Rv_Hu_HAS2_EMC: CAACCATGG-GATCTTCTTCTAAAAC	Tr_Hu_HAS2_EMC: TCCACACTTCGTCCCAGT-GCTCTGA
HAS3	Fw_Hu_HAS3_EMC: AAGGCCCTCGGCGATTC	Rv_Hu_HAS3_EMC: CCCCCGACTCCCCCTACT	T_Hu_HAS3_EMC: ACATCCAGGTGTGC-GACTCTGACACTGTG

Detection of PDGF-R phosphorylation

In order to assess PDGF-R activation, cultures were stimulated with PDGF-BB (50 ng/ml) for the indicated times and lysed (20 mM Tris pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol and protease inhibitors). Equivalent amounts of protein (~30 µg) were loaded on SDS-PAGE. Western blots were stained with either anti-PDGF-R or anti-phosphotyrosine antibodies. To determine the effect of imatinib mesylate and AMN107 on PDGF-BB-induced PDGF-R phosphorylation, immunoprecipitation using an anti-PDGF-R antibody was performed on cell lysates (~500 µg protein). Immune complexes were collected with a mix of protein A- and G-Sepharose (Sigma-Aldrich, Zwijndrecht, the Netherlands) and the level of PDGF-R phosphorylation was determined using an anti-phosphotyrosine antibody as described.[6]

Detection of c-Abl kinase activity

c-Abl kinase assays were essentially performed as described.[6] Briefly, cultures were stimulated for the indicated times and lysed at 4°C. Immunoprecipitation was performed with an anti-c-Abl antibody (#2862). Immune complexes were collected with a mix of protein A- and G-Sepharose and washed in lysis buffer and three times in kinase buffer (10mM HEPES pH 7.4, 50 mM $NaCl_2$, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0,5 mM DTT and protease inhibitors). Kinase reaction was performed in 10 μl kinasemix containing 2 µg GST-CRK, 100 µM ATP and 0,5 μCi gamma-^{32}P-dATP for 15 min at 37^{0}C. Reaction was stopped by adding loading buffer and samples were loaded on SDS-PAGE. Total c-Abl and actin protein were detected using anti-c-Abl (8E9) and anti-β-actin antibodies.

Detection of Smad activation

In order to assess Smad activation, cultures were treated with TGF-β_1 for the indicated times and lysed. Equivalent amounts of protein were loaded on SDS-PAGE and Western blots were subsequently stained with antibodies to phosphorylated-Smad3, Smad-3 and β-actin.

Statistical analysis

PDGF-B and TGF-β_1 mRNA levels in orbital tissue were analyzed using the Mann-Whitney test. Data concerning the effect of imatinib mesylate and AMN107 on orbital fibroblast proliferation, HAS expression levels and hyaluronan production were analyzed using the paired Student's t-test. A p-value <0.05 was considered significant.

RESULTS

PDGF-B and TGF-β_1 mRNA levels are elevated in GO orbital tissue

PDGF-B mRNA levels were significantly higher (~3 fold: $p<0.05$; Fig. 1) in GO as compared to control orbital tissue. Also, TGF-β_1 mRNA levels were significantly higher (~2 fold: $p<0.05$; Fig. 1) in GO as compared to control orbital tissue. No difference in PDGF-B and TGF-β_1 mRNA expression was observed between active or inactive GO patients and no correlation were observed between expression levels and clinical activity scores (CAS).[18]

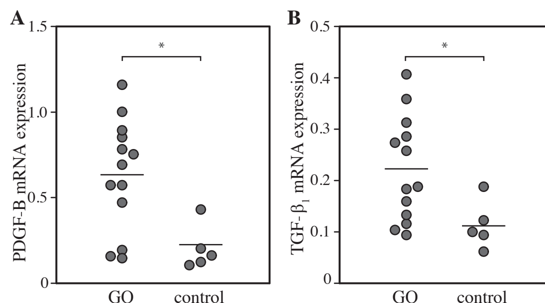


Figure 1 Relative mRNA expression for PDGF-B (A) and TGF-β_1 (B) in orbital tissue from GO patients and controls. mRNA expression levels were determined by RQ-PCR and normalized to the expression levels of Abelson. Each dot represents a single individual. The horizontal bars represent mean values. Data were analyzed using the Mann-Whitney test. * $p<0.05$

Imatinib mesylate and AMN107 inhibit PDGF-BB-induced orbital fibroblast proliferation and PDGF-R phosphorylation. TGF-β1 does not induce orbital fibroblast proliferation

PDGF-BB equally stimulated proliferation of orbital fibroblasts derived from GO and control patients with a mean induction of 37% and 45% proliferation above control, respectively (Fig. 2). No difference in PDGF-BB-induced proliferation was observed between fibroblasts

from active or inactive GO patients. Initial studies revealed that imatinib mesylate and AMN107 dose-dependently (range: 0.16-2.5 µg/ml) inhibited PDGF-BB-induced orbital fibroblast proliferation (data not shown). At the dose of 2.5 µg/ml, imatinib mesylate and AMN107 inhibited PDGF-BB-induced proliferation of GO and control orbital fibroblasts up to basal proliferation levels (GO: Imatinib and AMN107 both 5% proliferation above control; $p<0.05$, controls: Imatinib 6% and AMN107 8% proliferation above control, $p<0.05$; Fig. 2).

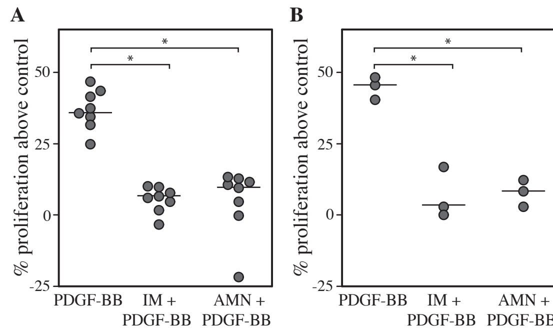


Figure 2 The effect of imatinib mesylate (IM; 2.5 µg/ml) and AMN107 (AMN; 2.5 µg/ml) on PDGF-BB (50 ng/ml) induced proliferation of GO (A) and control (B) orbital fibroblasts. Each dot represents a fibroblast strain obtained from a single individual. Horizontal bars represent mean values. Data were analyzed using the Students t-test. * $p<0.001$

With regard to mechanisms involved, PDGF-BB stimulated PDGF-R phosphorylation in a time dependent manner (Fig 3A). Imatinib mesylate and AMN107 both reduced PDGF-BB-induced PDGF-R phosporylation (Fig 3B), indicating efficient inhibition of PDGF-R tyrosine kinase activity in orbital fibroblasts by both tyrosine kinase inhibitors.
In contrast, stimulation with TGF-β_1 (range: 2.5–40 ng/ml) did not induce orbital fibroblast proliferation and was not further explored.

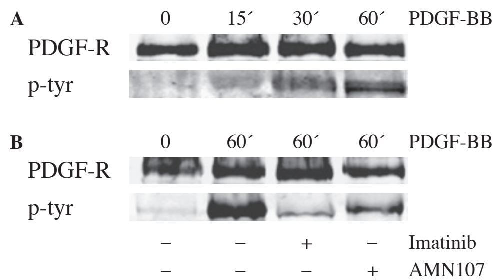


Figure 3 Orbital fibroblasts were stimulated with PDGF-BB (50 ng/ml), with or without imatinib mesylate (2.5 µg/ml) and AMN107 (2.5 µg/ml), for indicated times. (A) Western analysis was performed on lysates with antibodies against PDGF-R (*upper row)* and phosphorylated tyrosine residues *(lower row)*. (B) *Upper row:* Western analysis was performed with PDGF-R antibody on an aliquot prior to immunoprecipitation. *Lower row:* following PDGF-R immunoprecipitation, phosphorylated PDGF-R was determined with an antibody against phosphorylated tyrosine residues. Data depicted are from a representative experiment.

PDGF-BB induces HAS1 and HAS2 expression and TGF-β_1 induces *HAS1* expression

Hyaluronan synthesis is regulated by three hyaluronan synthases, encoded by individual genes; HAS1, HAS2 and HAS3.[22] We determined whether PDGF-BB and TGF-β_1 influenced the expression of these HAS genes in orbital fibroblasts. PDGF-BB stimulation increased HAS1 and HAS2 expression by ~6 fold in GO orbital fibroblasts ($p<0.01$), but did not influence HAS3 expression (Fig. 4). In control orbital fibroblasts PDGF-BB stimulation increased HAS2 expression ~3 fold, but did not affect HAS1 and HAS3 expression. TGF-β_1 stimulation increased HAS1 expression ~60 fold ($p<0.05$) in both GO and control fibroblasts, but did not affect HAS2 and HAS3 transcript levels (Fig. 4). No differences were observed between GO orbital fibroblasts from active or inactive disease with regard to PDGF-BB and TGF-β_1-induced HAS expression.

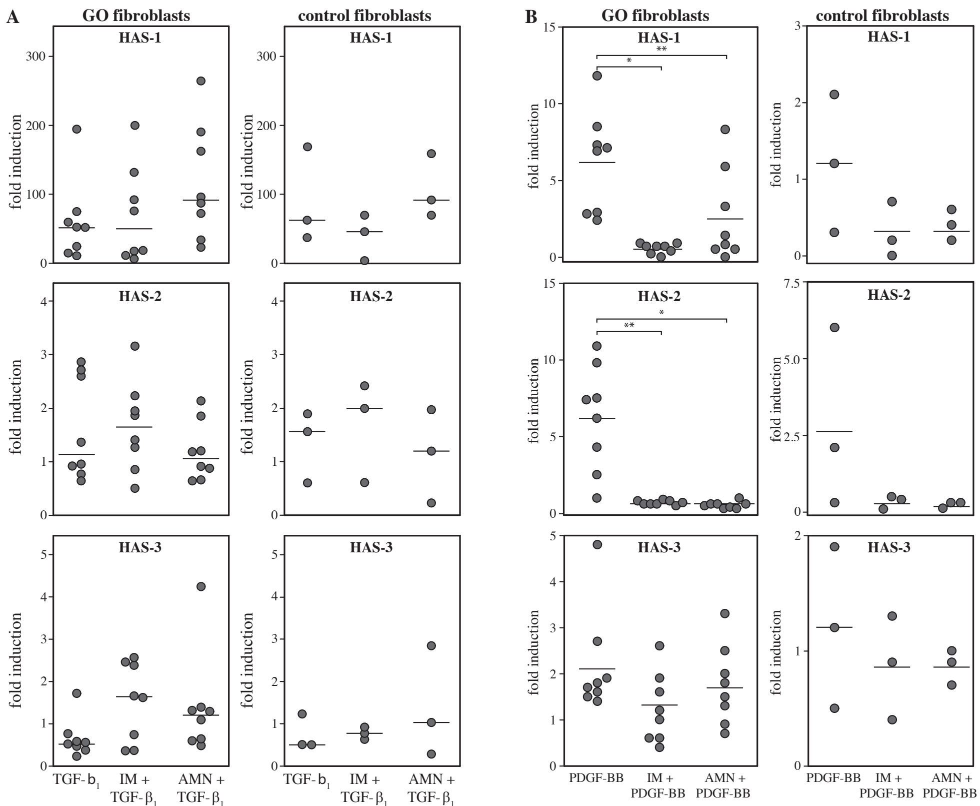


Figure 4 The effect of imatinib mesylate (IM; 2.5 µg/ml) and AMN107 (AMN; 2.5 µg/ml) on (A) TGF-β_1 (10 ng/ml) and (B) PDGF-BB (50 ng/ml) induced HAS expression in orbital fibroblasts. mRNA levels of HAS1, HAS2 and HAS3 were determined by RQ-PCR and normalized to the expression levels of Abelson.
The first row depicts HAS1 mRNA levels, the second row depicts HAS2 mRNA levels and the third row HAS3 mRNA levels in GO and control orbital fibroblasts. Each dot represents an orbital fibroblast strain obtained from a single individual. Data are presented as fold induction relative to unstimulated controls. Horizontal bars represent mean values. Data were analyzed using the Students t-test. * $p<0.001$, ** $p<0.05$

Imatinib mesylate and AMN107 inhibit PDGF-BB-induced HAS1/HAS2 expression and hyaluronan production

Imatinib mesylate reduced PDGF-BB-induced expression of HAS1 and HAS2 in GO orbital fibroblasts up to basal expression levels ($p<0.05$; Fig 4). AMN107 reduced PDGF-BB-induced HAS1 expression in GO orbital fibroblasts by ~3 fold ($p<0.05$) and HAS2 expression up to basal expression levels ($p<0.05$; Fig. 4). Both imatinib mesylate and AMN107 inhibited the PDGF-BB-induced HAS2 expression in control orbital fibroblasts (Fig. 4).

To examine whether treatment with imatinib mesylate and AMN107 reduced hyaluronan production, five GO orbital fibroblast strains (two from active and three from inactive GO) and three control orbital fibroblast strains were selected. PDGF-BB stimulated the production of hyaluronan by orbital fibroblasts derived from both GO (~3 fold induction; Fig. 5) and control patients (~4 fold induction; Fig. 5). Imatinib mesylate and AMN107 treatment efficiently reduced the PDGF-BB-induced hyaluronan production by GO and control orbital fibroblasts up to basal levels (both $p<0.05$; Fig. 5).

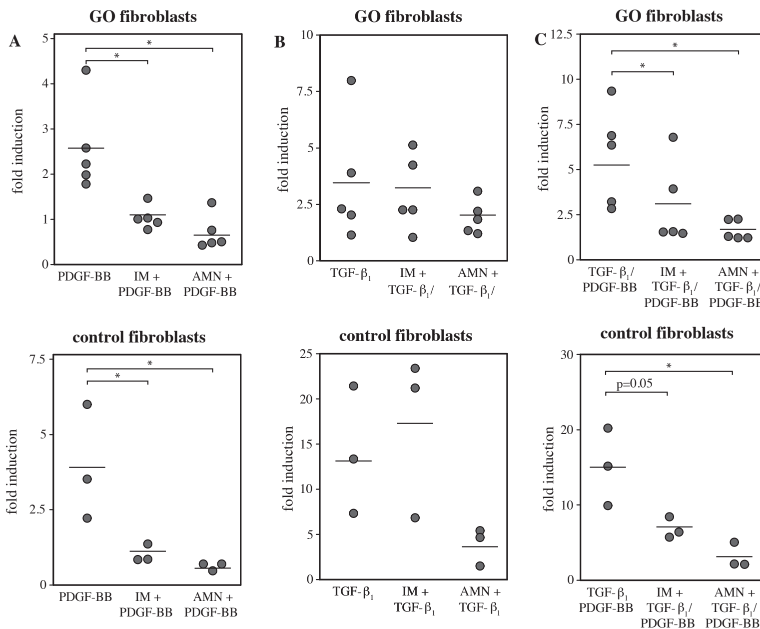


Figure 5 The effect of imatinib mesylate (IM; 2.5 μg/ml) and AMN107 (AMN; 2.5 μg/ml) on (A) PDGF-BB (50 ng/ml), (B) TGF-β_1 (10 ng/ml) and (C) PDGF-BB/TGF-β_1-induced hyaluronan production by orbital fibroblasts. Hyaluronan levels in culture supernatants were determined by ELISA. The first column depicts hyaluronan production in GO and control fibroblasts after PDGF-BB stimulation, the second column after TGF-β_1 stimulation and the third column after stimulation with both PDGF-BB and TGF-β_1. Each dot represents an orbital fibroblast strain obtained from a single individual. Data are presented as fold induction relative to unstimulated controls. Horizontal bars represent mean values. Data were analyzed using the Students t-test. * $p< 0.05$

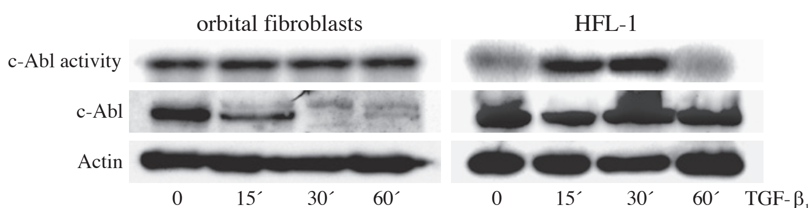


Figure 6 Orbital fibroblasts (left panel) and HFL-1 cells (right panel) were stimulated with TGF-β_1 (10ng/ml) for indicated times. *Upper rows:* following c-Abl immunoprecipitation, c-Abl kinase activity was determined using GST-CRK as a substrate. *Remaining rows:* Western analysis was performed with antibodies against c-Abl and β-actin on an aliquot prior to immunoprecipitation. Data depicted are from a representative experiment.

Imatinib mesylate and AMN107 do not inhibit TGF-β_1-induced HAS1 expression and hyaluronan production, since TGF-β_1 does not activate c-Abl kinase activity in orbital fibroblasts

Although TGF-β_1 clearly up regulated HAS1 expression in orbital fibroblasts (see before) and although it also stimulated hyaluronan production by both GO (~4 fold induction; Fig. 5) and control orbital fibroblasts (~14 fold induction; Fig. 5), imatinib mesylate and AMN107 neither affect the TGF-β_1-induced increase of HAS1 expression (Fig 4), nor reduced the TGF-β_1-induced hyaluronan production (Fig. 5). It must be noted that TGF-β_1-induced HAS1 expression could be blocked by the selective TGF-β_1 receptor I kinase inhibitor SB431542 in a dose dependent manner (data not shown), showing the systems ability to respond. Also imatinib mesylate and AMN107 reduced the hyaluronan production in orbital fibroblasts after co-stimulation by PDGF-BB and TGF-β_1.

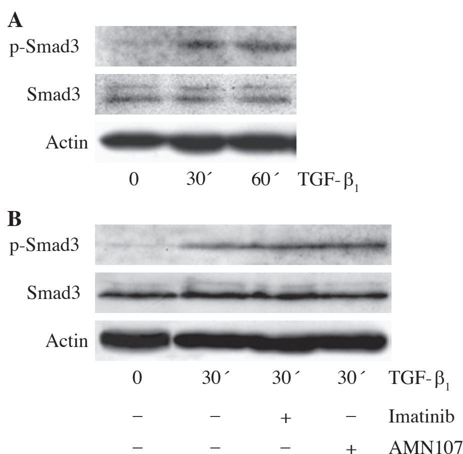


Figure 7 Orbital fibroblasts were stimulated with TGF-β_1 (10ng/ml) for indicated times (A). Western analysis was performed on lysates with antibodies against phosphorylated-Smad3, Smad3 and β-actin. Data depicted are from a representative experiment. Orbital fibroblasts were stimulated with TGF-β_1 (10ng/ml) for indicated times with or without imatinib mesylate (2.5 μg/ml) and AMN107 (2.5 μg/ml) for indicated times (B). Western analysis was performed on lysates with antibodies against phosphorylated-Smad3, Smad3 and β-actin. Data depicted are from a representative experiment.

To study this inability of the tyrosine kinase inhibitors regarding TGF-β_1-effects in orbital fibroblasts further, we studied the effects of TGF-β_1 stimulation on c-Abl kinase activity. It appeared that TGF-β_1 stimulation did not increase c-Abl kinase activity in orbital fibroblasts but was associated with a decrease in total c-Abl protein. This was in contrast to the effect of TGF-β_1 on lung fibroblasts where it induced c-Abl kinase activity without affecting total c-Abl levels (Fig 6). TGF-β_1 did induce Smad3 phosphorylation in orbital fibroblasts (Fig. 7A). This induction was not inhibited by imatinib mesylate and AMN107 (Fig. 7B). These data indicate that in orbital fibroblasts the Smad pathway is activated by TGF-β_1, while the c-Abl pathway is not.

DISCUSSION

Orbital fibroblast proliferation and hyaluronan accumulation are key features of GO and are thought to be driven by mediators present within the orbital tissue.[23-26]

PDGF-BB, a homo-dimeric protein consisting of two PDGF-B chains, is regarded as one of the most potent mitogens for fibroblasts and increased PDGF-BB production plays a key role in fibrosis of a variety of organ systems.[9] We here report, for the first time, increased levels of PDGF-B mRNA in orbital tissues from patients with GO In line with previous observations[15] we here also demonstrate that PDGF-BB is capable of inducing proliferation of orbital fibroblasts. We also found elevated levels of TGF-β_1 mRNA in GO orbital tissue. However, in our study, TGF-β_1 did not induce orbital fibroblast proliferation, which is opposed to Heufelder *et al*. who found TGF-β_1 to be mitogenic for GO orbital fibroblasts.[15] Unfortunately, we were unable to examine the actual orbital PDGF-BB and TGF-β_1 protein levels due to lack of material. However, several studies in fibrotic diseases showed clear correlations between PDGF-B and TGF-β_1 mRNA and protein levels.[27-30] PDGF-B and TGF-β_1 mRNA levels did not correlate with disease activity. Although the reason and underlying mechanism for this are unclear so far, the data do suggest that PDGF-BB and TGF-β_1 are involved in both active and inactive GO.

Next to proliferation, excessive hyaluronan production by orbital fibroblasts plays an important role in GO.[1, 5] Both TGF-β_1 and PDGF-BB are powerful stimulators of hyaluronan production by fibroblasts from a variety of organs and diseases characterized by tissue remodelling.[16, 31-32] We here demonstrate that PDGF-BB enhances HAS1 and HAS2 mRNA expression in GO orbital fibroblasts while TGF-β_1 increases HAS1 mRNA expression. This is in contrast with previous reports demonstrating that PDGF-BB does not induce HAS expression in orbital fibroblasts.[33] We take this discrepancy as due to differences in methodological approaches and consider the PDGF-BB-induced HAS expression found here as genuine, since we found it to co-exist with an increased hyaluronan production. In sum, our combined findings of increased orbital expression of PDGF-B and TGF-β_1 mRNA and

in vitro stimulation data strengthen the case for PDGF-BB and TGF-β_1 being involved in the increased hyaluronan synthesis in GO.

So far the fibroblast has not been a target for therapy in GO. Imatinib mesylate and AMN107 are tyrosine kinase inhibitors known to block PDGF and TGF-β_1 related tyrosine kinase activity in fibroblasts, making the drugs potential candidates to inhibit fibrosis and hyaluronan production associated with peri-orbital edema in GO.

Our studies show that imatinib mesylate and AMN107 inhibit the *in vitro* PDGF-BB-induced orbital fibroblast proliferation as well as the *in vitro* PDGF-BB-induced HAS expression and hyaluronan production by orbital fibroblasts by interfering with the PDGF-BB-induced autophosphorylation of its receptor.[9] These observations are in line with previous observations on these drugs in lung fibroblasts from systemic sclerosis patients[11]. However, in contrast to lung (and skin) fibroblasts,[6, 10-11] the tyrosine kinase inhibitors had no effect on TGF-β_1-induced extracellular matrix production from orbital fibroblasts, since TGF-β_1 did not induce c-Abl kinase activity in orbital fibroblasts. This suggests that TGF-β_1-induced hyaluronan production by orbital fibroblasts is regulated by a c-Abl independent signaling pathway and underscores previous notions that orbital fibroblasts are distinct from fibroblasts from other anatomical sites[34] and originate from a different embryonal site.[35]

Another major route by which TGF-β_1 regulates cell activation is the Smad signaling cascade and c-Abl-independent Smad signaling has been shown to be involved in hyaluronan production in different cell types.[31, 36-37] We indeed found this pathway activated in TGF-β_1-stimulated orbital fibroblasts and drugs targeting this pathway should be considered in GO.

Thus far, treatment with imatinib mesylate and AMN107 has been widely applied to treat BCR-ABL positive chronic myeloid leukemia[8]. In addition, imatinib mesylate has been successfully used to treat patients with gastrointestinal stromal tumors and mastocytosis by targeting c-Kit kinase activity.[38-39] Recently, we and others have shown that treatment with imatinib mesylate was associated with reversal of skin, renal and lung fibrosis in humans.[11, 40-42] These studies demonstrate that, besides preventing fibrosis development, imatinib mesylate and AMN107 may potentially also reverse established fibrosis.

Based on our data and the need for new therapies to treat GO, we suggest that imatinib mesylate and AMN107 should be considered as candidates for the treatment of GO patients, especially those with recent-onset marked impairment of ocular motility. Yet, it cannot be expected that these drugs affect TGF-β_1-driven hyaluronan production by orbital fibroblasts.

ACKNOWLEDGEMENTS

This work was supported by the ‘Stichting Wetenschappelijk Onderzoek Oogziekenhuis’.

REFERENCES

1. Burch HB, Wartofsky L. *Graves' ophthalmopathy: current concepts regarding pathogenesis and management*. Endocr Rev 1993;14:747-793.
2. Eckstein AK, Quadbeck B, et al. *Thyroid associated ophthalmopathy: evidence for CD4(+) gammadelta T cells; de novo differentiation of RFD7(+) macrophages, but not of RFD1(+) dendritic cells; and loss of gammadelta and alphabeta T cell receptor expression*. Br J Ophthalmol 2004;88:803-808.
3. Weetman AP, Cohen S, et al. *Immunohistochemical analysis of the retrobulbar tissues in Graves' ophthalmopathy*. Clin Exp Immunol 1989;75:222-227.
4. Khoo TK, Bahn RS. *Pathogenesis of Graves' ophthalmopathy: the role of autoantibodies*. Thyroid 2007;17:1013-1018.
5. Prabhakar BS, Bahn RS, et al. *Current perspective on the pathogenesis of Graves' disease and ophthalmopathy*. Endocr Rev 2003;24:802-835.
6. Daniels CE, Wilkes MC, et al. *Imatinib mesylate inhibits the profibrogenic activity of TGF-beta and prevents bleomycin-mediated lung fibrosis*. J Clin Invest 2004;114:1308-1316.
7. Wang S, Wilkes MC, et al. *Imatinib mesylate blocks a non-Smad TGF-beta pathway and reduces renal fibrogenesis in vivo*. Faseb J 2005;19:1-11.
8. Druker BJ, Talpaz M, et al. *Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia*. N Engl J Med 2001;344:1031-1037.
9. Bonner JC. *Regulation of PDGF and its receptors in fibrotic diseases*. Cytokine Growth Factor Rev 2004;15:255-273.
10. Distler JH, Jungel A, et al. *Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis*. Arthritis Rheum 2007;56:311-322.
11. van Daele PL, Dik WA, et al. *Is imatinib mesylate a promising drug in systemic sclerosis?* Arthritis Rheum 2008;58:2549-2552.
12. Akhmetshina A, Dees C, et al. *Dual inhibition of c-abl and PDGF receptor signaling by dasatinib and nilotinib for the treatment of dermal fibrosis*. Faseb J 2008;22:2214-2222.
13. Dik WA, McAnulty RJ, et al. *Short course dexamethasone treatment following injury inhibits bleomycin induced fibrosis in rats*. Thorax 2003;58:765-771.
14. McAnulty RJ, Laurent GJ. *Pathogenesis of lung fibrosis and potential new therapeutic strategies*. Exp Nephrol 1995;3:96-107.
15. Heufelder AE, Bahn RS. *Modulation of Graves' orbital fibroblast proliferation by cytokines and glucocorticoid receptor agonists*. Invest Ophthalmol Vis Sci 1994;35:120-127.
16. Imai Y, Odajima R, et al. *Effect of growth factors on hyaluronan and proteoglycan synthesis by retroocular tissue fibroblasts of Graves' ophthalmopathy in culture*. Acta Endocrinol (Copenh) 1992;126:541-552.
17. Korducki JM, Loftus SJ, et al. *Stimulation of glycosaminoglycan production in cultured human retroocular fibroblasts*. Invest Ophthalmol Vis Sci 1992;33:2037-2042.
18. Paridaens D, van den Bosch WA, et al. *The effect of etanercept on Graves' ophthalmopathy: a pilot study*. Eye 2005;19:1286-1289.
19. Dik WA, Nadel B, et al. *Different chromosomal breakpoints impact the level of LMO2 expression in T-ALL*. Blood 2007;110:388-392.
20. Smith TJ, Sempowski GD, et al. *Evidence for cellular heterogeneity in primary cultures of human orbital fibroblasts*. J Clin Endocrinol Metab 1995;80:2620-2625.
21. Dik WA, Versnel MA, et al. *Dexamethasone treatment does not inhibit fibroproliferation in chronic lung disease of prematurity*. Eur Respir J 2003;21:842-847.
22. Weigel PH, Hascall VC, et al. *Hyaluronan synthases*. J Biol Chem 1997;272:13997-14000.
23. Han R, Smith TJ. *T helper type 1 and type 2 cytokines exert divergent influence on the induction of prostaglandin E2 and hyaluronan synthesis by interleukin-1beta in orbital fibroblasts: implications for the pathogenesis of thyroid-associated ophthalmopathy*. Endocrinology 2006;147:13-19.
24. Heufelder AE, Bahn RS. *Detection and localization of cytokine immunoreactivity in retro-ocular connective tissue in Graves' ophthalmopathy*. Eur J Clin Invest 1993;23:10-17.

25. McLachlan SM, Prummel MF, et al. *Cell-mediated or humoral immunity in Graves' ophthalmopathy? Profiles of T-cell cytokines amplified by polymerase chain reaction from orbital tissue*. J Clin Endocrinol Metab 1994;78:1070-1074.
26. Wakelkamp IM, Bakker O, et al. *TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients*. Clin Endocrinol (Oxf) 2003;58:280-287.
27. Antoniades HN, Bravo MA, et al. *Platelet-derived growth factor in idiopathic pulmonary fibrosis*. J Clin Invest 1990;86:1055-1064.
28. Broekelmann TJ, Limper AH, et al. *Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis*. Proc Natl Acad Sci U S A 1991;88:6642-6646.
29. Zhang K, Flanders KC, et al. *Cellular localization of transforming growth factor-beta expression in bleomycin-induced pulmonary fibrosis*. Am J Pathol 1995;147:352-361.
30. Liu JY, Morris GF, et al. *Rapid activation of PDGF-A and -B expression at sites of lung injury in asbestos-exposed rats*. Am J Respir Cell Mol Biol 1997;17:129-140.
31. Eickelberg O, Kohler E, et al. *Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3*. Am J Physiol 1999;276:L814-824.
32. Hetzel M, Bachem M, et al. *Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts*. Lung 2005;183:225-237.
33. Kaback LA, Smith TJ. *Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 1999;84:4079-4084.
34. Smith TJ. *Orbital fibroblasts exhibit a novel pattern of responses to proinflammatory cytokines: potential basis for the pathogenesis of thyroid-associated ophthalmopathy*. Thyroid 2002;12:197-203.
35. Smith RS, Smith TJ, et al. *Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation*. Am J Pathol 1997;151:317-322.
36. Usui T, Amano S, et al. *Expression regulation of hyaluronan synthase in corneal endothelial cells*. Invest Ophthalmol Vis Sci 2000;41:3261-3267.
37. Nishitsuka K, Kashiwagi Y, et al. *Hyaluronan production regulation from porcine hyalocyte cell line by cytokines*. Exp Eye Res 2007;85:539-545.
38. Demetri GD, von Mehren M, et al. *Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors*. N Engl J Med 2002;347:472-480.
39. Droogendijk HJ, Kluin-Nelemans HJ, et al. *Imatinib mesylate in the treatment of systemic mastocytosis: a phase II trial*. Cancer 2006;107:345-351.
40. Distler JH, Manger B, et al. *Treatment of pulmonary fibrosis for twenty weeks with imatinib mesylate in a patient with mixed connective tissue disease*. Arthritis Rheum 2008;58:2538-2542.
41. Kay J, High WA. *Imatinib mesylate treatment of nephrogenic systemic fibrosis*. Arthritis Rheum 2008;58:2543-2548.
42. Sfikakis PP, Gorgoulis VG, et al. *Imatinib for the treatment of refractory, diffuse systemic sclerosis*. Rheumatology (Oxford) 2008;47:735-737.

Chapter 5

Platelet-derived growth factor-BB: a stimulus for cytokine production by orbital fibroblasts in Graves' ophthalmopathy

Leendert van Steensel [1], Dion Paridaens [2], Gemma M. Dingjan [1], Paul L.A. van Daele [1], P. Martin van Hagen [1,2], Robert W.A.M. Kuijpers [3], Willem A. van den Bosch [2], Hemmo A. Drexhage [1], Herbert Hooijkaas [1] and Willem A. Dik [1]

[1] Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
[2] Rotterdam Eye Hospital, Rotterdam, The Netherlands
[3] Department of Ophthalmology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

Invest Ophthalmol Vis Sci. 2010 Feb;51(2):1002-7

ABSTRACT

Purpose

Graves' ophthalmopathy (GO) is characterized by infiltration of immune cells into the orbit, a process in which cytokines play a central role. Orbital fibroblasts are potent producers of cytokines upon different stimuli. Recently, we showed increased expression of the PDGF-B chain in GO orbital tissue. The dimeric PDGF-BB molecule has been described to activate the NF-κB pathway, which is well recognized for its role in regulating cytokine production. This study was conducted to determine the role of PDGF-BB in the production of pro-inflammatory cytokines by orbital fibroblasts in GO.

Methods

Orbital, lung and skin fibroblasts were stimulated with PDGF-BB and cytokine production (IL-1β, IL-6, IL-8, IL-16, CCL2, CCL5, CCL7 and TNF-α) was measured by ELISA. Involvement of NF-κB activation through PDGF-signalling was investigated by Electrophoretic Mobility Shift Assay (EMSA), specific NF-κB inhibitors and the PDGF-receptor kinase inhibitor imatinib mesylate.

Results

IL-6, IL-8, CCL2, CCL5 and CCL7 production by orbital fibroblasts was increased by PDGF-BB stimulation while IL-16, IL-1β and TNF-α production was not affected. PDGF-BB induced NF-κB activity in orbital fibroblasts and both NF-κB inhibitors and imatinib mesylate reduced PDGF-BB induced cytokine production. Similar, but less vigorous effects of PDGF-BB on cytokine production were observed in lung and skin fibroblasts.

Conclusions

PDGF-BB is a potent inducer of pro-inflammatory cytokines via the NF-κB pathway in orbital fibroblasts whereas cytokine production by fibroblasts from other anatomical locations showed a moderate response. These data suggest a possible role for PDGF-BB in regulating orbital inflammation in GO and identify the PDGF-signalling cascade as therapeutic target in GO.

INTRODUCTION

Graves ophthalmopathy (GO), a frequent extra-thyroidal manifestation of Graves' disease, is histologically characterized by immune cells infiltrating the orbital tissue and producing cytokines, growth factors and immunoglobulins. These factors induce and maintain the inflammatory condition in the orbit, cause edema, stimulate orbital fibroblasts to produce extracellular matrix components (ECM) and attract additional inflammatory cells.[1]

Cytokines play a critical role in the regulation of inflammatory responses through attraction and activation of inflammatory cells. Numerous cytokines, such as IL-1β, IL-4, IL-6, IL-8, TNF-α, CCL2 and CXCL10, have been identified in orbital tissue and serum from GO patients and are therefore proposed to play an important role in GO.[2-12]

Immune cells are classically considered as main producers of cytokines. However, in recent years fibroblasts have been identified as another important source of cytokines and have therefore been suggested to play an important role in initiation and maintenance of inflammation.[13] Orbital fibroblasts are considered to play an important regulatory role in the pathophysiology of GO through production of cytokines such as IL-6, IL-8, IL-16, CCL2, CCL5, CXCL9, CXCL10 and CXCL11 when activated by CD40-CD154 ligation, IL-1β, TNF-α, IFN-γ or immunoglobulins.[5, 7, 10, 14-15] The produced cytokines and chemokines are known to attract and activate B cells, T cells, neutrophils, mast cells, monocytes and macrophages.[5-6, 8, 16] Nevertheless, further insight in fibroblast activating factors in GO is required to unravel the complex pathophysiology of this disease and to find new targets in the treatment of GO.

Platelet-derived growth factor (PDGF)-BB is a homo-dimeric protein centrally involved in wound healing and fibrotic diseases by stimulating chemotaxis, proliferation and ECM production by mesenchymal cells, such as fibroblasts.[17-18] PDGF has also been described to activate NF-κB signalling, a pathway involved in the production of several pro-inflammatory cytokines.[17] Although the latter suggests that PDGF can be involved in the regulation of inflammation, only sparse data supporting this is available. So far, PDGF-BB has been reported to induce expression of the JE and KC genes, which encode CCL2 and CXCL1, by murine fibroblasts.[19-21] In human, PDGF-BB induces CCL2 and IL-6 production by skin and lung fibroblasts and IL-8 production by human corneal fibroblasts.[22-24]

Recently, we demonstrated elevated PDGF-B mRNA expression in orbital tissue from GO patients.[25] For this current study we hypothesize that PDGF-BB stimulates cytokine production by orbital fibroblasts, thereby driving orbital inflammation in GO. We examined the effect of PDGF-BB on IL-1β, IL-6, IL-8, IL-16, CCL2, CCL5, CCL7 and TNF-α production by orbital fibroblasts from GO patients and controls as well as involvement of NF-κB activation. Since orbital fibroblasts are known to respond differently to stimuli than fibroblasts from other anatomical sites[13, 25], also lung and skin fibroblasts were investigated.

We show that PDGF-BB induces orbital fibroblasts to produce pro-inflammatory cytokines via a NF-κB dependent pathway. This response is stronger in orbital fibroblasts compared to lung and skin fibroblasts. Our current studies underscore the unique features of orbital fibroblasts and identify PDGF-BB as a regulatory factor of orbital inflammation in GO.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin and trypsin/EDTA were purchased from Cambrex BioWhittaker (Verviers, Belgium). Recombinant human PDGF-BB, recombinant human IL-16, biotinylated mouse anti-human IL-16 antibodies and CCL5 and CCL7 ELISA were obtained from R&D Systems (Abingdon, UK). Capture IL-16 antibodies for the IL-16 ELISA were kindly provided by W. Cruikshank (Boston, USA). IL-1β, IL-6, IL-8, CCL2 and TNF-α ELISA were obtained from Biosource (London, UK). The NF-κB inhibitors SC-514, SN50 and the control peptide SN50M were purchased from Calbiochem (Nottingham, UK). NF-κB p65 and p50 antibodies were obtained from Clontech (Mountain View, CA). Human fetal lung fibroblasts (HFL-1) were obtained from ATCC (Teddington, UK). Normal skin fibroblasts were kindly provided by Prof. E.P. Prens (Rotterdam, The Netherlands). Imatinib mesylate was kindly provided by Novartis Pharma (Basel, Switzerland).

Patients and controls

Orbital tissue was obtained from ten GO patients undergoing orbital decompression surgery and from five control patients without known thyroid disease and undergoing orbital surgery for other reasons (Table 1). All patients were euthyroid at the time undergoing orbital surgery. All tissues were obtained in the Rotterdam Eye Hospital (Rotterdam, The Netherlands) after informed consent and in accordance with the principles of the Declaration of Helsinki and after approval by the institutional review board at the Erasmus MC, University Medical Center (Rotterdam, The Netherlands).

Orbital fibroblast culture

Orbital fibroblast strains were established as described previously.[25] Briefly, orbital tissue was cut into small pieces and cultured in DMEM supplemented with 10% heat inactivated FCS and antibiotics (DMEM 10% FCS) in a humidified atmosphere of 5.0% CO_2 at 37°C. Once fibroblast monolayers were obtained, cultures were serially passaged after gentle treatment with trypsin/EDTA. Fibroblast strains used for experiments were between the 6th and 12th passage.

Table 1. Patients and controls

	Active GO (n=5)	Inactive GO (n=5)	Controls (n=5)
Age (range)	63.4 (32-79)	56.2 (43-69)	64.4 (49-78)
Sex (m/f)	0 / 5	0 / 5	1 / 4
Smoking	4 / 5	3 / 5	1 / 5
Graves' Disease	5 / 5	5 / 5	0/5
- RAI	4 / 5	5 / 5	-
- Surgery	1 / 5	0 / 5	-
- Strumazol	5 / 5	5 / 5	-
Treatment GO	5 / 5	5 / 5	0/5
- Surgery	5 / 5	5 / 5	-
- Prednison	5 / 5	5 / 5	-
- Radiation	1 / 5	2 / 5	-
Euthyroid	5 / 5	5 / 5	5/5
TSH-Receptor antibodies	5 / 5	5 / 5	0 / 5
TPO antibodies	3 / 5	4 / 5	0 / 5
Clinical Activity Score (CAS) (range)	5 (4 - 6)	2 (1 - 3)	-
NO-SPECS (range) #	4 (2 – 7)	3 (1 – 7)	-

TPO Thyroid peroxidase
NO-SPECS score: as described before [18]

Cytokine production by fibroblast cultures

Fibroblasts were seeded at 3.0 x10^5 cells/well into 6-well plates in DMEM 10% FCS and allowed to adhere. Hereafter, the fibroblasts were overnight incubated in DMEM supplemented with 1.0% FCS and antibiotics (DMEM 1.0% FCS). Subsequently, cells were washed and cultured in DMEM 1.0% FCS with or without PDGF-BB (50 ng/ml) for 24 hours. In order to examine NF-κB involvement, fibroblasts were pre-incubated with SC-514 (100 μM) 1 hour prior to PDGF-BB stimulation or with SN50 (15μM) or SN50M (15μM) for 15 minutes prior to PDGF-BB stimulation. To demonstrate involvement of PDGF-signalling, the PDGF-receptor tyrosine kinase inhibitor imatinib mesylate (2.5 μg/ml) was supplemented overnight prior to PDGF-BB stimulation as described previously.[25] Supernatants were harvested and cytokine levels were determined by ELISA according to the manufacturer's instructions. IL-16 ELISA was performed as previously described.[26]

NF-κB Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared by washing the cells twice with ice-cold PBS and subsequent scraping in 2 ml ice-cold PBS containing protease inhibitors. Cells were centrifuged and lysed with ice-cold lysisbuffer containing 10mM HEPES, 1.5mM $MgCl_2$, 10mM KCL, 0.5mM DTT and a protease inhibitor cocktail. After incubation and centrifugation, nuclei were lysed in ice-cold lysisbuffer containing 20mM HEPES, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and a protease inhibitor cocktail. Protein concentration was determined using the Bradford method. EMSA was performed as described previously.[27] Double-stranded γ-ATP-^{32}P-labeled oligonucleotide probes

specific for either NF-κB or OCT-1 were prepared using the following oligonucleotides: NF-κB sense 5'-AGTTGAGGGGACTTTCCCAGGC-3', NF-κB antisense 5'-GCCTGGGAAAGTCCCCTCAACT-3', OCT-1 sense 5'- TGTCGAATGCAAA-TCACTAGAA-3', OCT-1 antisense 5'- TTCTAGTGATTTGCATTCGACA-3'. To determine binding, equal amounts of nuclear extracts (10 μg) were incubated with labeled double-stranded oligonucleotide. Poly-dIdC was added to prevent unspecific binding of proteins to the probe. Specificity of the binding reaction was confirmed by a competition assay with a 4-fold excess of unlabeled double-stranded oligonucleotide probe. Supershift analysis was performed by pre-incubating nuclear protein extracts with antibodies specific for the p65 or the p50 NF-κB subunit for 30 min before incubation with labeled probe. Complexes were separated on a non-denaturing PAGE gel and bands were detected by a Typhoon scanner. Densitometric analysis was performed using ImageQuant 5.2 (Molecular Dynamics/GE Healthcare, Diegen, Belgium).

Statistical analysis

Data were analyzed using the paired Student's t-test. Differences between orbital fibroblasts and skin fibroblasts were analyzed using the Mann-Whitney test. A p-value <0.05 was considered significant.

RESULTS

Effect of PDGF-BB on cytokine production by orbital fibroblasts

PDGF-BB significantly induced IL-6, IL-8, CCL2, CCL5 and CCL7 production by orbital fibroblasts from both GO and control patients (all $p<0.05$; Fig. 1), but did not induce IL-1β, IL-16 and TNF-α production (data not shown). No differences in PDGF-BB-induced cytokine production were observed between orbital fibroblasts obtained from GO patients with active or inactive disease or control subjects.

Effect of PDGF-BB on cytokine production by lung and skin fibroblasts

Since orbital fibroblast have been shown to respond differently to certain stimuli than fibroblasts from other anatomic locations[13, 25], we examined whether the stimulatory effect of PDGF-BB on pro-inflammatory cytokine production was unique to orbital fibroblasts. Hereto, we stimulated HFL-1 (human fetal lung fibroblasts) and three normal skin fibroblast strains with PDGF-BB. In skin and lung fibroblasts, PDGF-BB induced IL-6, IL-8 and CCL2 production to a lesser extend than orbital fibroblasts (CCL2; $p<0.05$ for skin vs orbital fibroblasts). In contrast to orbital fibroblasts, CCL5 production was not induced by PDGF-BB in skin and lung fibroblasts ($p<0.05$ for skin vs orbital fibroblasts). Skin fibroblasts produced significantly ($p<0.05$) more CCL7 upon PDGF-BB stimulation than orbital fibroblasts.

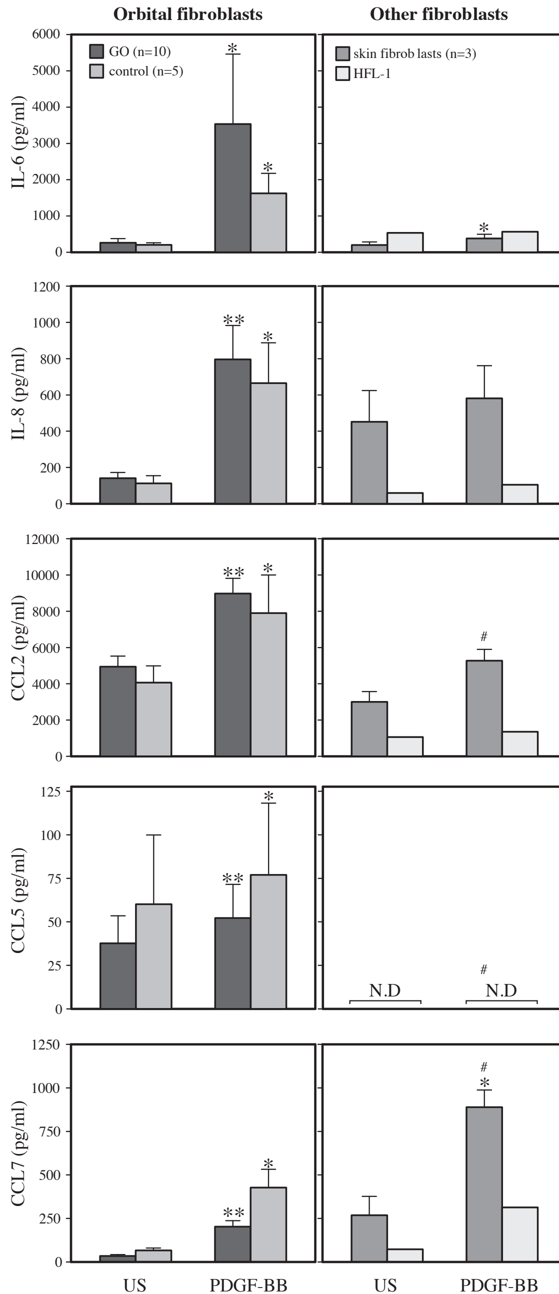


Figure 1 The production of IL-6, IL-8, CCL2, CCL5 and CCL7 by orbital (n=15), skin (n=3) and lung fibroblasts (n=1) upon 24 hours of stimulation with PDGF-BB (50 ng/ml). Each bar represents mean values and standard error of mean (SEM). N.D is not detectable, US is unstimulated. Data were analyzed using the paired Students t-test or the Mann-Whitney test. * $p<0.05$, ** $p<0.01$ vs unstimulated, # $p<0.05$ orbital vs skin fibroblasts.

5

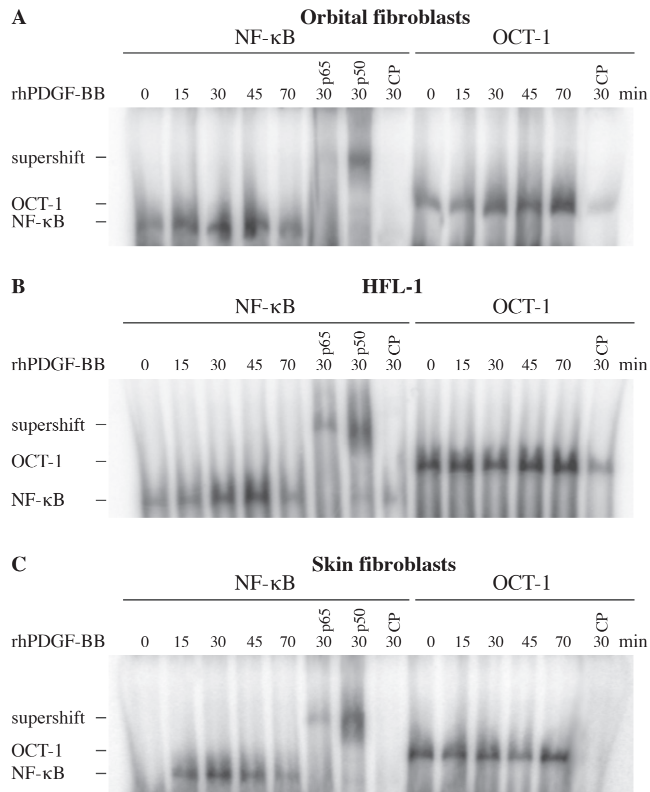


Figure 2 Induction of NF-κB activity following PDGF-BB (50 ng/ml) stimulation. Binding activity to an oligonucleotide containing a NF-κB consensus motif was assayed in nuclear extracts from orbital fibroblasts (A), lung fibroblasts (HFL-1; B) and skin fibroblasts (C). As loading control, the same nuclear extracts were analyzed for their binding activity to an oligonucleotide containing a consensus motif for the constitutively expressed transcription factor OCT-1. Fibroblasts were stimulated for 15, 30, 45 and 70 minutes with PDGF-BB. NF-κB and OCT-1 binding complexes are indicated on the left. Supershift indicates the motility retardation (supershift) induced when EMSA was carried out in the presence of antibodies to the p65 or p50 components of the NF-κB hetero-dimer. CP indicates EMSA carried out in the presence of excess unlabeled probe (cold probe). Data are from representative experiments.

Effect of PDGF-BB on NF-κB activity in fibroblasts

Since production of many cytokines is controlled by the NF-κB signalling pathway, we determined whether PDGF-BB induced nuclear translocation of active NF-κB in fibroblasts. Nuclear NF-κB activity was detectable between 15-45 minutes following PDGF-BB stimulation and was induced to comparable levels in orbital fibroblasts, lung fibroblasts and skin fibroblasts (Fig 2). Analysis for OCT-1, a constitutive active transcription factor, revealed equal loading between all tested samples (Fig 2). Addition of excess unlabeled probe (CP; either NF-κB or OCT-1) to the reaction mixture was associated with loss of

signal, demonstrating specificity of the DNA binding proteins (Fig 2). To further characterize the nuclear proteins binding to the NF-κB motif, nuclear extracts were pre-incubated with antibodies to p65 or p50, two protein subunits of the active NF-κB hetero-dimer. Incubation of nuclear extracts with these antibodies retarded mobility of the labeled NF-κB oligonucleotide probe, thereby supershifting activity to the top of the gel (Fig 2). This suggests that the PDGF-BB-induced nuclear NF-κB contained both the p65 and p50 subunits in all tested fibroblasts.

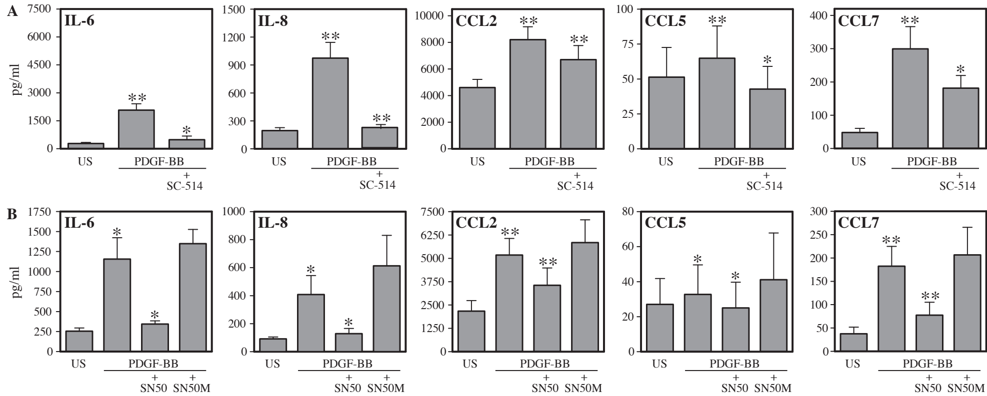


Figure 3 IL-6, IL-8, CCL2, CCL5 and CCL7 production by GO (n=7) and control (n=4) orbital fibroblasts is inhibited by blockade of the NF-κB pathway. Fibroblasts were stimulated for 24 hours with PDGF-BB (50 ng/ml) after (A) 60 minutes of pre-incubation with SC-514 (100 μM) or (B) 15 minutes of pre-incubation with SN50 (15 μM) and SN50M (15 μM). Each bar represents mean values and standard error of mean (SEM). US is unstimulated. Data were analyzed using the paired Students t-test. $p<0.05$, ** $p<0.01$ PDGF-BB vs unstimulated and PDGF-BB vs PDGF-BB + SC514 or PDGF-BB + SN50.

The effect of imatinib mesylate on PDGF-BB-induced cytokine production by orbital fibroblasts

Previously, we demonstrated that imatinib mesylate blocks PDGF-receptor phosphorylation and subsequent signalling in orbital fibroblasts.[25] Therefore, to confirm that PDGF receptor activation was required we tested whether imatinib mesylate blocked PDGF-BB-induced NF-κB activation and cytokine production. As expected, inhibition of PDGF receptor activation by imatinib mesylate prevented PDGF-BB induced NF-κB activation (Fig. 4A) and subsequent cytokine production (all $p<0.05$; Fig 4B).

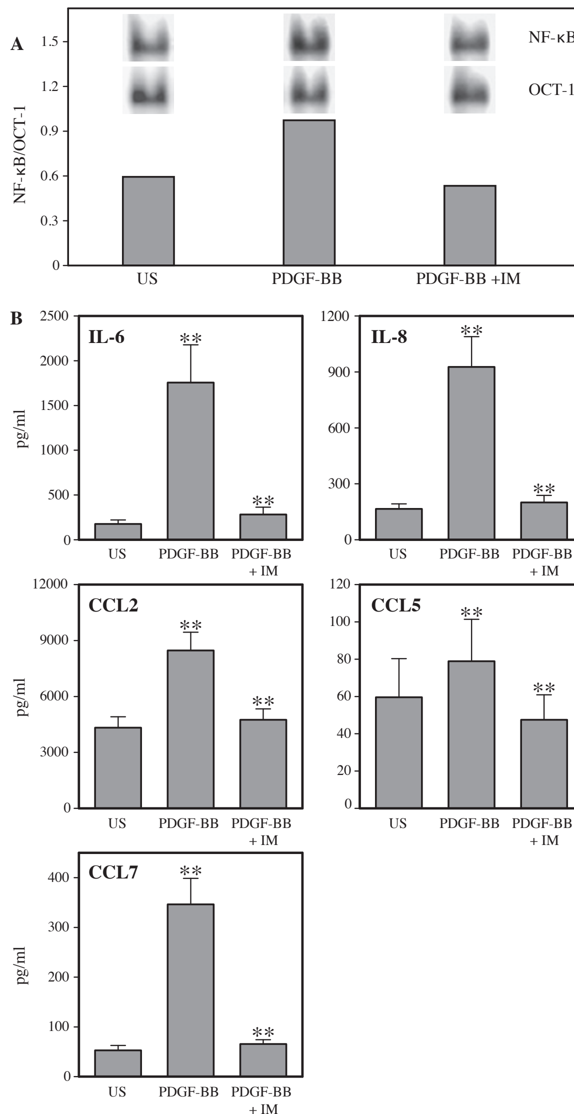


Figure 4 Imatinib mesylate (IM; 2.5 μg/ml) inhibits PDGF-BB (50 ng/ml) induced NF-κB activation and subsequent pro-inflammatory cytokine production. (A) Orbital fibroblasts were stimulated for 30 minutes with PDGF-BB with or without pre-incubation with imatinib mesylate. Subsequently, binding activity of nuclear extracts to an oligonucleotide containing a NF-κB or OCT-1 consensus motif was assayed and by densitometry the NF-κB/OCT-1 ratio was calculated. Data are from a representative experiment. (B) GO (n=7) and control (n=4) orbital fibroblasts were stimulated for 24 hours with PDGF-BB with or without pre-incubation with imatinib mesylate and subsequently IL-6, IL-8, CCL2, CCL5 and CCL7 levels were determined. Each bar represents mean values and standard error of mean (SEM). US is unstimulated. Data were analyzed using the paired Students t-test. * $p<0.05$, ** $p<0.01$ PDGF-BB vs unstimulated and PDGF-BB vs PDGF-BB + IM.

DISCUSSION

Orbital inflammation is a key feature of GO, and orbital fibroblasts are considered important in driving the inflammatory process through production of cytokines.[13] So far only few stimuli, such as CD40-CD154 mediated interactions between T cells and orbital fibroblasts, anti-IGF-receptor and anti-TSH-receptor antibodies, TNF-α, IFN-γ, IL-4 and IL-1β have been found to stimulate the production of cytokines by orbital fibroblasts.[5, 7, 10, 14-16]

Recently, we found increased PDGF-B mRNA expression in GO orbital tissue and demonstrated enhanced proliferation and hyaluronan production by orbital fibroblasts upon PDGF-BB stimulation, suggesting a role of the homo-dimeric growth factor PDGF-BB in the pathophysiology of GO.[25] Here we demonstrate for the first time that PDGF-BB induces production of IL-6, IL-8, CCL2, CCL5 and CCL7, cytokines relevant to the pathophysiology of GO, by orbital fibroblasts. IL-6 recruits and activates B cells and stimulates plasma cell differentiation and immunoglobulin production.[28] Furthermore, increased IL-6 mRNA levels have been described in orbital tissue from GO patients.[9] Also, IL-6 increases thyroid stimulating hormone receptor expression on orbital fibroblasts[29] and is considered as a determinant of orbital adipogenesis, another important component of the pathology of GO.[30] IL-8 is a powerful attractant for neutrophils.[31] Increased IL-8 mRNA levels have been detected in orbital tissue from GO patients[9] and elevated serum IL-8 levels have been associated with hyperthyroidism.[14] CCL2 and CCL7 are attractants for monocytes and macrophages[32], which are both abundantly present in the infiltrate in GO orbital tissue.[14, 33] In addition, increased CCL2 mRNA expression levels in GO orbital tissue correlate positively with macrophage infiltration into the adipose tissue.[14] CCL5 is well known for its ability to attract T cells.[34] Although expression of CCL5 in GO orbital tissue has so far never been demonstrated, a role for CCL5 in orbital T cell recruitment in GO has been suggested based on its production by orbital fibroblasts upon stimulation with immunoglobulins from patients with Graves' disease.[7, 35] Our current data suggest that PDGF-BB, through activation of orbital fibroblasts, induces production of several cytokines in orbital tissue, thereby regulating immune cell infiltration into the orbit in GO. Such a role for PDGF-BB is supported by studies in which pulmonary overexpression of the PDGF-B gene resulted in marked alveolitis, comprising mainly mononuclear cells and macrophages.[19, 36]

Inhibition of NF-κB activity completely abrogated PDGF-BB-induced IL-6, IL-8 and CCL5 production while CCL2 and CCL7 production were only partially reduced. This suggests that PDGF-BB induces also NF-κB independent activities that control CCL2 and CCL7 production or secretion by orbital fibroblasts. This is supported by the observation that PDGF-BB-induced NF-κB activity and cytokine production were completely blocked by the PDGF-receptor specific inhibitor imatinib mesylate. This strongly suggests that the observed effects truly depended on PDGF-signalling.

Our current data underscore the unique functional nature that has been attributed to the orbital fibroblast[16, 37] which may be a critical determinant for the development of GO.[13] Orbital fibroblasts produced more IL-6, IL-8, CCL2 and CCL5 upon PDGF-BB stimulation than skin and lung fibroblasts. This was not related to inability of skin and lung fibroblasts to activate NF-κB DNA binding activity upon PDGF-BB stimulation. Differences in NF-κB dimer induction are also unlikely to have contributed as PDGF-BB induced NF-κB p65/p50 hetero-dimer activity equally in orbital, skin and lung fibroblasts. Induction of p65/p50 activity is in line with previous observations of PDGF-BB-induced NF-κB activation in fibroblasts.[38] We can not exclude that specific nuclear modifications of NF-κB did enhance transcriptional activity[39-40] in orbital fibroblasts. In addition, the expression level and activity of other transcription factors may have influenced the transactivation potential of NF-κB.[40-41] In contrast to skin and lung fibroblasts, orbital fibroblasts had some basal expression of CCL5 which was enhanced upon PDGF-BB stimulation. This is opposite to previous studies demonstrating absence of basal CCL5 production by orbital fibroblasts.[7] Possibly this discrepancy is related to low basal production levels and sensitivity of assays used.

Collectively our previous observation of elevated orbital PDGF-B mRNA expression in GO[25] together with our current findings suggest that PDGF-BB, through activation of NF-κB signalling and subsequent cytokine production by orbital fibroblasts, is able to attract a variety of immune cells into the orbit. Thus besides stimulating the fibrotic component of GO,[25] PDGF-BB can be considered important in the initiation and maintenance of the inflammatory response in this disease as well. Therefore, we consider the PDGF system to be an attractive therapeutic target in GO, e.g. via the use of imatinib mesylate.

ACKNOWLEDGEMENTS

This work was supported by the 'Stichting Wetenschappelijk Onderzoek Oogziekenhuis (SWOO)' and the 'Nederlandse vereniging van Graves' patiënten (NVGP)'.

REFERENCES

1. Prabhakar BS, Bahn RS, et al. *Current perspective on the pathogenesis of Graves' disease and ophthalmopathy*. Endocr Rev 2003;24:802-835.
2. McLachlan SM, Prummel MF, et al. *Cell-mediated or humoral immunity in Graves' ophthalmopathy? Profiles of T-cell cytokines amplified by polymerase chain reaction from orbital tissue*. J Clin Endocrinol Metab 1994;78:1070-1074.
3. Forster G, Otto E, et al. *Analysis of orbital T cells in thyroid-associated ophthalmopathy*. Clin Exp Immunol 1998;112:427-434.
4. Hogaboam CM, Steinhauser ML, et al. *Novel roles for chemokines and fibroblasts in interstitial fibrosis*. Kidney Int 1998;54:2152-2159.
5. Sempowski GD, Rozenblit J, et al. *Human orbital fibroblasts are activated through CD40 to induce proinflammatory cytokine production*. Am J Physiol 1998;274:C707-714.
6. Hiromatsu Y, Yang D, et al. *Cytokine profiles in eye muscle tissue and orbital fat tissue from patients with thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 2000;85:1194-1199.
7. Pritchard J, Horst N, et al. *Igs from patients with Graves' disease induce the expression of T cell chemoattractants in their fibroblasts*. J Immunol 2002;168:942-950.
8. Kumar S, Bahn RS. *Relative overexpression of macrophage-derived cytokines in orbital adipose tissue from patients with graves' ophthalmopathy*. J Clin Endocrinol Metab 2003;88:4246-4250.
9. Wakelkamp IM, Bakker O, et al. *TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients*. Clin Endocrinol (Oxf) 2003;58:280-287.
10. Antonelli A, Rotondi M, et al. *Interferon-gamma-inducible alpha-chemokine CXCL10 involvement in Graves' ophthalmopathy: modulation by peroxisome proliferator-activated receptor-gamma agonists*. J Clin Endocrinol Metab 2006;91:614-620.
11. Lacka K, Manuszewska E, et al. *The effect of methylprednisolone pulse treatment on cytokine network in Graves ophthalmopathy*. Curr Eye Res 2007;32:291-297.
12. Gianoukakis AG, Khadavi N, et al. *Cytokines, Graves' disease, and thyroid-associated ophthalmopathy*. Thyroid 2008;18:953-958.
13. Smith TJ. *Unique properties of orbital connective tissue underlie its involvement in Graves' disease*. Minerva Endocrinol 2003;28:213-222.
14. Chen MH, Chen MH, et al. *Role of macrophage infiltration in the orbital fat of patients with Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2008;69:332-337.
15. Antonelli A, Ferrari SM, et al. *Monokine induced by interferon gamma (IFNgamma) (CXCL9) and IFNgamma inducible T-cell alpha-chemoattractant (CXCL11) involvement in Graves' disease and ophthalmopathy: modulation by peroxisome proliferator-activated receptor-gamma agonists*. J Clin Endocrinol Metab 2009;94:1803-1809.
16. Hwang C, Afifiyan N, et al. *Orbital fibroblasts from patients with thyroid-associated ophthalmopathy over-express CD40: CD154 hyper-induces IL-6, IL-8 and MCP-1*. Invest Ophthalmol Vis Sci 2008.
17. Pahl HL. *Activators and target genes of Rel/NF-kappaB transcription factors*. Oncogene 1999;18:6853-6866.
18. Bonner JC. *Regulation of PDGF and its receptors in fibrotic diseases*. Cytokine Growth Factor Rev 2004;15:255-273.
19. Hoyle GW, Li J, et al. *Emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor*. Am J Pathol 1999;154:1763-1775.
20. Alberta JA, Auger KR, et al. *Platelet-derived growth factor stimulation of monocyte chemoattractant protein-1 gene expression is mediated by transient activation of the phosphoinositide 3-kinase signal transduction pathway*. J Biol Chem 1999;274:31062-31067.
21. Cochran BH, Reffel AC, et al. *Molecular cloning of gene sequences regulated by platelet-derived growth factor*. Cell 1983;33:939-947.
22. Distler O, Pap T, et al. *Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects on monocyte chemotaxis and collagen synthesis*. Arthritis Rheum 2001;44:2665-2678.

23. Vij N, Sharma A, et al. *PDGF-driven proliferation, migration, and IL8 chemokine secretion in human corneal fibroblasts involve JAK2-STAT3 signaling pathway*. Mol Vis 2008;14:1020-1027.
24. Takemura H, Suzuki H, et al. *Enhanced interleukin 6 production by cultured fibroblasts from patients with systemic sclerosis in response to platelet derived growth factor*. J Rheumatol 1998;25:1534-1539.
25. van Steensel L, Paridaens D, et al. *Imatinib mesylate and AMN107 inhibit PDGF-signaling in orbital fibroblasts: a potential treatment for Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2009;50:3091-3098.
26. Little FF, Lynch E, et al. *Tumor necrosis factor-alpha-induced synthesis of interleukin-16 in airway epithelial cells: priming for serotonin stimulation*. Am J Respir Cell Mol Biol 2003;28:354-362.
27. Pandey R, DeStephan CM, et al. *NKp30 ligation induces rapid activation of the canonical NF-kappaB pathway in NK cells*. J Immunol 2007;179:7385-7396.
28. Hirano T. *Interleukin 6 and its receptor: ten years later*. Int Rev Immunol 1998;16:249-284.
29. Jyonouchi SC, Valyasevi RW, et al. *Interleukin-6 stimulates thyrotropin receptor expression in human orbital preadipocyte fibroblasts from patients with Graves' ophthalmopathy*. Thyroid 2001;11:929-934.
30. Bahn RS. *Thyrotropin receptor expression in orbital adipose/connective tissues from patients with thyroid-associated ophthalmopathy*. Thyroid 2002;12:193-195.
31. Kobayashi Y. *The role of chemokines in neutrophil biology*. Front Biosci 2008;13:2400-2407.
32. Proost P, Wuyts A, et al. *Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1*. J Leukoc Biol 1996;59:67-74.
33. Eckstein AK, Quadbeck B, et al. *Thyroid associated ophthalmopathy: evidence for CD4(+) gammadelta T cells; de novo differentiation of RFD7(+) macrophages, but not of RFD1(+) dendritic cells; and loss of gammadelta and alphabeta T cell receptor expression*. Br J Ophthalmol 2004;88:803-808.
34. Schall TJ, Bacon K, et al. *Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES*. Nature 1990;347:669-671.
35. Pritchard J, Han R, et al. *Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway*. J Immunol 2003;170:6348-6354.
36. Yoshida M, Sakuma J, et al. *A histologically distinctive interstitial pneumonia induced by overexpression of the interleukin 6, transforming growth factor beta 1, or platelet-derived growth factor B gene*. Proc Natl Acad Sci U S A 1995;92:9570-9574.
37. Chen B, Tsui S, et al. *IL-1 beta induces IL-6 expression in human orbital fibroblasts: identification of an anatomic-site specific phenotypic attribute relevant to thyroid-associated ophthalmopathy*. J Immunol 2005;175:1310-1319.
38. Olashaw NE. *Inducible activation of RelB in fibroblasts*. J Biol Chem 1996;271:30307-30310.
39. Chen LF, Mu Y, et al. *Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB*. Embo J 2002;21:6539-6548.
40. Hayden MS, Ghosh S. *Shared principles in NF-kappaB signaling*. Cell 2008;132:344-362.
41. Gerritsen ME, Williams AJ, et al. *CREB-binding protein/p300 are transcriptional coactivators of p65*. Proc Natl Acad Sci U S A 1997;94:2927-2932.

Chapter 6

Whole orbital tissue culture identifies imatinib mesylate and adalimumab as potential therapeutics for Graves' ophthalmopathy

Leendert van Steensel [1], P. Martin van Hagen [1], Dion Paridaens [2],
Robert W.A.M. Kuijpers [3], Willem A. van den Bosch [2],
Hemmo A. Drexhage [1], Herbert Hooijkaas [1] and Willem A. Dik [1]

[1] Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
[2] Rotterdam Eye Hospital, Rotterdam, The Netherlands
[3] Department of Ophthalmology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

Br J Ophthalmol. 2011 May;95(5):735-8.

ABSTRACT

Background and Aims

Biologicals and small inhibitory molecules are used to treat inflammatory diseases, but their efficacy varies upon clinical application. Using a whole orbital tissue culture system we tested the potential efficacy of imatinib mesylate (a tyrosine kinase inhibitor that blocks PDGF-receptor, c-Abl and c-Kit activity) and adalimumab (an anti-TNF-α antibody) for the treatment of Graves' ophthalmopathy (GO).

Methods

Orbital fat tissue from GO patients (n = 10) was cultured with or without imatinib mesylate or adalimumab. PDGF-B and TNF-α mRNA expression levels were determined in the primary orbital tissue and IL-6 and hyaluronan were measured in tissue culture supernatants.

Results

Imatinib mesylate significantly ($p=0.005$) reduced IL-6 and hyaluronan production. The inhibition of hyaluronan production correlated positively and significantly ($p<0.05$) with the PDGF-B mRNA level in the primary tissue. Adalimumab also significantly ($p=0.005$) reduced IL-6 production. The amount of IL-6 inhibition correlated positively with the TNF-α mRNA level in the primary tissue, but this was not significant.

Conclusions

Imatinib mesylate can be expected to reduce inflammation and tissue remodelling in GO, while adalimumab can mainly be expected to reduce inflammation. This *in vitro* tissue culture model may, in future, prove valuable to test novel therapeutics for their presumed effect in GO as well as in other inflammatory diseases.

INTRODUCTION

Advances in the understanding of the pathophysiology of inflammatory disorders with regard to cellular involvement as well as the contribution of (soluble) mediators has led to development of biologicals that target cytokines (e.g. adalimumab; a human anti-tumor necrosis factor (TNF)-α antibody), B cells (e.g. rituximab; an anti-CD20 antibody) or cell-cell interactions (e.g abatacept; a fusion protein that blocks CD80/CD86-induced co-stimulation). Furthermore, small inhibitory molecules directed against specific signaling molecules have been developed, for example imatinib mesylate that targets the tyrosine kinase activity of c-ABL, c-Kit and the platelet-derived growth factor (PDGF)-receptor.[1-2] The application of these therapeutics has improved the treatment results in various inflammatory disorders, such as rheumatoid arthritis, psoriasis, systemic sclerosis as well as specific uveitis entities.[3-7] Nevertheless, despite the promising results of these therapeutics, a substantial number of patients do not respond, often without a clear explanation, and have to be switched to other therapeutics.

New therapeutics are introduced with a label that describes their application for specific disorders within specific limits. All use outside of this description is off-label and can only be performed if sufficient expectations exist that the agent may indeed prove effective for the disorder. Effective *in vitro* screening systems may prove helpful to that purpose, especially for rare immune mediated diseases in which the achievement of randomized controlled trials is extremely difficult.[8]

Graves' ophthalmopathy (GO) is an inflammatory fibro-proliferative disease of the orbit, that is characterized by inflammation and excessive orbital fibroblast proliferation and extracellular matrix production (especially hyaluronan), which together cause orbital tissue expansion and proptosis.[9-10] Besides autoantibodies against the thyroid stimulating hormone receptor and the insulin-like growth factor-1 receptor, also cytokines, including TNF-α and interleukin (IL)-6, as well as growth factors, such as PDGF, play a role in the pathophysiology of GO.[9, 11-12] In line with the growing importance of biological therapies in clinical practice, several small studies with biologicals have been performed in GO. We demonstrated that TNF-α targeting by etanercept (a TNF-receptor Fc fusion protein) in GO patients resulted in reduction of orbital inflammation.[13] A comparable reduction in inflammation was observed in a GO patient treated with the chimeric anti-TNF-α antibody infliximab.[14] Furthermore, promising results have been obtained with rituximab in GO patients.[15]

Recently, we demonstrated an elevated PDGF-B mRNA expression in GO orbital tissue.[11] We showed that PDGF-BB enhanced proliferation as well as cytokine and hyaluronan production by orbital fibroblasts.[11-12] These effects were all efficiently blocked through inhibition of PDGF receptor activity by the tyrosine kinase inhibitors imatinib mesylate and nilotinib.[11-12] Although these data suggest that inhibition of PDGF signaling could be

effective in GO, these results were obtained using a single cell (orbital fibroblast) and single mediator (PDGF-BB) culture system, while the orbital pathophysiological process of GO comprises a variety of cell types (e.g. fibroblasts, adipocytes, T cells, B cells, mast cells and macrophages)[9, 16-17] and mediators (e.g. IL-6, TNF-α and TGF-β_1).[11, 13, 18]

Therefore, to determine the potential efficacy of imatinib mesylate in a physiologically more relevant culture system, we cultured whole orbital tissue from GO patients in the presence or absence of imatinib mesylate and determined the effect on the production of IL-6 and hyaluronan, two important markers in GO. IL-6 is a factor highly expressed in orbital tissue and serum from GO patients[18-20] and is known to recruit and activate B cells as well as plasma cells.[21] Furthermore, IL-6 enhances the differentiation of orbital fibroblasts into mature adipocytes and the expression of the thyroid stimulating hormone receptor on these adipocytes.[22] Hyaluronan is the most important glycosaminoglycan produced in the orbit and its production is strongly enhanced in the orbit of GO patients and thereby contributes markedly to orbital tissue expansion and peri-orbital edema.[9] The same set of experiments was performed with adalimumab in order to examine the effect of specific anti-TNF-α therapy.

METHODS

Patients

Orbital fat tissue was obtained from ten patients with GO who underwent orbital decompression surgery for burnt-out ophthalmopathy (Clinical Activity Scores 0/7).[13] All patients were euthyroid and had not received steroid or other immunosuppressive treatment for at least 3 months at the moment of surgery. All tissues were obtained in the Rotterdam Eye Hospital (Rotterdam, the Netherlands) after informed consent had been obtained in accordance with the principles of the Declaration of Helsinki and after approval by the institutional review board at the Erasmus MC, University Medical Center (Rotterdam, the Netherlands).

Orbital tissue culture

Of each patient, the orbital tissue was cut into 4 equal pieces of which one (further referred to as primary tissue) was immediately snap-frozen for mRNA extraction. The other three pieces were cultured in medium (Dulbecco's modified Eagle's medium containing 1% fetal calf serum and antibiotics), medium with imatinib mesylate (2.5 μg/ml, Novartis Pharma, Basel, Switzerland) or medium with adalimumab (10 μg/ml, Abbott, Hoofddorp, the Netherlands) for 48 hours. Hereafter, medium was removed and fresh medium containing the same therapeutic as before was added for another 24 hours. Subsequently, cultured tissues were dried on filtration paper, snap-frozen and weighted. The collected culture supernatants were subjected to IL-6 ELISA (Biosource, London, UK) and hyaluronan ELISA (R&D Systems, Abingdon, UK).[11-12]

PDGF-B and TNF-α mRNA expression

RNA was isolated using Rneasy colums (Qiagen, Hilden, Germany) from snap-frozen primary tissue and reverse transcribed into cDNA. PDGF-B and TNF-α mRNA expression was determined by real-time quantitative PCR (RQ-PCR, 7900 PCR system; Applied Biosystems, Foster City, CA). Transcript levels were normalized to the control gene Abelson.[11]

Statistics

The effect of imatinib mesylate and adalimumab on IL-6 and hyaluronan levels in culture supernatants were analyzed using the paired Wilcoxon rank sum test. Inhibition indices (calculated as: 1 - the production levels achieved with medication / production levels without medication) were correlated to mRNA expression levels in primary tissue using Spearman's correlation test. A p-value < 0.05 was considered significant.

RESULTS

The effect of imatinib mesylate on IL-6 and hyaluronan production by GO orbital tissues

Imatinib mesylate reduced IL-6 and hyaluronan production in all samples (Fig 1A, both p=0.005). Similar results were observed when IL-6 and hyaluronan production were corrected for orbital tissue weight (data not shown). The IL-6 inhibition index showed a positive correlation (*r*=0.406) with the PDGF-B mRNA level in the primary tissue, but this proved not significant (Fig 1B, p=0.244). The hyaluronan inhibition index correlated positively and significantly (*r*=0.733, p=0.02) with the PDGF-B mRNA level in the primary tissue (Fig 1B).

The effect of adalimumab on IL-6 and hyaluronan production by GO orbital tissues

Adalimumab reduced the IL-6 production in all samples (Fig 2A, p=0.005). The hyaluronan production was reduced in 8 of 10 tissues, but this reduction proved not significant (Fig 2A, p=0.06). Similar results were observed when IL-6 and hyaluronan production were corrected for orbital tissue weight (data not shown). The IL-6 inhibition index showed a positive correlation (*r*=0.612) with the TNF-α mRNA level in the primary tissue but this proved not significant (Fig 2B, p=0.06). No positive correlation was observed between the hyaluronan inhibition index and the TNF-α mRNA levels within the primary tissue (Fig 2B, *r*=-0.491, p=0.15).

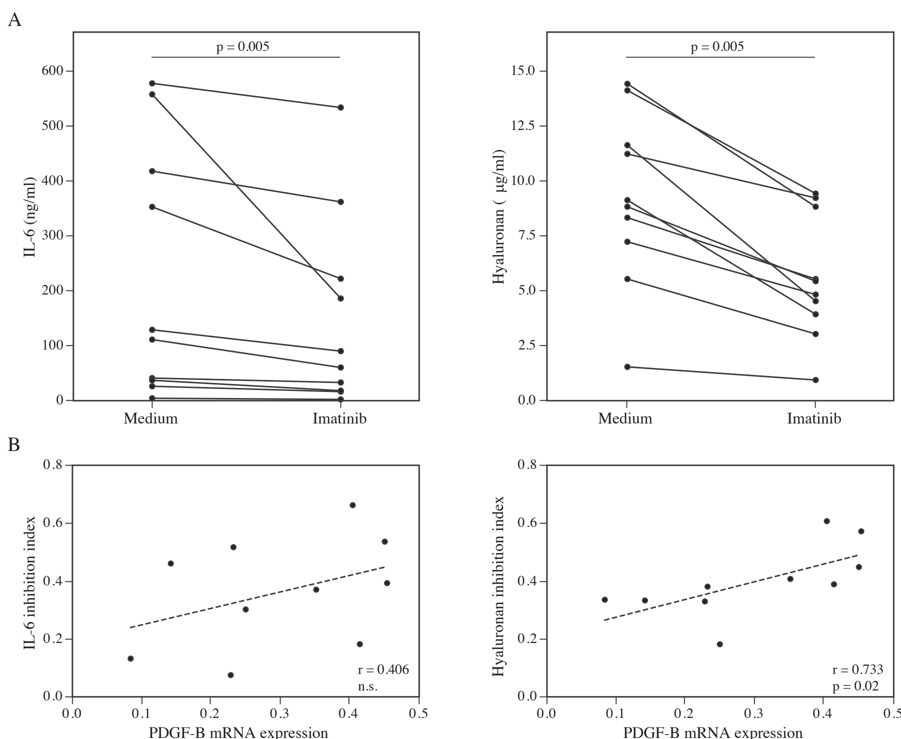


Figure 1 B The effect of imatinib mesylate (2.5 μg/ml) on cultured GO orbital tissues (n=10). (A) IL-6 and hyaluronan production is significantly reduced by imatinib mesylate. (B) A positive correlation exists between the IL-6 and hyaluronan inhibition indexes and PDGF-B mRNA expression in the primary tissue, but is only significant for the hyaluronan inhibition index. Each dot represents a single orbital tissue. The effect of imatinib mesylate on IL-6 and hyaluronan production was analyzed using the paired Wilcoxon rank sum test. Correlations were analyzed using Spearman's correlation test. A p-value < 0.05 was considered significant.

DISCUSSION

In this study we use a whole orbital tissue culture system to demonstrate that imatinib mesylate inhibits the production of both IL-6 and hyaluronan, two important components of the pathological process of GO, while adalimumab only reduces IL-6 production.

Previously, we showed that PDGF-BB induced IL-6 and hyaluronan production by orbital fibroblasts *in vitro*, which was blocked by imatinib mesylate through inhibition of PDGF-receptor activation.[11-12] In our current study, imatinib mesylate reduced IL-6 and hyaluronan production by whole orbital tissues, but a significant positive correlation existed only between the PDGF-B mRNA levels in the primary orbital tissue and the suppression of hyaluronan production. This suggests that hyaluronan production in GO tissue is strongly controlled by PDGF-receptor activation while IL-6 production involves other stimuli as well

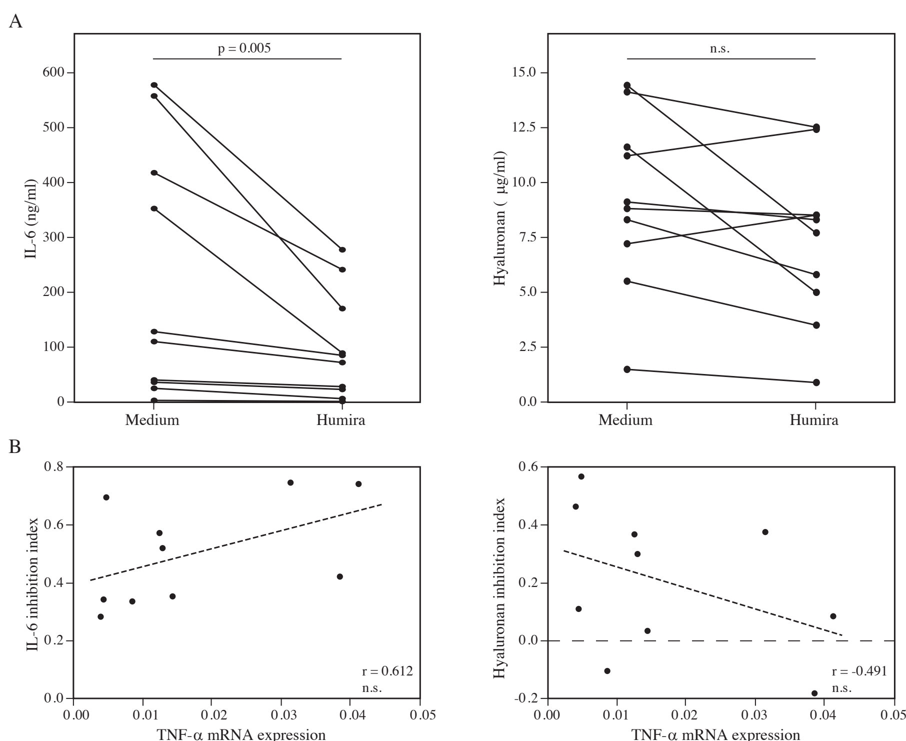


Figure 2 The effect of adalimumab (10 µg/ml) on cultured GO orbital tissues (n=10). (A) IL-6 production is significantly reduced in tissues by adalimumab, while hyaluronan production is reduced in 8 of 10 orbital tissues. (B) A positive correlation exists between the IL-6 inhibition index and TNF-α mRNA expression in the primary tissue and a negative correlation exists between the hyaluronan inhibition index and TNF-α mRNA expression in the primary tissue, but neither are significant. Each dot represents an individual orbital tissue. The effect of adalimumab on IL-6 and hyaluronan production was analyzed using the paired Wilcoxon rank sum test. Correlations were analyzed using Spearman's correlation test. A p-value < 0.05 was considered significant.

(e.g. TNF-α). However, imatinib mesylate also inhibits the tyrosine kinases c-Abl and c-Kit.[23] Therefore, we cannot exclude that inhibition of these tyrosine kinases also contributed to the inhibitory effects of imatinib mesylate in our whole tissue cultures. Nevertheless, our previous studies[11-12], as well as the current study show that imatinib mesylate may potentially reduce inflammation and tissue remodeling in GO. Furthermore, involvement of PDGF-receptor inhibition, at least on orbital fibroblasts but possibly also on other cell types, can be expected herein. Considering this, other tyrosine kinase inhibitors that block PDGF-receptor activation, such as nilotinib and dasatinib, or PDGF neutralizing antibodies can be expected to be effective in the treatment of GO as well.

Adalimumab reduced IL-6 production, but larger series may be needed to demonstrate a correlation between IL-6 inhibition and the TNF-α mRNA level in the primary tissue. This

observation is in accordance with previous studies which demonstrated that TNF-α-directed treatment with either etanercept or infliximab reduced peri-ocular inflammation in GO patients.[13-14] The concordance of the observed clinical effects of TNF-α-directed treatment in GO patients and our *in vitro* data underscores the strength of the whole orbital tissue culture approach used in this study to screen for potential treatment efficacy.

In our study we used orbital tissue from patients with burnt-out disease, suggesting that imatinib mesylate and adalimumab are effective in this stage of disease. However, imatinib mesylate can be expected to inhibit active disease as well, as target molecules for imatinib mesylate (e.g. PDGF) are equally elevated in active and burnt-out disease.[11] Furthermore, similar levels of TNF-α have been reported in active and burnt-out GO and TNF inhibition by etanercept reduced signs of active GO.[13, 18] Therefore, we expect that TNF-α inhibition by adalimumab will reduce disease activity in active GO as well.

The use of a whole orbital tissue culture has the advantage that it adequately represents the multicellular (e.g. adipocytes, fibroblasts and various immune cells) and multifactorial (e.g. autoantibodies, cytokines, growth factors) pathological process in GO. Therefore, our whole orbital tissue culture is a more suitable approach to test therapeutics than single cell systems. In addition, using specific immune targeted therapies in whole tissue cultures could give more insight into key mediators involved in the pathological process of diseases. Furthermore, it could provide a rationale to start off-label use of novel therapeutics, for instance in rare immune mediated diseases in which achievement of randomized controlled trials is extremely difficult. Clearly, a prerequisite of a whole tissue culture approach is that tissue samples can be obtained relatively easy. Besides GO, this is often the case in idiopathic orbital inflammation and in other inflammatory disorders such as rheumatoid arthritis, psoriasis and systemic sclerosis.

In conclusion, we show that a whole orbital tissue culture system may prove of value to test for possible effects of novel therapeutics in GO. Based on our *in vitro* results using physiologically relevant concentrations of imatinib mesylate and adalimumab,[6, 24] imatinib mesylate can be expected to reduce inflammation as well as tissue remodeling in GO. Adalimumab (and other TNF-α neutralizing therapies) may reduce inflammation in GO effectively, but cannot be expected to affect orbital hyaluronan production as effective as imatinib mesylate.

ACKNOWLEDGEMENTS

This work was supported by the ‘Stichting Wetenschappelijk Onderzoek Oogziekenhuis (SWOO)’, the ‘Prof. Dr. Henkes Stichting’ and the ‘Nederlandse Vereniging van Graves’ Patienten (NVGP).

REFERENCES

1. Ong VH, Denton CP. *Innovative therapies for systemic sclerosis*. Curr Opin Rheumatol 2010;22:264-272.
2. Buch MH. *Sequential use of biologic therapy in rheumatoid arthritis*. Curr Opin Rheumatol 2010;22:321-329.
3. Kunz M. *Current treatment of psoriasis with biologics*. Curr Drug Discov Technol 2009;6:231-240.
4. Sfikakis PP, Gorgoulis VG, et al. *Imatinib for the treatment of refractory, diffuse systemic sclerosis*. Rheumatology (Oxford) 2008;47:735-737.
5. van Daele PL, Dik WA, et al. *Is imatinib mesylate a promising drug in systemic sclerosis?* Arthritis Rheum 2008;58:2549-2552.
6. Akhmetshina A, Venalis P, et al. *Treatment with imatinib prevents fibrosis in different preclinical models of systemic sclerosis and induces regression of established fibrosis*. Arthritis Rheum 2009;60:219-224.
7. Imrie FR, Dick AD. *Biologics in the treatment of uveitis*. Curr Opin Ophthalmol 2007;18:481-486.
8. Baeten D, van Hagen PM. *Use of TNF blockers and other targeted therapies in rare refractory immune-mediated inflammatory diseases: evidence-based or rational?* Ann Rheum Dis 2010;69:2067-2073.
9. Bahn RS. *Graves' ophthalmopathy*. N Engl J Med 2010;362:726-738.
10. van Steensel L, Dik WA. *The orbital fibroblast: a key player and target for therapy in graves' ophthalmopathy*. Orbit 2010;29:202-206.
11. van Steensel L, Paridaens D, et al. *Imatinib mesylate and AMN107 inhibit PDGF-signaling in orbital fibroblasts: a potential treatment for Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2009;50:3091-3098.
12. van Steensel L, Paridaens D, et al. *Platelet-derived growth factor-BB: a stimulus for cytokine production by orbital fibroblasts in Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2010;51:1002-1007.
13. Paridaens D, van den Bosch WA, et al. *The effect of etanercept on Graves' ophthalmopathy: a pilot study*. Eye (Lond) 2005;19:1286-1289.
14. Durrani OM, Reuser TQ, et al. *Infliximab: a novel treatment for sight-threatening thyroid associated ophthalmopathy*. Orbit 2005;24:117-119.
15. El Fassi D, Nielsen CH, et al. *B lymphocyte depletion with the monoclonal antibody rituximab in Graves' disease: a controlled pilot study*. J Clin Endocrinol Metab 2007;92:1769-1772.
16. Weetman AP, Cohen S, et al. *Immunohistochemical analysis of the retrobulbar tissues in Graves' ophthalmopathy*. Clin Exp Immunol 1989;75:222-227.
17. Boschi A, Daumerie C, et al. *Quantification of cells expressing the thyrotropin receptor in extraocular muscles in thyroid associated orbitopathy*. Br J Ophthalmol 2005;89:724-729.
18. Wakelkamp IM, Bakker O, et al. *TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients*. Clin Endocrinol (Oxf) 2003;58:280-287.
19. Molnar I, Balazs C. *High circulating IL-6 level in Graves' ophthalmopathy*. Autoimmunity 1997;25:91-96.
20. Wakelkamp IM, Gerding MN, et al. *Both Th1- and Th2-derived cytokines in serum are elevated in Graves' ophthalmopathy*. Clin Exp Immunol 2000;121:453-457.
21. Hirano T. *Interleukin 6 and its receptor: ten years later*. Int Rev Immunol 1998;16:249-284.
22. Jyonouchi SC, Valyasevi RW, et al. *Interleukin-6 stimulates thyrotropin receptor expression in human orbital preadipocyte fibroblasts from patients with Graves' ophthalmopathy*. Thyroid 2001;11:929-934.
23. Druker BJ, Talpaz M, et al. *Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia*. N Engl J Med 2001;344:1031-1037.
24. Karmiris K, Paintaud G, et al. *Influence of trough serum levels and immunogenicity on long-term outcome of adalimumab therapy in Crohn's disease*. Gastroenterology 2009;137:1628-1640.

Chapter 7

Orbit-infiltrating mast cells, monocytes and macrophages produce PDGF isoforms that orchestrate orbital fibroblast activation in Graves' ophthalmopathy

Leendert van Steensel [1], Dion Paridaens [2], Marjan van Meurs [1], P. Martin van Hagen [1], Willem A. van den Bosch [2], Robert W.A.M. Kuijpers [3], Hemmo A. Drexhage [1], Herbert Hooijkaas [1] and Willem A. Dik [1]

[1]Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
[2]Rotterdam Eye Hospital, Rotterdam, The Netherlands
[3]Department of Ophthalmology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

J Clin Endocrinol Metab. 2012 in press

ABSTRACT

Purpose

Platelet-derived growth factors (PDGF) are regulators of fibroblast activity that may be involved in the pathophysiology of Graves' ophthalmopathy (GO). We unraveled the expression and origin of PDGF family members in GO orbital tissue and investigated the effect of PDGF isoforms on IL-6 and hyaluronan production and proliferation by orbital fibroblasts.

Methods

PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGF-Rα and PDGF-Rβ expression was determined by RQ-PCR and PDGF-A and PDGF-B protein expression was determined by Western blot in orbital tissues. Orbital tissues were immunohistochemically stained for PDGF-A and PDGF-B expression, together with stainings for T cells, monocytes, B cells, macrophages and mast cells. Effects of PDGF-AA, PDGF-AB and PDGF-BB on orbital fibroblast proliferation and IL-6 and hyaluronan production were examined. Finally, effects of PDGF-BB and PDGF-AA neutralizing antibodies on IL-6 and hyaluronan production in GO whole orbital tissue cultures were tested.

Results

GO orbital tissue showed increased PDGF-A and PDGF-B mRNA and protein levels. Increased numbers of PDGF-A and PDGF-B-positive monocytes, macrophages and mast cells were present in GO orbital tissue. PDGF-BB stimulated proliferation and hyaluronan and IL-6 production by orbital fibroblasts the most, followed by PDGF-AB and PDGF-AA. Finally, in particular imatinib mesylate and PDGF-BB neutralizing antibodies reduced IL-6 and hyaluronan production by whole orbital tissue cultures from GO patients.

Conclusions

In GO, mast cells, monocytes and macrophages may activate orbital fibroblasts via secretion of especially PDGF-AB and PDGF-BB. Pre-clinical studies with whole orbital tissue cultures show that blocking PDGF-B chain containing isoforms can be a promising treatment for GO.

INTRODUCTION

Graves' ophthalmopathy (GO) is an extra-thyroidal complication of Graves' disease (GD) that results in clinical symptoms and signs of the orbits in ~50% of GD patients.[1] Well-known signs of GO include dry and gritty eyes, photophobia, chemosis, double vision, upper eyelid retraction, edema of peri-orbital tissues and conjunctivae, and proptosis. In the most severe cases, sight-threatening corneal ulceration or compressive optic neuropathy can develop.[1] Although GO results in a considerable physical and mental burden for many of the patients, no clear improvement in medical therapy has been achieved during the last two decades, mainly because the pathobiology is still poorly understood.

The pathobiological picture of GO consists of an intricate interplay between orbit-infiltrated immune cells and their products, such as cytokines and growth factors, and orbital fibroblasts and extra-ocular muscle cells.[1-3] Cytokines and growth factors activate orbital fibroblasts to produce inflammatory mediators, to proliferate and to secrete the glycosaminoglycan hyaluronan.[4-7] Consequently, orbital fibroblast activation is recognized as a main determinant of orbital tissue expansion and the resultant eye ball protrusion in GO.[1, 8] Nevertheless, the cellular origins of cytokines and growth factors and the relative contribution of these factors to orbital fibroblast activation in GO thus far remain largely unknown. However, such insight is not only crucial to delineate GO pathology, but also to improve treatment strategies.

Platelet-derived growth factors (PDGF) are amongst the most powerful fibroblast activating growth factors identified to date. PDGF comprise a family of five isoforms, consisting of homo- or heterodimerized PDGF-A, PDGF-B, PDGF-C or PDGF-D chains, which signal through a homo- or heterodimeric receptor of PDGF-receptor α (PDGF-Rα) or PDGF-receptor β (PDGF-Rβ) subunits (Fig. 1A).[9-11] PDGF play a prominent role in tissue repair by stimulating proliferation, cytokine production and extra-cellular matrix production by fibroblasts[11], although the amplitude by which fibroblast effector functions are activated can differ between the various PDGF isoforms.[11-12] Excessive production and activity of PDGF is implicated in pathological conditions that involve excessive fibroblast activity, such as fibrotic diseases of lungs, liver, skin and kidneys.[10-11] However, different PDGF isoforms can be involved in different organ systems.[10-13]

To date, no detailed data are present on the involvement of the different PDGF family members in GO, although some studies suggest that PDGF may be important in GO pathophysiology.[4, 14-15] Therefore, we here investigated: 1) the expression of all PDGF family members and receptors in GO orbital tissue, 2) the contribution of different intra-orbital leukocyte populations to PDGF production in GO orbital tissue, and 3) the effect of different PDGF isoforms on orbital fibroblast effector functions.

This study is the first to demonstrate a comprehensive role for the PDGF system in GO. We show that macrophages, monocytes and mast cells stimulate orbital fibroblast effector

functions in GO via the release of PDGF-AA, PDGF-AB and PDGF-BB, and as such may contribute to the pathophysiology of GO. Of the three PDGF isoforms, PDGF-BB most potently activated orbital fibroblasts to proliferate and to produce IL-6 and hyaluronan, while PDGF-AA was the least potent. Thus, especially PDGF-B chain containing PDGF isoforms may contribute to orbital tissue expansion in GO, and may be an attractive therapeutic target for the treatment of GO.

METHODS

Patients and control subjects

Orbital tissue was obtained from euthyroid GO patients during orbital decompression surgery and from healthy controls without inflammatory orbital or thyroid disease that underwent eyelid surgery for cosmetic reasons. All patients were euthyroid and had not received steroid or other immunosuppressive treatment for at least 3 months prior to surgery. All orbital tissues were obtained in the Rotterdam Eye Hospital (Rotterdam, The Netherlands) after informed consent and in accordance with the principles of the Declaration of Helsinki. Approval was obtained from the local medical ethics committee. Orbital fibroblast strains were established as described before.[14]

Detection of PDGF and PDGF-receptor mRNA in orbital tissue

Messenger RNA was isolated from orbital tissue from 17 GO patients and 13 controls and reverse transcribed into cDNA as described previously.[14] PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGF-Rα and PDGF-Rβ mRNA levels were determined by real-time quantitative PCR using an Applied Biosystems 7900 PCR machine (Foster City, CA, USA). Transcript levels were normalized to the control gene Abelson.[14] Primer-probe combinations used are listed in Table 1 or were published previously.[14]

Detection of PDGF protein in orbital tissue

Snap-frozen orbital tissue from 8 GO patients and from 4 controls was lysed (20 mM Tris [pH 8.0], 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol and protease inhibitors). Western blot analysis was essentially performed as described previously.[14] Briefly, equivalent amounts of protein (~30 μg) were loaded and run on SDS-PAGE gels and blotted. Blots were stained using a polyclonal rabbit-anti-human PDGF-A (SC-128; Santa-Cruz Biotechnologies, Heidelberg, Germany) or PDGF-B antibody (SC-127, Santa Cruz) and a secondary horseradish peroxidase (HRP)-labeled swine-anti-rabbit antibody (Dako, Heverlee, Belgium). As loading control, the blots were stained with a monoclonal mouse-anti-human β-actin antibody (AB6276; Abcam, Cambridge, UK) and a secondary HRP-

labeled rabbit-anti-mouse antibody (Dako). Densitometric analysis was performed using ImageJ Software (NIH, Bethesda, Maryland, USA) and results are shown as PDGF / β-actin ratio.

Table 1. RQ-PCR primer – probe combinations

Gene	Forward primer	Reverse primer	Probe
PDGF-A	Fw_Hu_PDGFA_EMC: CGGGGTCCATGCCACTAA	Rv_Hu_PDGFA_EMC: GGGGCCAGATCAGGAA-GTTG	T_Hu_PDGFA_EMC: AGCTTCCTCGAT-GCTTCTCTTCCTCCG
PDGF-C	Fw_Hu_PDGFC_EMC: ACCAGGGTTCTGCATC-CACTA	Rv_Hu_PDGFC_EMC: AAGGGGGTAGCACTGAA-GGAC	T_Hu_PDGFC_EMC: ACATTGTCATGC-CACAATTCACAGAAGCTG
PDGF-D	Fw_Hu_PDGFD_EMC: TTGCGGATGCTCTGGACA	Rv_Hu_PDGFD_EMC: TTCCTGGGAGTGCAACT-GTAA	T_Hu_PDGFD_EMC: ATACCATGACCGGAAGT-CAAAAGTTGACCTG
PDGF-RA	Fw_Hu_PDGFRA_EMC: TGAAGGCAGGCACATTTA-CATCTA	Rv_Hu_PDGFRA_EMC: TACAGGAGTCTCGG-GATCAGTTG	T_Hu_PDGFRA_EMC: TGCCAGACCCAGATG-TAGCCTTTGTACCTC
PDGF-RB	Fw_Hu_PDGFRB_EMC: GGGGACAGGGAGGTG-GATT	Rv_Hu_PDGFRB_EMC: ATTCCCGATCACAATG-CACA	T_Hu_PDGFRB_EMC: TCTACAGACTCCAGGTGT-CATCCATCAACGTC
IL-6	Fw_hu_IL6_EMC: TAGCCGCCCCACACAGA	Rv_hu_IL6_EMC: GTGCCTCTTTGCT-GCTTTCAC	T_hu_IL6_EMC: AGCCACTCACCTCTTCA-GAACGAATTGACA

Immunohistochemical detection of PDGF in orbital tissue

Immunohistochemical visualization of PDGF-A and PDGF-B protein chains in orbital tissues was performed on orbital tissues from 2 healthy controls and 3 GO patients, of which two had active disease. Frozen 7 µm tissue sections were put onto glass slides and kept overnight at room temperature in a humidified atmosphere. Thereafter, slides were air-dried, fixed in acetone containing 0.02% H_2O_2 and air-dried for 10 min. Slides were then washed with PBS and incubated overnight in humidified atmosphere with a rabbit-anti-human PDGF-A antibody (SC-128, Santa Cruz), a rabbit-anti-human PDGF-B antibody (DP-145, Acris Antibodies GmbH, Herford, Germany) or an appropriate rabbit IgG isotype control (Santa Cruz). Incubation with a secondary biotin-labeled donkey-anti-rabbit antibody (Dako) and tertiary HRP-labeled avidin–biotin complex (ABC/HRP; Vector laboratories Ltd, Peterborough, UK) were performed for 1 hour at room temperature. HRP activity was revealed by incubation with 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich, Zwijndrecht, the Netherlands) for 10 min at room temperature, leading to a bright red precipitate. Slides were counterstained with haematoxylin and embedded with glycerol-gelatin.

The same procedure was followed for the double stainings, using the following antibodies: rabbit-anti-human PDGF-A antibody (SC-128, Santa Cruz), rabbit-anti-human

PDGF-B antibody (SC-127, Santa Cruz), mouse-anti-human CD3 antibody (F7-2-38; Dako) to identify T cells, mouse-anti-human CD14 antibody (My-4; Beckman-Coulter, Woerden, The Netherlands) to identify monocytes, mouse-anti-human CD20 (C26, Dako) to identify B cells, mouse-anti-human CD68 antibody (EBM11, Dako) to identify macrophages, mouse-anti-human CD117 antibody (YB5.B8, BD Biosciences, Erembodegem, Belgium) to identify mast cells, secondary alkaline phosphatase (AP)-labeled goat-anti-rabbit antibody (Dako), secondary biotin-labeled horse-anti-mouse antibody (Vector), tertiary AP-labeled rabbit-anti-goat antibody (Southern Biotech, Birmingham, USA) and tertiary ABC/HRP. Alkaline phosphatase activity was revealed using Fast Blue B Base (Sigma-Aldrich), leading to a blue precipitate for PDGF-A and PDGF-B. HRP activity was revealed by incubation with AEC, leading to a bright red precipitate for CD3, CD14, CD20, CD68 and CD117. Slides were examined and photographed using a Zeiss AxioCam microscope.

The effect of different PDGF-isoforms on hyaluronan and IL-6 expression by orbital fibroblasts

For mRNA studies, fibroblasts were seeded into 6-well plates and allowed to grow confluent in DMEM supplemented with 10% FCS and antibiotics (DMEM 10% FCS; all Cambrex BioWhittaker, Verviers, Belgium). Hereafter, fibroblast monolayers were incubated in DMEM containing 1% FCS and antibiotics (DMEM 1% FCS) for 16 hours. Subsequently, DMEM 1% FCS with or without PDGF-AA, PDGF-AB, or PDGF-BB (50 ng/ml; R&D systems, Abingdon, UK) was added for the indicated time points. Hereafter, mRNA was isolated, reverse transcribed into cDNA and the expression of the three different hyaluronan synthases (HAS1-3) and IL-6 was determined by RQ-PCR and normalized to the control gene Abelson.[14] Primer-probe combinations used are listed in Table 1 or were published previously.[14]

To determine the effect of the three PDGF isoforms on hyaluronan and IL-6 production by orbital fibroblasts, fibroblast monolayers were incubated in DMEM 1% FCS with or without PDGF-AA, PDGF-AB, or PDGF-BB (10 and 50 ng/ml) for 24 hours. Supernatants were collected and subjected to IL-6 (Invitrogen, London, UK) or hyaluronan (R&D systems) ELISA.

The effect of different PDGF-isoforms on orbital fibroblast proliferation

The effect of PDGF-AA, PDGF-AB and PDGF-BB on orbital fibroblast proliferation was investigated as described previously.[14] Briefly, fibroblasts were seeded at 6 x10^3 cells/well into 96-well plates in 50 μL DMEM 1% FCS and allowed to adhere. Hereafter, medium was changed into DMEM 1% FCS with or without PDGF-AA, PDGF-AB or PDGF-BB (10 and 50 ng/ml) at six replicates per condition. Proliferation was assessed after 24 hours using a colorimetric assay based on the uptake and subsequent release of methylene blue dye. The

level of proliferation was expressed as percentage change in mean absorbance from that of cells exposed to DMEM 1% FCS alone.

Inhibition of PDGF-receptor activation with the tyrosine kinase inhibitor imatinib mesylate

To demonstrate the involvement of PDGF-receptor signalling, the PDGF-receptor tyrosine kinase inhibitor imatinib mesylate (2.5 µg/ml; Novartis Pharma, Basel, Switzerland) was supplemented 16 hours before and together with the PDGF stimulation. Hereafter, proliferation, IL-6 production or hyaluronan production were determined as mentioned before.

The effect of PDGF neutralizing antibodies on IL-6 and hyaluronan production by whole orbital tissue cultures

Orbital tissue cultures were essentially performed as described previously.[16] Briefly, orbital tissues from 3 GO patients were each cut into 7 pieces and incubated in DMEM 1% FCS with or without PDGF-AA neutralizing antibodies (40 and 20 µg/ml; R&D systems), PDGF-BB neutralizing antibodies (4 and 2 µg/ml; R&D systems) or imatinib mesylate (2.5 µg/ml) for 48 hours. As the neutralizing capacity of the PDGF-BB neutralizing antibody (ND_{50} = 1 µg/ml) was ten-fold higher than the neutralizing capacity of the PDGF-AA neutralizing antibody (ND_{50} = 10 µg/ml), ten-fold lower concentrations of the PDGF-BB neutralizing antibody were used. Culture supernatants were subjected to IL-6 and hyaluronan ELISA. Detected values were corrected for the orbital tissue weight and expressed as inhibition index (calculated as: 1 - the production levels achieved with medication / production levels without medication).[16]

Statistical analysis

Messenger RNA and protein levels in orbital tissue specimens were analyzed using a two-tailed Mann-Whitney test. Data from fibroblast stimulation experiments were analyzed using a repeated measurements ANOVA and subsequent paired comparison of the means. A p-value < 0.05 was considered significant.

RESULTS

PDGF expression in orbital tissue

The involvement of the different PDGF family members in GO was first determined by measuring mRNA expression levels of all four PDGF chains and of the two PDGF-receptor variants in orbital tissue from GO patients and healthy controls. PDGF-C, PDGF-D, PDGF-

Rα and PDGF-Rβ mRNA expression levels did not differ between GO patients and controls. GO orbital tissue did contain significantly increased PDGF-A and PDGF-B mRNA levels (p<0.05; Fig 1B). In line with this, GO orbital tissue also contained significantly increased amounts of the PDGF-A and PDGF-B protein chains (p<0.05; Fig. 1C).

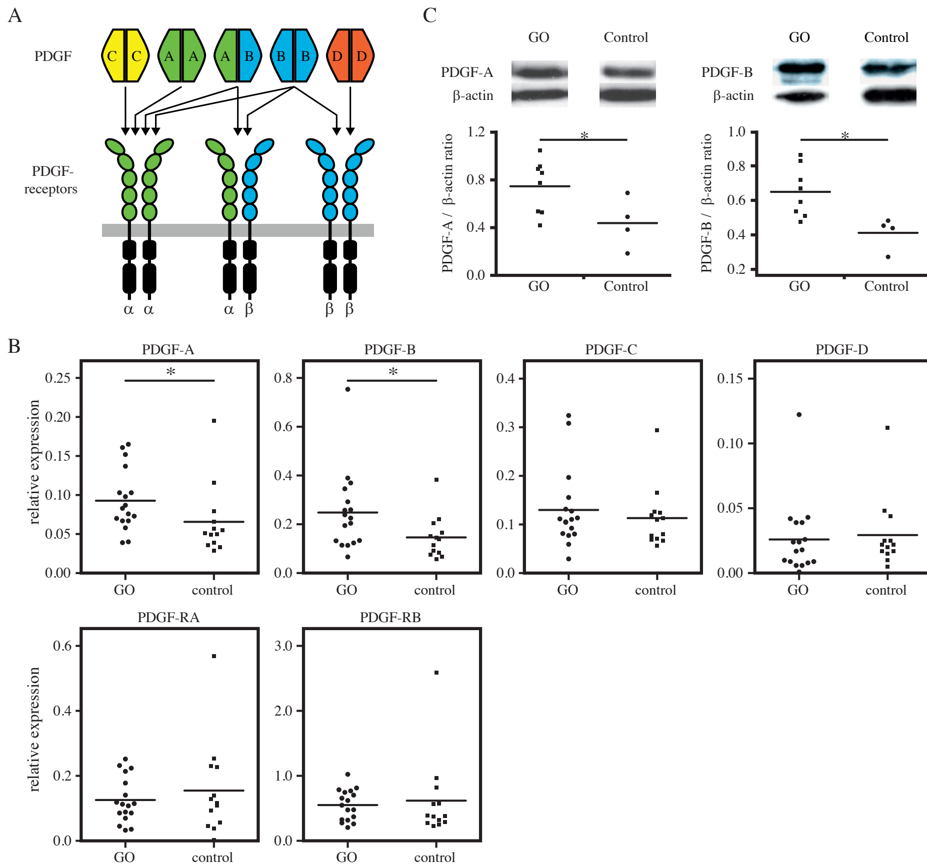


Figure 1 PDGF and PDGF-receptors in orbital tissue. A: PDGF isoforms, PDGF-receptors, and their specific interactions. B: PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGF-Rα and PDGF-Rβ mRNA transcript levels were determined in orbital tissue from GO patients (n=17) and healthy controls (n=13) by real-time quantitative PCR. Data are presented as relative expression compared to the control gene Abelson. C: Western blot analysis was performed on lysates from orbital tissue from GO patients (n=8) and healthy controls (n=4) using antibodies against PDGF-A (*left panel*) and PDGF-B (*right panel*). Antibodies against β-actin were used as loading control. Representative blots from a GO patient and a healthy control are shown. Densitometric analysis was performed and results are depicted as PDGF / β-actin ratio. Each data point represents a single individual. Horizontal bars represent mean values. Data were analyzed using the two-tailed Mann-Whitney test. * $p < 0.05$.

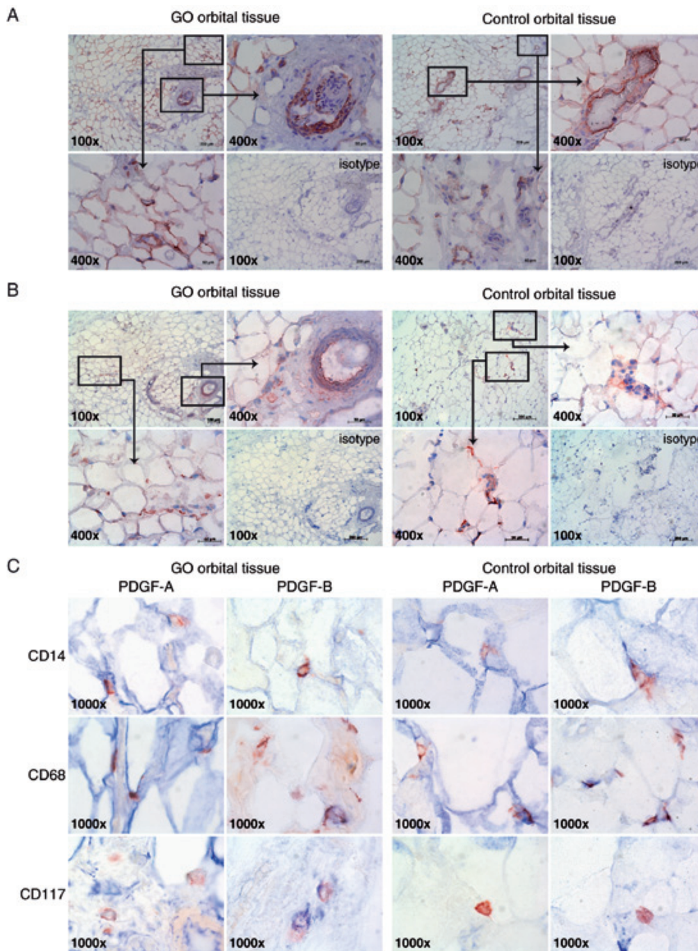


Figure 2 PDGF-A and PDGF-B localization in orbital tissue from GO patients and healthy controls. Orbital tissues from GO patients (n=3) and healthy controls (n=2) were immunohistochemically stained for PDGF-A and PDGF-B alone or PDGF-A and PDGF-B in combination with staining for CD14, CD68 and CD117. A: single staining for PDGF-A (*red staining*) and B: single staining for PDGF-B (*red staining*) shows positive staining around vessels and in between fibroblast-like cells and adipocytes in orbital tissue. C: Double stainings showing co-localization of PDGF-A and PDGF-B (*blue staining)* with CD14 (monocyte marker; *first row*), CD68 (macrophage marker; *second row*) and CD117 (mast cell marker; *third row*) (*all red staining)*. Representative pictures from a GO patient and a healthy control are shown.

PDGF-A and PDGF-B protein localization in orbital tissue

To identify the localization and potential cellular sources that account for the increased levels of PDGF-A and PDGF-B in orbital tissue, immunohistochemical studies were conducted. These revealed localization of PDGF-A and PDGF-B protein chains around vessels and diffuse localization between spindle-shaped fibroblasts and adipocytes (Fig 2A and 2B).

To identify which type of leucocytes are the source of PDGF in GO, double staining for PDGF-A or PDGF-B in combination with staining for CD3 (as a T cell marker), CD20 (as a B cell marker), CD14 (as a monocyte marker), CD68 (as a macrophage marker) or CD117 (as a mast cell marker) was performed. T cells and few B-cells were detectable in orbital tissue from GO and controls, but never co-localized with PDGF-A or PDGF-B (data not shown). Monocytes, macrophages and mast cells were detected in both GO orbital tissue and control orbital tissue and stained positive for PDGF-A and PDGF-B (Fig 2C). Moreover, monocyte and mast cell numbers appeared to be elevated in GO orbital tissues compared to control orbital tissue upon visual examination, but were not further quantified.

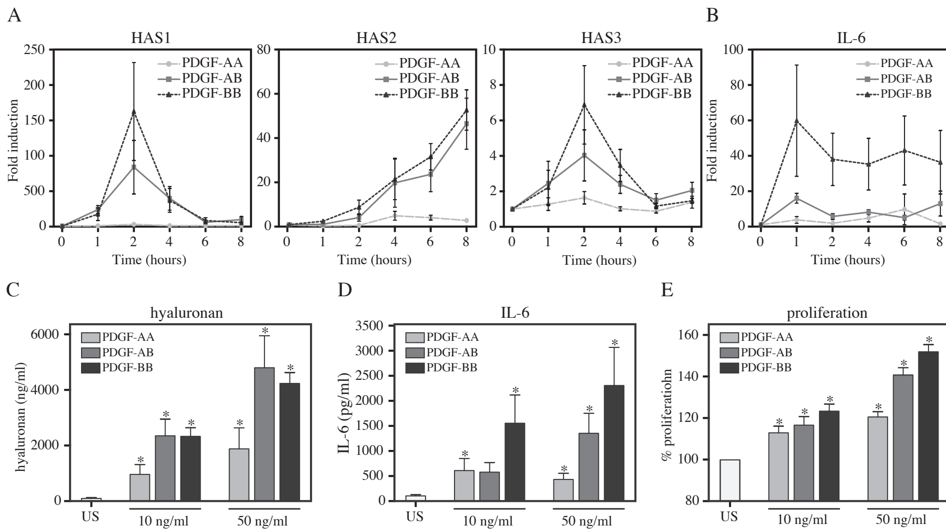


Figure 3 The effect of PDGF-isoforms on orbital fibroblast activation. Orbital fibroblasts (n=3) were stimulated with or without PDGF-AA, PDGF-AB or PDGF-BB (50 ng/ml) for the indicated periods. mRNA expression of HAS1, HAS2, HAS3 (A) and IL-6 (B) was determined and depicted as the mean fold increase relative to the expression in unstimulated orbital fibroblasts. C and D: orbital fibroblasts (n=10) were stimulated for 24 hours with or without PDGF-AA, PDGF-AB or PDGF-BB (10 and 50 ng/ml) and hyaluronan and IL-6 production was measured by ELISA. E: orbital fibroblasts (n=10) were cultured for 24 hours in the presence or absence of PDGF-AA, PDGF-AB or PDGF-BB (10 and 50 ng/ml) and orbital fibroblast proliferation was determined using a colorometric, methylene blue based, assay. The level of proliferation is depicted as percentage change in mean absorbance from that of unstimulated cells. Each data point or bar represents mean ± SEM. Data were analyzed using a repeated measurements ANOVA and subsequent comparison of the means. * $p<0.05$ US: unstimulated

The effect of different PDGF-isoforms on hyaluronan production, IL-6 production and proliferation by orbital fibroblasts

The data above implicate that especially PDGF-AA, PDGF-AB and PDGF-BB isoforms can be expected in GO. Excessive production of hyaluronan and IL-6 by orbital fibroblasts and enhanced orbital fibroblast proliferation are key events in the pathophysiology of GO.[8, 14-15,]

[17] Therefore, we examined how these processes are affected by PDGF-AA, PDGF-AB and PDGF-BB.

PDGF-AB and PDGF-BB stimulation transiently increased HAS1 and HAS3 mRNA expression levels (with maximum levels 2 hours after stimulation) and gradually increased HAS2 mRNA levels during the time period tested (Fig 3A). PDGF-AA marginally enhanced the expression of only HAS2 (Fig 3A). All three PDGF isoforms stimulated hyaluronan production in a dose-dependent manner (Fig 3C). Furthermore, all three PDGF isoforms enhanced IL-6 mRNA levels (Fig 3B) and dose-dependently stimulated IL-6 production (Fig 3D) and orbital fibroblast proliferation (Fig. 3E). PDGF isoforms containing the PDGF-B chain appeared to be the most potent stimulators for all examined orbital fibroblast effector functions, with PDGF-BB usually being the most potent factor, followed by PDGF-AB and PDGF-AA.

The effect of imatinib mesylate on orbital fibroblast activation

To determine whether the observed effects were truly due to PDGF-receptor activation, the PDGF-receptor tyrosine kinase inhibitor imatinib mesylate was used. Imatinib mesylate potently blocked PDGF-AA, PDGF-AB and PDGF-BB-induced hyaluronan production (Fig 4A), IL-6 production (Fig 4B) and proliferation (Fig 4C) by orbital fibroblasts.

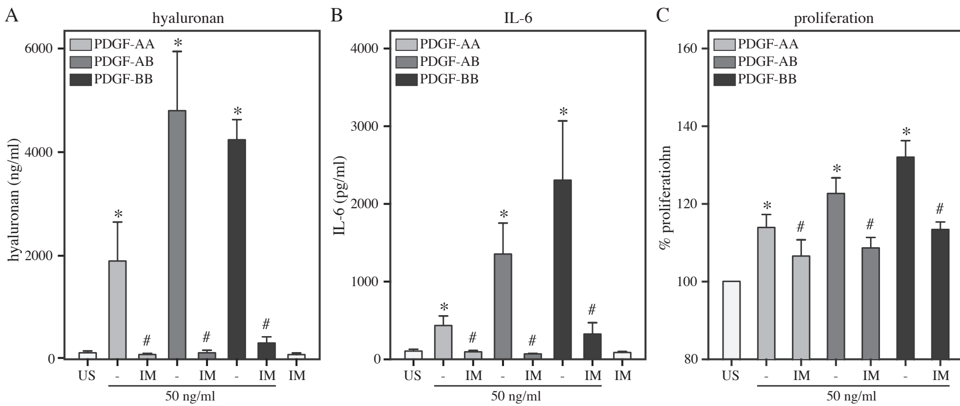


Figure 4 The effect of the PDGF-receptor tyrosine kinase inhibitor imatinib mesylate on orbital fibroblast activation. Orbital fibroblasts (n=10) were stimulated for 24 hours with PDGF-AA, PDGF-AB or PDGF-BB (50 ng/ml) with or without pre-incubation with imatinib mesylate (2.5 µg/ml). A: hyaluronan production and B: IL-6 production was determined by ELISA. C: orbital fibroblast proliferation was determined using a colorometric, methylene blue based, assay. Each bar represents the mean ± SEM. Data were analyzed using a repeated measurements ANOVA and subsequent comparison of the means. * $p < 0.05$ vs unstimulated, # $p < 0.05$ vs PDGF stimulated; US: unstimulated, IM: imatinib mesylate

The effect of PDGF neutralizing antibodies on whole orbital tissue cultures

The previous data indicate that PDGF family members that contain the PDGF-B chain are the most potent stimulators of hyaluronan and cytokine production in GO, and can therefore be considered attractive therapeutic targets. To test this we used our recently published whole orbital tissue culture approach[16] and tested the effect PDGF-AA and PDGF-BB neutralizing antibodies and imatinib mesylate herein. PDGF-BB neutralizing antibody inhibited IL-6 and hyaluronan production by orbital tissues, while the effect of the PDGF-AA neutralizing antibody was variable (Fig 5). Imatinib mesylate blocked IL-6 and hyaluronan production by the orbital tissues (Fig 5).

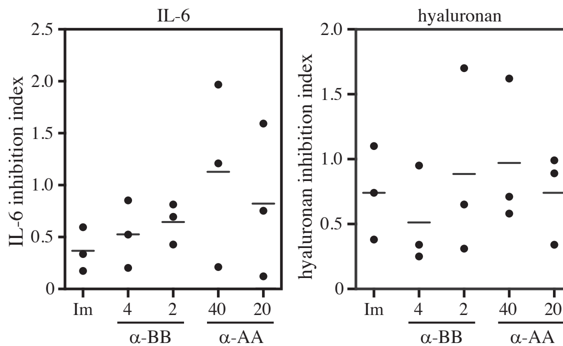


Figure 5 Inhibition of PDGF-activity in complete orbital tissue from GO patients. The effect of imatinib mesylate (2.5 μg/ml), PDGF-AA neutralizing antibodies (20 and 40 μg/ml) or PDGF-BB neutralizing antibodies (2 and 4 μg/ml) on GO orbital tissues (n=3) that were cultured for 48 hours. IL-6 production (*left panel*) and hyaluronan production (*right panel)* were determined in culture supernatants by ELISA. Each dot represents a single orbital tissue. Detected values were corrected for the orbital tissue weight and expressed as inhibition index.[16]

DISCUSSION

The intra-orbital events in GO comprise the recruitment of immune cells and the activation of orbital fibroblasts. Cytokines and growth factors secreted by the orbital infiltrate activate orbital fibroblasts to proliferate and to secrete inflammatory mediators and hyaluronan, which together cause orbital tissue expansion.[1-3, 8] In this study, we describe that monocytes, macrophages and mast cells are capable to dictate orbital fibroblast activity in GO in a PDGF-dependent manner.

GO orbital tissue was characterized by overexpression of PDGF-A and PDGF-B mRNA, of which the latter is in line with our previous observation.[14] In addition, the PDGF-A and PDGF-B protein chain levels were also markedly increased in GO orbital tissue. PDGF-C, PDGF-D, PDGF-Rα and PDGF-Rβ mRNA levels did not differ between GO and control

orbital tissue, despite the fact that increased levels of these PDGF family members have been found in other fibro-proliferative disorders, such as in skin and kidneys.[11, 18-19] GO orbital tissue is thus characterized by a restricted increase of PDGF-A and PDGF-B chains, pointing at specific involvement of PDGF-AA, PDGF-AB and PDGF-BB isoforms in the pathophysiology of GO.

Key effector functions of activated fibroblasts in GO are cytokine production (amongst which IL-6), hyaluronan production and proliferation. PDGF-BB most potently stimulated these processes by orbital fibroblasts, while PDGF-AA was the least effective. These differences in stimulatory capacity between the PDGF isoforms can be related to differences in PDGF-receptor expression.[20-21] In line with this, we found that PDGF-Rβ was expressed at a higher level in orbital tissue than PDGF-Rα (Fig 1B), which was also true for orbital fibroblasts (data not shown). Moreover, PDGF-BB signals through all PDGF receptor dimers (αα, αβ, and ββ), while PDGF-AB activates αα and αβ dimers and PDGF-AA only acts through αα dimers (Fig 1).[9-10] Besides IL-6, PDGF-BB also stimulates IL-8, CCL2, CCL5 and CCL7 production by orbital fibroblasts.[15]

In orbital tissue, PDGF-A and PDGF-B immunoreactivity was detected in monocytes, macrophages and mast cells, which are well-known producers of PDGF.[18, 20, 22] Monocyte infiltration into orbital tissue and their subsequent maturation into macrophages have been reported previously as important events in early and advanced GO.[23-26] In early GO, activated orbital fibroblasts secrete cytokines such as IL-6 and the glycosaminoglycan hyaluronan which lead to inflammation and edema, respectively. Orbital fibroblast proliferation most likely attributes to tissue remodeling processes in advanced GO. In this study we demonstrate that orbital monocytes and macrophages are also capable of PDGF-A and PDGF-B chain production and that especially PDGF-AB and PDGF-BB stimulates IL-6 and hyaluronan production by orbital fibroblasts and orbital fibroblast proliferation.

In line with previous studies, we found increased numbers of mast cells in GO orbital tissues that locate predominantly adjacent to orbital fibroblasts.[27-28] Although this suggests a role for mast cells in the activation of orbital fibroblasts in GO, very limited insight is available on this interaction. We demonstrate for the first time that PDGF-expressing mast cell numbers are increased in GO orbital tissue. Together with the observed effects of PDGF on orbital fibroblasts, this points at a role for mast cells in orbital fibroblast activation in GO. This is further supported by the finding that mast cell-derived prostaglandin D_2 enhances hyaluronan production by orbital fibroblasts.[29] Overall, these data indicate that mast cells are regulators of orbital fibroblast activity in GO and may be considered as treatment targets in GO. This latter notion is supported by a recent study in which clinical improvement was observed in a small cohort of GO patients treated with Cetirizine and Montelukast.[30]

It must be noted that we also observed PDGF-A and PDGF-B immunoreactivity around

vessels and diffusely between fibroblast-shaped cells and adipocytes. Cells such as smooth muscle cells, vascular endothelial cells, fibroblasts, adipocytes and fibrocytes can also produce PDGF [22, 31-36], and we do not exclude that such cells (besides monocytes, macrophages and mast cells) also contribute to the PDGF production in GO orbital tissue.

Inhibition of PDGF signalling with PDGF receptor tyrosine kinase inhibitors, such as imatinib mesylate, showed promising results in various fibro-proliferative disorders.[37-39] Previously, imatinib mesylate was found to block PDGF-BB-induced cytokine production, hyaluronan production and proliferation by orbital fibroblast.[14-15] Moreover, imatinib mesylate reduced IL-6 and hyaluronan production in cultures of whole orbital tissue from GO patients.[16] However, imatinib mesylate not only targets PDGF receptor tyrosine kinase activity[40] and may therefore interfere with other biological processes as well. We found that a PDGF-BB neutralizing antibody consistently, and equally effective as imatinib mesylate, reduced IL-6 and hyaluronan production by GO orbital tissue explants. In contrast, a PDGF-AA neutralizing antibody only showed variable effects, most likely due to the high cross-reactivity (~40%) for PDGF-AB. Therefore, specific targeting of PDGF-B chain containing PDGF isoforms can be valuable in GO. However, so far, PDGF neutralizing antibodies are not yet available for clinical application.

In conclusion, we show for the first time a comprehensive role for the PDGF system in GO. In GO, mast cells, monocytes and macrophages are capable to activate orbital fibroblasts via production of especially PDGF-AB and PDGF-BB. Specific inhibition of PDGF-B chain containing PDGF isoforms or the producing cell types may therefore be novel and promising therapeutic approaches for the treatment of GO.

ACKNOWLEDGEMENTS

This work was supported by 'Stichting Wetenschappelijk Onderzoek Oogziekenhuis' (SWOO) and the 'Nederlandse Vereniging van Graves Patienten' (NVGP).

REFERENCES

1. Bahn RS. *Graves' ophthalmopathy*. N Engl J Med 2010;362:726-738.
2. Weetman AP. *Determinants of autoimmune thyroid disease*. Nat Immunol 2001;2:769-770.
3. Drexhage HA. *Are there more than antibodies to the thyroid-stimulating hormone receptor that meet the eye in Graves' disease?* Endocrinology 2006;147:9-12.
4. Heufelder AE, Bahn RS. *Modulation of Graves' orbital fibroblast proliferation by cytokines and glucocorticoid receptor agonists*. Invest Ophthalmol Vis Sci 1994;35:120-127.
5. Wang HS, Tung WH, et al. *TGF-beta induced hyaluronan synthesis in orbital fibroblasts involves protein kinase C betaII activation in vitro*. J Cell Biochem 2005;95:256-267.
6. Han R, Smith TJ. *T helper type 1 and type 2 cytokines exert divergent influence on the induction of prostaglandin E2 and hyaluronan synthesis by interleukin-1beta in orbital fibroblasts: implications for the pathogenesis of thyroid-associated ophthalmopathy*. Endocrinology 2006;147:13-19.
7. Hwang CJ, Afifiyan N, et al. *Orbital fibroblasts from patients with thyroid-associated ophthalmopathy overexpress CD40: CD154 hyperinduces IL-6, IL-8, and MCP-1*. Invest Ophthalmol Vis Sci 2009;50:2262-2268.
8. van Steensel L, Dik WA. *The orbital fibroblast: a key player and target for therapy in graves' ophthalmopathy*. Orbit 2010;29:202-206.
9. Heldin CH, Westermark B. *Mechanism of action and in vivo role of platelet-derived growth factor*. Physiol Rev 1999;79:1283-1316.
10. Andrae J, Gallini R, et al. *Role of platelet-derived growth factors in physiology and medicine*. Genes Dev 2008;22:1276-1312.
11. Bonner JC. *Regulation of PDGF and its receptors in fibrotic diseases*. Cytokine Growth Factor Rev 2004;15:255-273.
12. Clark JG, Madtes DK, et al. *Effects of platelet-derived growth factor isoforms on human lung fibroblast proliferation and procollagen gene expression*. Exp Lung Res 1993;19:327-344.
13. Rosenkranz S, Kazlauskas A. *Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes*. Growth Factors 1999;16:201-216.
14. van Steensel L, Paridaens D, et al. *Imatinib mesylate and AMN107 inhibit PDGF-signaling in orbital fibroblasts: a potential treatment for Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2009;50:3091-3098.
15. van Steensel L, Paridaens D, et al. *Platelet-derived growth factor-BB: a stimulus for cytokine production by orbital fibroblasts in Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2010;51:1002-1007.
16. van Steensel L, van Hagen PM, et al. *Whole orbital tissue culture identifies imatinib mesylate and adalimumab as potential therapeutics for Graves' ophthalmopathy*. Br J Ophthalmol 2011;95:735-738.
17. Wiersinga WM. *Autoimmunity in Graves' Ophthalmopathy: The Result of an Unfortunate Marriage Between TSH Receptors and IGF-1 Receptors?* J Clin Endocrinol Metab 2011.
18. Trojanowska M. *Role of PDGF in fibrotic diseases and systemic sclerosis*. Rheumatology (Oxford) 2008;47 Suppl 5:v2-4.
19. Ostendorf T, Eitner F, et al. *The PDGF family in renal fibrosis*.Pediatr Nephrol 2011; DOI 10.1007/s00467-011-1892-z.
20. Bonner JC, Osornio-Vargas AR, et al. *Differential proliferation of rat lung fibroblasts induced by the platelet-derived growth factor-AA, -AB, and -BB isoforms secreted by rat alveolar macrophages*. Am J Respir Cell Mol Biol 1991;5:539-547.
21. Caniggia I, Liu J, et al. *Fetal lung epithelial cells express receptors for platelet-derived growth factor*. Am J Respir Cell Mol Biol 1993;9:54-63.
22. Nagaoka I, Trapnell BC, et al. *Upregulation of platelet-derived growth factor-A and -B gene expression in alveolar macrophages of individuals with idiopathic pulmonary fibrosis*. J Clin Invest 1990;85:2023-2027.
23. Eckstein AK, Quadbeck B, et al. *Thyroid associated ophthalmopathy: evidence for CD4(+) gammadelta T cells; de novo differentiation of RFD7(+) macrophages, but not of RFD1(+) dendritic cells; and loss of gammadelta and alphabeta T cell receptor expression*. Br J Ophthalmol 2004;88:803-808.
24. Quadbeck B, Stucke M, et al. *Dysregulation of TNF/TNFR superfamily members: a systemic link between intra- and extrathyroidal manifestations in Graves' disease*. Scand J Immunol 2006;64:523-530.

25. Chen MH, Liao SL, et al. *Role of macrophage infiltration in the orbital fat of patients with Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2008;69:332-337.
26. Kumar S, Bahn RS. *Relative overexpression of macrophage-derived cytokines in orbital adipose tissue from patients with graves' ophthalmopathy*. J Clin Endocrinol Metab 2003;88:4246-4250.
27. Boschi A, Daumerie C, et al. *Quantification of cells expressing the thyrotropin receptor in extraocular muscles in thyroid associated orbitopathy*. Br J Ophthalmol 2005;89:724-729.
28. Wegelius O, Asboe-Hansen G, et al. *Retrobulbar connective tissue changes in malignant exophthalmos*. Acta Endocrinol (Copenh) 1957;25:452-456.
29. Guo N, Baglole CJ, et al. *Mast cell-derived prostaglandin D2 controls hyaluronan synthesis in human orbital fibroblasts via DP1 activation: implications for thyroid eye disease*. J Biol Chem 2010;285:15794-15804.
30. Lauer SA, Silkiss RZ, et al. *Oral montelukast and cetirizine for thyroid eye disease*. Ophthal Plast Reconstr Surg 2008;24:257-261.
31. Gay S, Jones RE, Jr., et al. *Immunohistologic demonstration of platelet-derived growth factor (PDGF) and sis-oncogene expression in scleroderma*. J Invest Dermatol 1989;92:301-303.
32. Gressner AM, Bachem MG. *Cellular sources of noncollagenous matrix proteins: role of fat-storing cells in fibrogenesis*. Semin Liver Dis 1990;10:30-46.
33. Homma S, Nagaoka I, et al. *Localization of platelet-derived growth factor and insulin-like growth factor I in the fibrotic lung*. Am J Respir Crit Care Med 1995;152:2084-2089.
34. Pallua N, Pulsfort AK, et al. *Content of the growth factors bFGF, IGF-1, VEGF, and PDGF-BB in freshly harvested lipoaspirate after centrifugation and incubation*. Plast Reconstr Surg 2009;123:826-833.
35. Reilkoff RA, Bucala R, et al. *Fibrocytes: emerging effector cells in chronic inflammation*. Nat Rev Immunol 2011;11:427-435.
36. Hiromatsu Y, Toda S. *Mast cells and angiogenesis*. Microsc Res Tech 2003;60:64-69.
37. van Daele PL, Dik WA, et al. *Is imatinib mesylate a promising drug in systemic sclerosis?* Arthritis Rheum 2008;58:2549-2552.
38. Rhee CK, Lee SH, et al. *Effect of Nilotinib on Bleomycin-Induced Acute Lung Injury and Pulmonary Fibrosis in Mice*. Respiration 2011.
39. Shaker ME, Zalata KR, et al. *Comparison of imatinib, nilotinib and silymarin in the treatment of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis*. Toxicol Appl Pharmacol 2011;252:165-175.
40. Waller CF. *Imatinib mesylate*. Recent Results Cancer Res 2010;184:3-20.

Chapter 8

PDGF enhances orbital fibroblast responses to TSHR stimulating autoantibodies in Graves' ophthalmopathy patients

Leendert van Steensel [1], Herbert Hooijkaas [1], Dion Paridaens [2,3,4], Willem A. van den Bosch [2], Robert W.A.M. Kuijpers [4], Hemmo A. Drexhage [1], P. Martin van Hagen [1,5] and Willem A. Dik [1]

[1]Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
[2]Rotterdam Eye Hospital, Rotterdam, The Netherlands
[3]Department of Ophthalmology, Geneva General Hospital, Geneva, Switzerland
[4]Department of Ophthalmology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
[5]Department of Internal Medicine, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

Submitted for publication

ABSTRACT

Purpose

Thyroid stimulating hormone receptor (TSHR) stimulating autoantibodies contribute to Graves' ophthalmopathy (GO), the orbital manifestation of Graves' disease (GD). TSHR autoantibody levels and orbital TSHR expression levels correlate positively with GO disease activity. Platelet-derived growth factors (PDGF) are increased in GO and potently activate orbital fibroblast effector functions. Therefore we investigated the possible relationship between PDGF and TSHR expression on orbital fibroblasts and how that influences the immunopathological effects of TSHR autoantibodies on orbital fibroblast activity.

Methods

Orbital fibroblasts were stimulated with PDGF-AA, PDGF-AB and PDGF-BB and TSHR expression was determined by flowcytometry. Stimulatory effects of bTSH and GD immunoglobulins on orbital fibroblasts (with or without PDGF-BB pre-incubation) were determined by IL-6, IL-8, CCL2, CCL5, CCL7 and hyaluronan ELISA. The TSHR blocking antibody K1-70 and the cAMP inhibitor H89 were used to determine involvement of TSHR signalling.

Results

PDGF-AB and PDGF-BB stimulation increased TSHR expression on orbital fibroblasts, while PDGF-AA did not. Furthermore, stimulation with bTSH and immunoglobulins from GD patients (GD-IgG) induced IL-6, IL-8, CCL2 and hyaluronan production by orbital fibroblasts, and PDGF-BB pre-incubation enhanced this response of orbital fibroblasts. Blocking studies with a TSHR blocking antibody and a cAMP inhibitor inhibited these effects, indicating the involvement of TSHR signalling and thus of TSHR stimulating autoantibodies herein.

Conclusion

These findings indicate that PDGF-B containing PDGF isoforms amplify the immunopathological effects of TSHR stimulating autoantibodies in GO patients by stimulating TSHR expression on orbital fibroblasts.

INTRODUCTION

Graves' disease (GD) is an autoimmune disorder in which autoantibodies directed at the thyroid stimulating hormone receptor (TSHR) activate the thyroid gland and cause hyperthyroidism. Graves' ophthalmopathy (GO) is a frequent extra-thyroidal manifestation of GD, characterized by orbital inflammation and tissue expansion.[1] The clinical signs of GO may comprise any of the following: dry and gritty eyes, photophobia, chemosis, edema of peri-orbital tissues and conjunctivae, proptosis and double vision.[1-2] Although novel therapeutics such as rituximab and TNF-targeting therapies showed promising results in GO[3-4], treatment still mostly consists of corticosteroid treatment or surgical decompression of the orbits.[1-2, 5] The limited availability of treatment options is likely related to our incomplete understanding of the complex regulation of the (immuno)biological process that occurs in the orbital tissue of GO patients.

Increased orbital fibroblast activity is a central pathophysiological event in GO.[5-6] Activated orbital fibroblasts produce cytokines and chemokines that subsequently recruit and activate immune cells.[6-8] Moreover, activated orbital fibroblasts play a prominent role in orbital tissue expansion through increased production of the glycosaminoglycan hyaluronan, enhanced proliferation and increased adipogenic differentiation.[1, 5-6] Different growth factors and cytokines have been implicated in the activation of orbital fibroblasts in GO.[5-6, 9]

Also stimulatory autoantibodies against the THSR and the insulin-like growth factor 1 receptor (IGF-1R) may be involved in orbital fibroblast activation in GO.[10] So far, the contributory role of TSHR stimulatory autoantibodies to orbital fibroblast activation in GO is most convincing.[6, 11] TSHR is expressed in orbital tissue and at even higher levels in GO orbital tissue. This increased expression is confined to orbital fibroblasts, which seems to be a unique feature for orbital fibroblasts, since fibroblasts from other anatomical sites generally do not express TSHR.[12-17] In line with this, TSH or TSHR stimulating autoantibodies increase hyaluronan and IL-6 production as well as adipocytic differentiation by orbital fibroblasts.[18-20] These observations may (at least partially) explain the positive correlation between TSHR autoantibody serum titers and orbital TSHR expression with disease activity in GO patients.[11,15,21]

The platelet-derived growth factor (PDGF) family comprises four chains that can form five different dimeric isoforms.[22] Previously, we demonstrated that PDGF-A and PDGF-B chain expression is increased in GO orbital tissue and that these chains are produced by monocytes, macrophages and mast cells that have infiltrated the orbital tissue.[23-24] In addition, we showed that PDGF is important in the pathophysiology of GO. Especially PDGF-AB and PDGF-BB stimulate proliferation, hyaluronan production and cytokine production by orbital fibroblasts.[24] Considering the important contribution of PDGF, orbital TSHR expression and TSHR stimulatory autoantibodies to the pathogenesis of GO, we examined whether a relationship exists between PDGF and orbital TSHR expression and whether this influences

the effect of TSHR autoantibodies on orbital fibroblast activation.

We demonstrate that PDGF-AB and PDGF-BB enhance TSHR expression on orbital fibroblasts, with PDGF-BB being the most potent. This increased TSHR expression results in an increased capacity of immunoglobulins from GD patients (GD-IgG) to stimulate cytokine/chemokine production by orbital fibroblasts in a TSHR-dependent manner. Our data point at an important role for PDGF-B chain containing PDGF isoforms in the regulation of enhanced orbital TSHR expression in GO and the subsequent capacity of TSHR stimulating autoantibodies to stimulate inflammatory mediator and hyaluronan production by orbital fibroblasts in GO.

METHODS

Patients and control subjects

Orbital tissue was obtained from euthyroid GO patients that underwent orbital decompression surgery. Control orbital tissue was obtained from patients without inflammatory orbital or thyroid disease who underwent surgery for cosmetic reasons. All orbital tissues were obtained in the Rotterdam Eye Hospital (Rotterdam, The Netherlands) after informed consent and in accordance with the principles of the Declaration of Helsinki. Approval was obtained from the local medical ethics committee. Orbital fibroblast strains were established as described before [23]. The dermal fibroblast strain (C5RO)[25] was kindly provided by Dr. M. van der Burg (Rotterdam, the Netherlands).

Detection of TSHR expression by fibroblasts

For mRNA studies, fibroblasts were seeded into 6-well plates and allowed to grow confluent in DMEM supplemented with 10% FCS and antibiotics (DMEM 10% FCS; all Cambrex BioWhittaker, Verviers, Belgium). Messenger RNA was isolated, reverse transcribed into cDNA and TSHR mRNA expression was determined by RQ-PCR and normalized to the control gene Abelson.[23] TSHR mRNA was detected with a commercially available primer-probe combination mix (HS01053847_m1, Applied Biosystems, Foster City, CA, USA).

TSHR protein expression was studied by flowcytometric analysis. Heretoo, orbital fibroblasts were seeded into 10 cm petri-dishes and allowed to grow confluent in DMEM 10% FCS. Hereafter, fibroblast monolayers were incubated in DMEM 1% FCS for 16 hours. Subsequently, medium was replaced with DMEM 1% FCS with or without PDGF-AA, PDGF-AB or PDGF-BB (50 ng/ml, R&D systems, Abingdon, UK) for 24 hours. Similar experiments were performed in the presence of the PDGF receptor tyrosine kinase inhibitor imatinib mesylate (2.5 μg/ml, Novartis Pharma, Basel, Switzerland).

Fibroblasts were harvested, washed with phosphate-buffered saline supplemented with 0.2% bovine serum albumin and 0.1% sodium azide and incubated for 30 min at 4°C with a monoclonal mouse-anti-human TSHR antibody (MCA1571; AbDSerotec, Düsseldorf, Germany) or a mouse IgG_{2a} isotype control (G155-178; BD Biosciences, San Jose, CA, USA). Subsequently, cells were washed twice and incubated for 30 min at 4°C with a FITC-labeled rat-anti-mouse antibody (Dako, Heverlee, Belgium). Finally, cells were washed and TSHR expression was determined using a LSR II Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo FACS analysis software 7.5.4 (TreeStar, Ashland, OR, USA).

Immunoglobulin purification

Immunoglobulin (IgG) fractions were isolated from sera from patients with GD (GD-IgG), Sjögren's syndrome (SjS-IgG) or healthy volunteers (h-IgG) using a protein-G affinity column.

Stimulation of fibroblasts with bovine TSH or immunoglobulins

Fibroblasts were seeded into 6-well plates and allowed to grow confluent in DMEM 10% FCS. Hereafter, the medium was replaced with DMEM 1% FCS for 16 hours. Subsequently, cells were incubated in DMEM 1% FCS with or without PDGF-BB (50 ng/ml) for 24 hours. Thereafter, fibroblast monolayers were washed with DMEM 1% FCS and incubated with DMEM 1% FCS in the presence or absence of bovine (b)TSH (5 or 10 mU/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands), GD-IgG, SjS-IgG, h-IgG or a clinical intravenous immunoglobulin (IVIG) preparation (Nanogam, Amsterdam, the Netherlands) (all 20 ng/ml) for 24 hours. Culture supernatants were subjected to IL-6, IL-8, CCL2 (all Invitrogen, London, UK), CCL5, CCL7 and hyaluronan (all R&D systems) ELISA. Because PDGF can induce the production of these mediators by itself[26], results are presented as the delta production, which is calculated by subtraction of the production by unstimulated orbital fibroblasts from the production by bTSH- or IgG-stimulated orbital fibroblasts. Similar calculations were done for orbital fibroblasts that were pre-incubated with PDGF-BB.

Blocking experiments were performed with the monoclonal TSHR blocking antibody K1-70[27] (500 ng/ml; RSR Limited, Cardiff, UK) and with H89 (10 μM; Sigma-Aldrich), an inhibitor of protein kinase A (PKA) which is a downstream kinase in the cyclic adenosine monophosphate (cAMP) pathway.[28] Hereto, the orbital fibroblasts were incubated with K1-70 or H89 for 30 minutes prior to and during the 24 hours of stimulation with bTSH or IgG.

IGF-1R mRNA expression and the effect of recombinant human IGF-1 on IL-6 production by orbital fibroblasts

Orbital fibroblasts were grown confluent in DMEM 10% FCS in 6-well plates. Hereafter, medium was replaced with DMEM 1% FCS for 16 hours. Subsequently, cells were cultured

in DMEM 1% FCS with or without PDGF-BB (50 ng/ml) for 24 hours.

For mRNA studies, mRNA was isolated, reverse transcribed into cDNA, and TSHR and IGF-1R mRNA expression were determined by RQ-PCR and normalized to the control gene Abelson [23]. IGF-1R mRNA expression was determined using the following primer-probe combination: forward primer; AGCCGATGTGTGAGAAGACCA, reverse primer; GGGGTGGCAGCACTCATTG, probe; TGGGCACATTTCTGGCAGCGGTT.

To determine the effect of recombinant human (rh)IGF-1 on IL-6 production by orbital fibroblasts, fibroblast monolayers were incubated with DMEM 1% FCS in the presence or absence of bTSH (10 mU/ml) or rhIGF-1 (50 ng/ml; R&D Systems) for 24 hours. Culture supernatants were subjected to IL-6 ELISA. Because of residual stimulatory activity of the PDGF pre-incubation, results are presented as the delta production, which was calculated by subtraction of the production by unstimulated orbital fibroblasts from the production by bTSH or rhIGF-1-stimulated orbital fibroblasts. Similar calculations were done for orbital fibroblasts that were pre-incubated with PDGF-BB.

Statistical analysis

Messenger RNA and protein levels in orbital tissue specimens were analyzed using a two-tailed Mann-Whitney test. Data from fibroblast stimulation experiments were analyzed using a paired two-tailed Student's t-test. A p-value <0.05 was considered significant.

RESULTS

PDGF-AB and PDGF-BB stimulate TSHR expression by orbital fibroblasts

Orbital fibroblasts cultured under basal conditions expressed TSHR mRNA while dermal fibroblasts did not express TSHR mRNA (Fig. 1A), which is in line with previous findings.[15] GO orbital fibroblasts showed a trend towards higher basal TSHR mRNA expression than control orbital fibroblasts (Fig. 1A).

Of the three PDGF isoforms tested, PDGF-AB and PDGF-BB significantly increased TSHR protein expression by orbital fibroblasts, while PDGF-AA had no effect (Fig. 1B and C). Orbital fibroblasts from GO patients and from healthy controls did not differ in their response to PDGF stimulation (Supplemental Fig 1). The PDGF receptor tyrosine kinase inhibitor imatinib mesylate efficiently inhibited PDGF-induced TSHR expression by orbital fibroblasts (Fig. 1D), indicating that the increase of TSHR expression was due to activation of PDGF-receptor signalling.

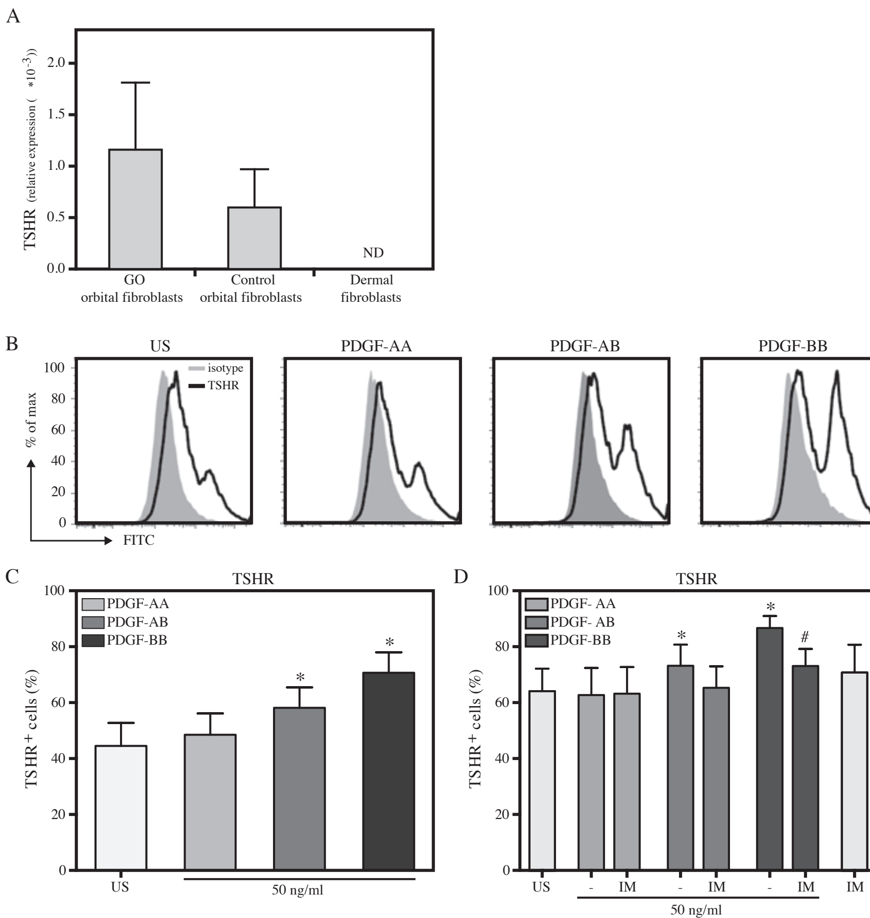


Figure 1 TSHR expression by fibroblasts A: TSHR mRNA expression by GO orbital fibroblasts (n=8), control orbital fibroblasts (n=4) and dermal fibroblasts (n=3). B and C: TSHR protein expression by orbital fibroblasts after stimulation with PDGF-AA, PDGF-AB and PDGF-BB (all 50 ng/ml) for 24 hours. B: Histograms showing TSHR protein expression by representative orbital fibroblasts line. C: Mean TSHR protein expression of orbital fibroblast lines (n=10) after PDGF stimulation. D: Mean TSHR expression on PDGF-stimulated orbital fibroblasts (n=6) in the presence or absence of imatinib mesylate. Data are presented as mean ± SEM and were analyzed using the Students t-test. * $p < 0.05$. ND: non detectable, US: unstimulated

PDGF-BB stimulation enhances the capacity of bovine TSH to stimulate cytokine and hyaluronan production by orbital fibroblasts

To examine whether PDGF induced expression of a truly functional TSHR, orbital fibroblasts were stimulated with bTSH after an initial pre-incubation period in the presence or absence of PDGF-BB (the PDGF isoform that proved most effective to enhance TSHR expression). Bovine TSH dose-dependently increased IL-6 production by orbital fibroblasts (Fig. 2A). Pre-incubation of orbital fibroblasts with PDGF-BB significantly increased the bTSH-

induced IL-6 production (p<0.05, Fig. 2A). Similar results were obtained when recombinant human TSH was used instead of bTSH (Supplemental Fig. 2). Bovine TSH did not induce IL-6 production by dermal fibroblasts, also not after PDGF-BB pre-incubation (Fig. 2A). This latter is in line with the observed lack of TSHR expression by dermal fibroblasts.

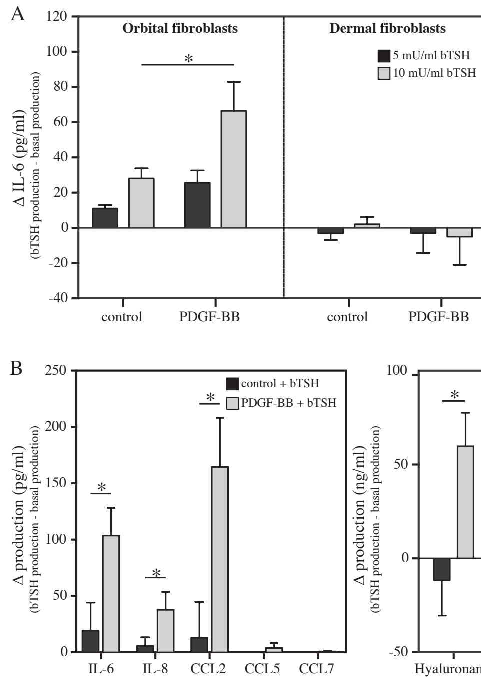


Figure 2 PDGF-BB enhances the responsiveness of orbital fibroblasts to bTSH stimulation A: Orbital fibroblasts (n=8) and dermal fibroblasts (n=6) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours, after which medium with or without bTSH (5 and 10 mU/ml) was added for another 24 hours. Supernatants were subjected to IL-6 ELISA. B: Orbital fibroblasts (n=6) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours, after which medium with or without bTSH (10 mU/ml) was added for 24 hours. Supernatants were subjected to IL-6, IL-8, CCL2, CCL5, CCL7 and hyaluronan ELISA. Data are presented as the mean delta (Δ) protein production ± SEM and were analyzed using the Students t-test. * $p < 0.05$

We also investigated the impact of bTSH on the production of hyaluronan and of various other cytokines (IL-8, CCL2, CCL5 and CCL7) that can be produced by orbital fibroblasts. Bovine TSH slightly increased IL-8 and CCL2 production by orbital fibroblasts, while it somewhat decreased hyaluronan production by orbital fibroblasts, but these effects were not statistically significant (Fig 2B). Pre-incubation with PDGF-BB significantly increased bTSH-induced IL-8, CCL2 and hyaluronan production by orbital fibroblasts (p<0.05; Fig. 2B). CCL5 and CCL7 production was not stimulated by bTSH, also not after PDGF-BB pre-incubation (Fig. 2B).

Figure 3 PDGF-BB increases the responsiveness of orbital fibroblasts to GD-IgG stimulation A: Orbital fibroblasts (n=6) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours, after which medium with or without GD-IgG, h-IgG, SjS-IgG and IVIG (all 20 ng/ml) was added for 24 hours. Supernatants were subjected to IL-6 ELISA. B: Orbital fibroblasts (n=6) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours, after which medium with or without GD-IgG or h-IgG (both 20 ng/ml) was added for 24 hours. Supernatants were subjected to IL-8, CCL2, CCL5, CCL7 and hyaluronan ELISA. Data are presented as the mean delta (Δ) protein production ± SEM and were analyzed using the Students t-test. * $p < 0.05$; GD-IgG: immunoglobulins from GD patients, h-IgG: immunoglobulins from healthy controls, IVIG: intravenous immunoglobulin preparation, SjS-IgG: immunoglobulins from Sjögren's syndrome patients

PDGF-BB stimulation enhances the capacity of immunoglobulins from GD patients to stimulate cytokine and hyaluronan production by orbital fibroblasts

GD patients have autoantibodies capable of activating TSHR signalling. Therefore, we investigated whether PDGF-BB also affected the response of orbital fibroblasts to stimulation

with IgG from GD patients (GD-IgG). IgG from 2 healthy controls (h-IgG), 2 Sjögren's syndrome patients (SjS-IgG) and a clinical IVIG preparation served as controls. Stimulation with h-IgG, SjS-IgG and IVIG did not increase IL-6 production by orbital fibroblasts, also not after PDGF-BB pre-incubation (Fig 3A). In contrast, 3 of 5 GD-IgG induced IL-6 production by orbital fibroblasts and PDGF-BB pre-incubation significantly enhanced the GD-IgG-induced IL-6 production by orbital fibroblasts ($p<0.05$, Fig. 3A).

Next we determined whether PDGF-BB also enhanced the capacity of GD-IgG to stimulate IL-8, CCL2, CCL5 and hyaluronan production by orbital fibroblasts. Unlike h-IgG, GD-IgG stimulated CCL2 and CCL5 production by orbital fibroblasts (Fig 3B). No effect of GD-IgG was observed on IL-8 and hyaluronan production. PDGF-BB pre-incubation however consistently increased the GD-IgG-induced IL-8, CCL2 and CCL5 production by orbital fibroblasts, while no such effect was observed for hyaluronan production or for h-IgG stimulation (Fig 3B).

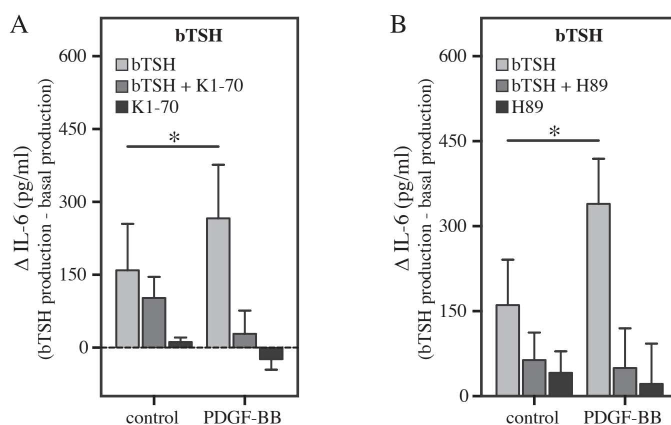


Figure 4 Bovine TSH-stimulated IL-6 production by orbital fibroblasts is inhibited by the TSHR blocking antibody K1-70 and the cAMP signalling inhibitor H89 A: Orbital fibroblasts (n=5) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours. Hereafter, the orbital fibroblasts were incubated with K1-70 (500 ng/ml) for 30 minutes prior to and during the stimulation with bTSH (10 mU/ml). Supernatants were subjected to IL-6 ELISA. B: Orbital fibroblasts (n=6) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours. Hereafter, the orbital fibroblasts were incubated with H89 (10 μM) for 30 minutes prior to and during the stimulation with bTSH (10 mU/ml). Supernatants were subjected to IL-6 ELISA. Data are presented as the mean Δ IL-6 production ± SEM and were analyzed using the Students t-test. * $p < 0.05$

Activation of TSHR and cAMP signalling in orbital fibroblasts regulates the response of PDGF-BB pre-incubated orbital fibroblasts to GD-IgG

To examine whether the effect that GD-IgG exerted on orbital fibroblasts was truly mediated via TSHR activation, several blocking studies with the monoclonal TSHR blocking antibody K1-70 or the cAMP pathway (PKA) inhibitor H89 were performed.

K1-70 almost completely inhibited bTSH-induced IL-6 production by PDGF-BB-pre-incubated orbital fibroblasts, while it only slightly decreased bTSH-induced IL-6 production by orbital fibroblasts that were not pre-incubated with PDGF-BB (Fig 4A). Similarly, H89 strongly inhibited bTSH-induced IL-6 production by orbital fibroblasts that were pre-incubated with PDGF-BB, while it only slightly reduced the bTSH-induced IL-6 production by non-pre-incubated orbital fibroblasts (Fig 4B).

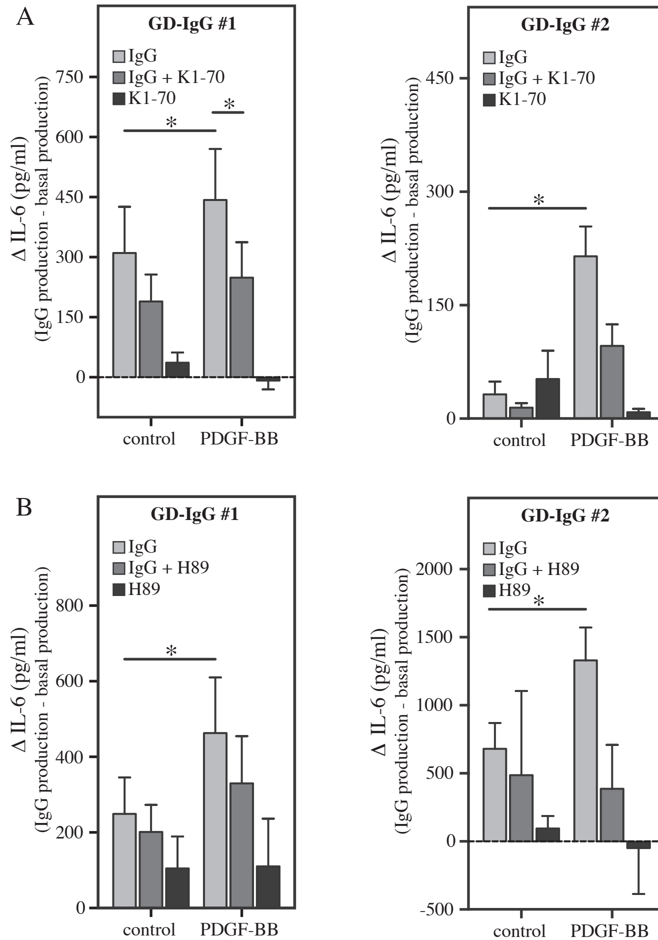


Figure 5 GD-IgG-stimulated IL-6 production by orbital fibroblasts is inhibited by the TSHR blocking antibody K1-70 and the cAMP signalling inhibitor H89 A: Orbital fibroblasts (n=5) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours. Hereafter, the orbital fibroblasts were incubated with K1-70 (500 ng/ml) for 30 minutes prior to and during the stimulation with GD-IgG (20 ng/ml). Supernatants were subjected to IL-6 ELISA. B: Orbital fibroblasts (n=6) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours. Hereafter, the orbital fibroblasts were incubated with H89 (10 μM) for 30 minutes prior to and during the stimulation with GD-IgG (20 ng/ml). Supernatants were subjected to IL-6 ELISA. Data are presented as the mean delta (Δ) IL-6 production ± SEM and were analyzed using the Students t-test. * $p < 0.05$; GD-IgG: immunoglobulins from GD patients

K1-70 also consistently reduced GD-IgG-induced IL-6 production by orbital fibroblasts that were pre-incubated with PDGF-BB. This inhibitory effect of K1-70 was never complete but was significant ($p<0.05$) or approached significance (Fig 5A). K1-70 also inhibited the GD-IgG-induced IL-6 production by orbital fibroblasts that were not pre-incubated with PDGF-BB, but this inhibitory effect was never complete nor significant (Fig 5A). H89 consistently reduced the GD-IgG-induced IL-6 production as well, but this never reached significance. H89, however, most efficiently reduced the GD-IgG-induced IL-6 production by orbital fibroblasts that were pre-incubated with PDGF-BB. K1-70 and H89 did not affect the basal IL-6 production by orbital fibroblasts (Fig 4 and 5).

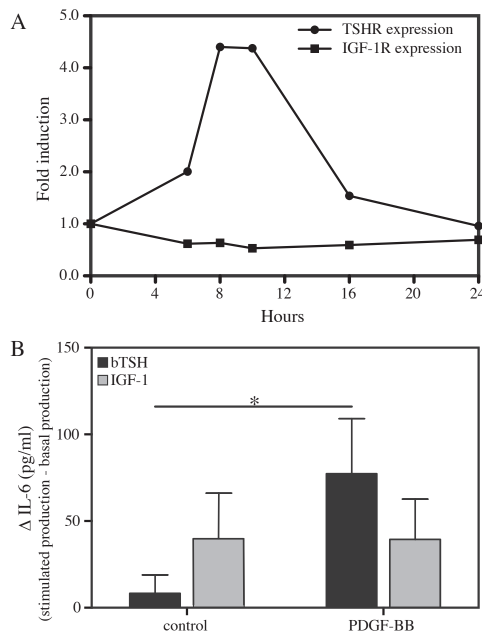


Figure 6 PDGF-BB pre-incubation does not affect IGF-1R mRNA expression nor the capacity of rhIGF-1 to stimulate IL-6 production by orbital fibroblasts A: Orbital fibroblasts (n=2) were stimulated with PDGF-BB (50 ng/ml) for the indicated periods and TSHR and IGF-1R mRNA expression was determined. B: Orbital fibroblasts (n=7) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours, after which medium with or without bTSH (10 mU/ml) or rhIGF-1 (50 ng/ml) was added for 24 hours. Supernatants were subjected to IL-6 ELISA. Data are presented as the mean delta (Δ) protein production ± SEM and were analyzed using the Students t-test. * $p < 0.05$

PDGF-BB does not affect IGF-1R expression and IGF-1-induced IL-6 production by orbital fibroblasts

Stimulatory antibodies against the IGF-1R can also be present in GO patients and may stimulate cytokine/chemokine production by orbital fibroblasts.[7] To rule out the possibility that IGF-1R stimulating autoantibodies interfered with our studies, we determined the

effect of PDGF-BB on IGF-1R mRNA expression and IGF-1-induced IL-6 production by orbital fibroblasts. PDGF-BB stimulation of orbital fibroblasts did not alter IGF-1R mRNA levels over a period of 24 hours, which strongly contrasted with the stimulatory effect on TSHR mRNA expression by orbital fibroblasts (Fig 6A). Stimulation with rhIGF-1 slightly increased IL-6 production by orbital fibroblasts, but this effect of rhIGF-1 on IL-6 production was not enhanced by pre-incubating the orbital fibroblasts with PDGF-BB. This was clearly the opposite of the enhancing effect that PDGF-BB pre-incubation exerted on the capacity of bTSH to induce IL-6 production by orbital fibroblasts (Fig 6B).

DISCUSSION

TSHR stimulating autoantibodies are generally considered to contribute to the occurrence and severity of GO.[1, 11] Several lines of evidence suggest that this is related to TSHR activation in orbital fibroblasts: 1) orbital TSHR expression is increased in GO and TSHR activation on orbital fibroblasts induces cytokine and hyaluronan production by these cells, and 2) a positive correlation exists between the clinical activity of GO and serum TSHR autoantibody levels.[11, 15, 18-20] Despite this important correlation between orbital TSHR expression and TSHR stimulating autoantibodies in GO, few data are available on the regulation of TSHR expression on orbital fibroblasts.[29-32]

We previously showed that PDGF-A and PDGF-B chains are increased in GO orbital tissue and that at least monocytes, macrophages and mast cells contribute to the production of these PDGF chains.[23-24] Consequently, the PDGF-AA, PDGF-AB and PDGF-BB dimeric isoforms can be formed in GO orbital tissue, and we showed that especially PDGF-AB and PDGF-BB isoforms potently stimulate proliferation, hyaluronan production and cytokine/chemokine production by orbital fibroblasts.[24] PDGF plays thus an important role in orbital fibroblast activation in GO, which is further supported by our observations that the PDGF receptor tyrosine kinase inhibitor imatinib mesylate and PDGF-BB neutralizing antibodies inhibit hyaluronan production by GO whole orbital tissue cultures.[24, 33]

Here, we demonstrate, to our knowledge for the first time, that PDGF-AB and PDGF-BB, but not PDGF-AA, increase TSHR expression by orbital fibroblasts. This renders the orbital fibroblasts more susceptible to activation by TSHR stimulating autoantibodies. The differences between the PDGF isoforms to increase TSHR expression by orbital fibroblasts are in line with the differential stimulatory potential of PDGF isoforms on orbital fibroblast proliferation, hyaluronan production and cytokine production that we previously found.[24]

Upregulation of TSHR expression by orbital fibroblasts has thus far exclusively been related to the differentiation of orbital fibroblasts into adipocytes.[30-31] However, this differentiation process is slow (~10 days)[30], while the stimulatory effect of PDGF-AB and PDGF-BB on TSHR expression is rapid (~1 day). Whether a relation exists between

PDGF stimulation and adipocytic differentiation by orbital fibroblasts is thus far unknown, but contrasting effects of PDGF on adipocytic differentiation by pre-adipocytes from other anatomical locations and species have been described.[34-37] The *in vitro* stimulatory effect of PDGF on TSHR expression by orbital fibroblasts may be transient, as we found that TSHR mRNA expression reached baseline levels again 24 hours after PDGF-BB stimulation (Fig 6A). The equally increased expression of the PDGF-B chain in orbital tissue from GO patients with active and inactive disease[23-24], suggests that orbital fibroblasts are exposed to PDGF-BB throughout the whole disease course. We can therefore not exclude that continuous *in vivo* exposure of orbital fibroblasts to PDGF contributes to maintenance of elevated TSHR expression by orbital fibroblasts in GO patients.

Chemokines and cytokines regulate the orbital inflammatory process in GO by recruiting and activating immune cells as well as orbital fibroblasts.[1, 6] Moreover, excessive hyaluronan deposition is a major contributor to orbital tissue expansion in GO.[1, 6] Orbital fibroblasts are centrally involved in the production of these factors in GO and TSHR activation on orbital fibroblasts induces the production of IL-6 and hyaluronan.[18-20, 38] In line with this, we found that bTSH stimulated IL-6 production by orbital fibroblasts. In addition, we show for the first time that bTSH also stimulates the production of IL-8 and CCL2. Pre-incubation of orbital fibroblasts with PDGF-BB not only further enhanced the effect of bTSH on the production of these cytokines, but also allowed bTSH to stimulate hyaluronan production by orbital fibroblasts. A synergistic interaction between PDGF-BB and bTSH is unlikely to be involved in this as bTSH stimulation never induced CCL5 and CCL7 production by orbital fibroblasts, while we previously found that PDGF-BB by itself does stimulate CCL5 and CCL7 production by orbital fibroblasts.[26]

The enhanced stimulatory capacity of bTSH on PDGF-BB pre-incubated orbital fibroblasts is in line with the increased TSHR expression by orbital fibroblasts after stimulation with PDGF-BB. Furthermore, inhibition of TSHR or its downstream cAMP signalling pathway consistently reduced the capacity of bTSH to stimulate IL-6 production in PDGF-BB pre-incubated orbital fibroblasts. This illustrates that the increased responsiveness of PDGF-BB pre-incubated orbital fibroblasts to bTSH stimulation directly relates to PDGF-BB-enhanced TSHR expression. This phenomenon is unique to orbital fibroblasts, as bTSH did not induce IL-6 production by dermal fibroblasts, also not after PDGF-BB pre-incubation. This observation is in line with the fact that dermal fibroblasts do not express TSHR and underscores previous notions that orbital fibroblasts display unique characteristics that contribute to the pathophysiology of GO.[5, 9, 26]

We show for the first time that GD-IgG stimulate IL-6, CCL2 and CCL5 production by orbital fibroblasts. IL-6 activates B cells and regulates plasma cell differentiation[39], while CCL2 and CCL5 recruit and activate various leukocytes, amongst which monocytes, macrophages, mast cells and T cells.[40-42] All these leukocytes are present in increased numbers in GO

orbital tissue.[16, 24, 43-45] This points at a role for GD-IgG in the regulation of orbital leukocyte recruitment and activation through stimulation of cytokine/chemokine production by orbital fibroblasts. The GD-IgG-induced production of these cytokines was further enhanced when orbital fibroblasts were pre-incubated with PDGF-BB. Inhibition of TSHR or its downstream cAMP signalling pathway consistently inhibited the effects of GD-IgG on IL-6 production by orbital fibroblasts, also when orbital fibroblasts were pre-incubated with PDGF-BB. This indicates that the GD-IgG-induced effects are mediated by TSHR stimulating autoantibodies, which is in line with our finding that IgG from healthy individuals, Sjögren's syndrome patients and IVIG, which all lack TSHR stimulating autoantibodies, never stimulated IL-6 production by orbital fibroblasts. Involvement of IGF-1R stimulating autoantibodies in the increased responsiveness of orbital fibroblasts to GD-IgG is unlikely, as PDGF-BB did not influence IGF-1R mRNA expression nor the capacity of rhIGF-1 to stimulate IL-6 production by orbital fibroblasts.

TSHR affinity differences can exist between bTSH and TSHR stimulating autoantibodies and result in differences in downstream TSHR signalling.[38, 46] Furthermore, differences exist in the capability of GD-IgG preparations to activate TSHR downstream signalling pathways other than the cAMP pathway, such as the AKT/PI3K pathway.[47-49] This can be related to the relative distribution of TSHR stimulatory, inhibitory or neutral autoantibodies in GD-IgG preparations.[50] Differences in downstream TSHR signalling may have contributed to the slightly different cytokine profile that is produced by orbital fibroblasts after stimulation with bTSH or GD-IgG as well as to the incomplete inhibition of GD-IgG-induced IL-6 production obtained with the cAMP inhibitor H89. In addition, recent reports demonstrate that GD-IgG only induce hyaluronan production by orbital fibroblasts that are differentiated into adipocytes and thus express higher TSHR levels than undifferentiated orbital fibroblasts.[38, 51] This suggests that TSHR expression by orbital fibroblasts has to exceed a certain level in order to become efficiently activated by GD-IgG or that changes occur in TSHR downstream signalling upon adipogenic differentiation of orbital fibroblasts.

Altogether, we show that PDGF-AB and PDGF-BB increase TSHR expression on orbital fibroblasts. This enhanced TSHR expression potentiates the capacity of TSHR stimulating autoantibodies to augment the production of various cytokines and hyaluronan by orbital fibroblasts. Our data point at an important role for PDGF-AB and PDGF-BB in amplifying the immunopathological effects of TSHR stimulating autoantibodies in GO. From our current findings, together with our previous observations that PDGF isoforms also directly stimulate proliferation, cytokine production and hyaluronan production by orbital fibroblasts[23-24, 26], the concept emerges that PDGF-B containing PDGF isoforms are master regulators of several pathophysiological processes in GO. Consequently, PDGF-B chain containing PDGF isoforms should be considered as attractive treatment targets in GO.

ACKNOWLEDGEMENTS

We thank Benjamin Schrijver and Jaap Buth for their technical help with the experiments. This work was supported by 'Stichting Wetenschappelijk Onderzoek Oogziekenhuis', 'Prof. Dr. Henkes Stichting', 'Jan Dekkerstichting en Ludgardine Bouwmanstichting' and 'Nederlandse Vereniging van Graves' Patiënten'.

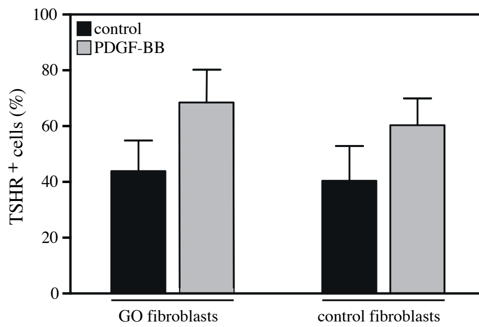
REFERENCES

1. Bahn RS. *Graves' ophthalmopathy*. N Engl J Med 2010;362:726-738.
2. Prabhakar BS, Bahn RS, et al. *Current perspective on the pathogenesis of Graves' disease and ophthalmopathy*. Endocr Rev 2003;24:802-835.
3. Hegedus L, Smith TJ, et al. *Targeted biological therapies for Graves' disease and thyroid-associated ophthalmopathy. Focus on B-cell depletion with Rituximab*. Clin Endocrinol (Oxf) 2011;74:1-8.
4. Paridaens D, van den Bosch WA, et al. *The effect of etanercept on Graves' ophthalmopathy: a pilot study*. Eye (Lond) 2005;19:1286-1289.
5. Smith TJ, Tsai CC, et al. *Unique attributes of orbital fibroblasts and global alterations in IGF-1 receptor signaling could explain thyroid-associated ophthalmopathy*. Thyroid 2008;18:983-988.
6. van Steensel L, Dik WA. *The orbital fibroblast: a key player and target for therapy in graves' ophthalmopathy*. Orbit 2010;29:202-206.
7. Pritchard J, Han R, et al. *Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway*. J Immunol 2003;170:6348-6354.
8. Hwang CJ, Afifiyan N, et al. *Orbital fibroblasts from patients with thyroid-associated ophthalmopathy overexpress CD40: CD154 hyperinduces IL-6, IL-8, and MCP-1*. Invest Ophthalmol Vis Sci 2009;50:2262-2268.
9. Heufelder AE, Bahn RS. *Modulation of Graves' orbital fibroblast proliferation by cytokines and glucocorticoid receptor agonists*. Invest Ophthalmol Vis Sci 1994;35:120-127.
10. Khoo TK, Bahn RS. *Pathogenesis of Graves' ophthalmopathy: the role of autoantibodies*. Thyroid 2007;17:1013-1018.
11. Wiersinga WM. *Autoimmunity in Graves' Ophthalmopathy: The Result of an Unfortunate Marriage Between TSH Receptors and IGF-1 Receptors?* J Clin Endocrinol Metab 2011;96:2386-2394.
12. Bahn RS, Dutton CM, et al. *Thyrotropin receptor expression in cultured Graves' orbital preadipocyte fibroblasts is stimulated by thyrotropin*. Thyroid 1998;8:193-196.
13. Bahn RS, Dutton CM, et al. *Thyrotropin receptor expression in Graves' orbital adipose/connective tissues: potential autoantigen in Graves' ophthalmopathy*. J Clin Endocrinol Metab 1998;83:998-1002.
14. Bell A, Gagnon A, et al. *Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts*. Am J Physiol Cell Physiol 2000;279:C335-340.
15. Wakelkamp IM, Bakker O, et al. *TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients*. Clin Endocrinol (Oxf) 2003;58:280-287.
16. Boschi A, Daumerie C, et al. *Quantification of cells expressing the thyrotropin receptor in extraocular muscles in thyroid associated orbitopathy*. Br J Ophthalmol 2005;89:724-729.
17. Stadlmayr W, Spitzweg C, et al. *TSH receptor transcripts and TSH receptor-like immunoreactivity in orbital and pretibial fibroblasts of patients with Graves' ophthalmopathy and pretibial myxedema*. Thyroid 1997;7:3-12.
18. Zhang L, Baker G, et al. *Biological effects of thyrotropin receptor activation on human orbital preadipocytes*. Invest Ophthalmol Vis Sci 2006;47:5197-5203.
19. Zhang L, Bowen T, et al. *Thyrotropin receptor activation increases hyaluronan production in preadipocyte fibroblasts: contributory role in hyaluronan accumulation in thyroid dysfunction*. J Biol Chem 2009;284:26447-26455.
20. Kumar S, Schiefer R, et al. *A stimulatory thyrotropin receptor antibody (M22) and thyrotropin increase interleukin-6 expression and secretion in Graves' orbital preadipocyte fibroblasts*. Thyroid 2010;20:59-65.
21. Gerding MN, van der Meer JW, et al. *Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2000;52:267-271.
22. Bonner JC. *Regulation of PDGF and its receptors in fibrotic diseases*. Cytokine Growth Factor Rev 2004;15:255-273.
23. van Steensel L, Paridaens D, et al. *Imatinib mesylate and AMN107 inhibit PDGF-signaling in orbital fibroblasts: a potential treatment for Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2009;50:3091-3098.

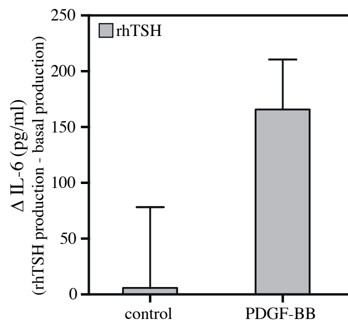
24. van Steensel L, Paridaens D, et al. *Orbit-infiltrating mast cells, monocytes and macrophages produce PDGF isoforms that orchestrate orbital fibroblast activation in Graves' ophthalmopathy*. J Clin Endocrinol Metab 2012;in press.
25. van der Burg M, Ijspeert H, et al. *A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous end-joining*. J Clin Invest 2009;119:91-98.
26. van Steensel L, Paridaens D, et al. *Platelet-derived growth factor-BB: a stimulus for cytokine production by orbital fibroblasts in Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2010;51:1002-1007.
27. Evans M, Sanders J, et al. *Monoclonal autoantibodies to the TSH receptor, one with stimulating activity and one with blocking activity, obtained from the same blood sample*. Clin Endocrinol (Oxf) 2010;73:404-412.
28. Latif R, Morshed SA, et al. *The thyroid-stimulating hormone receptor: impact of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies on multimerization, cleavage, and signaling*. Endocrinol Metab Clin North Am 2009;38:319-341, viii.
29. Pasquali D, Pierantoni GM, et al. *Fenofibrate increases the expression of high mobility group AT-hook 2 (HMGA2) gene and induces adipocyte differentiation of orbital fibroblasts from Graves' ophthalmopathy*. J Mol Endocrinol 2004;33:133-143.
30. Valyasevi RW, Erickson DZ, et al. *Differentiation of human orbital preadipocyte fibroblasts induces expression of functional thyrotropin receptor*. J Clin Endocrinol Metab 1999;84:2557-2562.
31. Valyasevi RW, Harteneck DA, et al. *Stimulation of adipogenesis, peroxisome proliferator-activated receptor-gamma (PPARgamma), and thyrotropin receptor by PPARgamma agonist in human orbital preadipocyte fibroblasts*. J Clin Endocrinol Metab 2002;87:2352-2358.
32. Valyasevi RW, Jyonouchi SC, et al. *Effect of tumor necrosis factor-alpha, interferon-gamma, and transforming growth factor-beta on adipogenesis and expression of thyrotropin receptor in human orbital preadipocyte fibroblasts*. J Clin Endocrinol Metab 2001;86:903-908.
33. van Steensel L, van Hagen PM, et al. *Whole orbital tissue culture identifies imatinib mesylate and adalimumab as potential therapeutics for Graves' ophthalmopathy*. Br J Ophthalmol 2011;95:735-738.
34. Bachmeier M, Loffler G. *Influence of growth factors on growth and differentiation of 3T3-L1 preadipocytes in serum-free conditions*. Eur J Cell Biol 1995;68:323-329.
35. Krieger-Brauer HI, Kather H. *Antagonistic effects of different members of the fibroblast and platelet-derived growth factor families on adipose conversion and NADPH-dependent H2O2 generation in 3T3 L1-cells*. Biochem J 1995;307 (Pt 2):549-556.
36. Artemenko Y, Gagnon A, et al. *Anti-adipogenic effect of PDGF is reversed by PKC inhibition*. J Cell Physiol 2005;204:646-653.
37. Molgat AS, Gagnon A, et al. *Preadipocyte apoptosis is prevented by macrophage-conditioned medium in a PDGF-dependent manner*. Am J Physiol Cell Physiol 2009;296:C757-765.
38. van Zeijl CJ, Fliers E, et al. *Thyrotropin receptor-stimulating Graves' disease immunoglobulins induce hyaluronan synthesis by differentiated orbital fibroblasts from patients with Graves' ophthalmopathy not only via cyclic adenosine monophosphate signaling pathways*. Thyroid 2011;21:169-176.
39. Hirano T. *Interleukin 6 and its receptor: ten years later*. Int Rev Immunol 1998;16:249-284.
40. Castellani ML, De Lutiis MA, et al. *Impact of RANTES, MCP-1 and IL-8 in mast cells*. J Biol Regul Homeost Agents 2010;24:1-6.
41. Schall TJ, Bacon K, et al. *Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES*. Nature 1990;347:669-671.
42. Proost P, Wuyts A, et al. *Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1*. J Leukoc Biol 1996;59:67-74.
43. Kahaly G, Hansen C, et al. *Immunohistochemical staining of retrobulbar adipose tissue in Graves' ophthalmopathy*. Clin Immunol Immunopathol 1994;73:53-62.
44. Pappa A, Lawson JM, et al. *T cells and fibroblasts in affected extraocular muscles in early and late thyroid associated ophthalmopathy*. Br J Ophthalmol 2000;84:517-522.
45. Chen MH, Liao SL, et al. *Role of macrophage infiltration in the orbital fat of patients with Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2008;69:332-337.
46. Laurent E, Van Sande J, et al. *Unlike thyrotropin, thyroid-stimulating antibodies do not activate phospholipase C in human thyroid slices*. J Clin Invest 1991;87:1634-1642.

47. Drexhage HA. *Autoimmunity and thyroid growth. Where do we stand?* Eur J Endocrinol 1996;135:39-45.
48. Di Cerbo A, Corda D. *Signaling pathways involved in thyroid hyperfunction and growth in Graves' disease*. Biochimie 1999;81:415-424.
49. Kumar S, Nadeem S, et al. *A stimulatory TSH receptor antibody enhances adipogenesis via phosphoinositide 3-kinase activation in orbital preadipocytes from patients with Graves' ophthalmopathy*. J Mol Endocrinol 2011;46:155-163.
50. Ponto KA, Kanitz M, et al. *Clinical relevance of thyroid-stimulating immunoglobulins in graves' ophthalmopathy*. Ophthalmology 2011;118:2279-2285.
51. van Zeijl CJ, Fliers E, et al. *Effects of thyrotropin and thyrotropin-receptor-stimulating Graves' disease immunoglobulin G on cyclic adenosine monophosphate and hyaluronan production in nondifferentiated orbital fibroblasts of Graves' ophthalmopathy patients*. Thyroid 2010;20:535-544.

SUPPLEMENTAL DATA



Supplemental figure 1 PDGF-induced TSHR expression is not different between GO and control orbital fibroblasts. GO (n=4) and control (n=3) orbital fibroblasts were stimulated with PDGF-BB (50 ng/ml) for 24 hours and TSHR expression was determined by flowcytometry. Data are presented as mean ± SEM.



Supplemental figure 2 PDGF-BB increases the capacity of recombinant human TSH (rhTSH) to stimulate IL-6 production by orbital fibroblasts. Orbital fibroblasts (n=3) were pre-incubated with PDGF-BB (50 ng/ml) for 24 hours, after which medium with or without rhTSH (10 mU/ml) was added for another 24 hours. Supernatants were subjected to IL-6 ELISA. Data are presented as the mean delta (Δ) IL-6 production ± SEM.

Chapter 9

General discussion

Graves' ophthalmopathy (GO) causes a considerable physical and mental burden for patients and medical treatment has not improved much in the last two decades. Activation of orbital fibroblasts is crucial in the pathophysiology of GO as it contributes to excessive orbital tissue expansion and inflammation.[1-2] More insight into factors that drive orbital fibroblast activation and subsequent pathophysiological processes is needed as this may lead to the development of novel treatment options for GO patients.

Platelet-derived growth factors (PDGF) are strongly involved in fibro-proliferative/fibrotic disorders of other organs where they stimulate fibroblasts to proliferate and to produce extracellular matrix (ECM) components and inflammatory mediators.[2] Similarly, orbital fibroblasts contribute to GO through increased proliferation, ECM production (mainly hyaluronan) and production of inflammatory mediators. This thesis demonstrates that PDGF fulfills a central role in the pathophysiology of GO.

PDGF: A MASTER REGULATOR OF ORBITAL FIBROBLAST ACTIVATION IN GRAVES' OPHTHALMOPATHY

Orbital inflammation and tissue expansion contribute to the clinical symptoms of GO.[1, 3] Important (immuno)pathological processes that contribute to GO development and severity and to which orbital fibroblast activity contributes include: 1) orbital inflammation; orbital fibroblasts are activated by inflammatory mediators such as cytokines, growth factors and thyroid stimulating hormone receptor (TSHR) or insulin-like growth factor-1 receptor (IGF-1R) stimulating autoantibodies, but orbital fibroblasts themselves are also an important source of pro-inflammatory cytokines/chemokines, 2) orbital fibrosis; comprising proliferation and hyaluronan production by orbital fibroblasts, and 3) orbital adipogenesis; increased differentiation of orbital fibroblasts into adipocytes.

It has been postulated that mediators that activate fibroblasts and thereby contribute to fibro-proliferative disorders such as GO, should fit three basic criteria: 1) their expression (or the expression of their target molecules (e.g. specific receptors)) has to be increased in the affected tissue of patients, 2) they should exhibit strong fibroblast-activating properties, and 3) their inhibition should attenuate pathophysiological or clinical characteristics.[4] PDGF fulfills these criteria in GO, as described hereafter.

Increased orbital expression of PDGF-AA, PDGF-AB and PDGF-BB in Graves' ophthalmopathy

Of all PDGF family members, expression of the PDGF-A and PDGF-B chains was clearly increased in orbital tissue from GO patients, while PDGF-C, PDGF-D, PDGF-receptor α and PDGF-receptor β expression was comparable to control orbital tissue (chapter 4 and

7). PDGF-A and PDGF-B chains were equally expressed in orbital tissues from patients with active and inactive GO, suggesting that PDGF contributes to GO during all stages of disease. This increased production of the PDGF-A and PDGF-B chains likely results in high levels of dimeric PDGF-AA, PDGF-AB and PDGF-BB isoforms that can influence the pathophysiological process in GO orbital tissues, as discussed later.

The cause of increased PDGF-A and PDGF-B chain expression in GO orbital tissue remains unclear so far. Several cell types, including monocytes, macrophages and mast cells have been identified as PDGF-producers.[5-7] In this thesis, orbit-infiltrated monocytes, macrophages and mast cells were identified as producers of PDGF-A and PDFG-B chains (chapter 7). In line with other studies[8-11], monocyte, macrophage and mast cell numbers were elevated in GO orbital tissue, which may account for the PDGF elevation detected in GO orbital tissue (chapter 7). Monocyte, macrophage and mast cell numbers are equally increased during active and inactive stages of GO, which parallels that of PDGF expression in GO orbital tissue. This further strengthens the existence of a positive correlation between PDGF expression levels and monocyte, macrophage and mast cell numbers in GO.

From the data presented in chapter 8 it cannot be concluded that the PDGF expression in GO orbital tissue is increased on a per-leukocyte basis. Gross visual examination of double stainings for mast cells and PDGF did however reveal a more intense PDGF staining in mast cells in GO orbital tissue than in mast cells in control orbital tissue (chapter 7). This may suggest that higher amounts of PDGF are produced per mast cell in GO, but this requires further studies.

PDGF expression and activity as well as PDGF-receptor expression is influenced by cytokines and growth factors.[12-19] For instance, IL-1β, IFN-γ, TNF-α and TGF-β, that are all increased in GO orbital tissue, can stimulate PDGF production or upregulate PDGF receptor expression by several different cell types.[20-27] TGF-β_1 did not directly affect orbital fibroblast proliferation, nor did it amplify the effect of PDGF-BB on hyaluronan production by orbital fibroblasts after 24 hours of co-stimulation (chapter 4). However, TGF-β_1 did increase PDGF-A and PDGF-B mRNA expression by orbital fibroblasts (Van Steensel *et al.,* unpublished observations) and may thus increase orbital fibroblast proliferation and hyaluronan production via stimulation of PDGF production by orbital fibroblasts. This however likely takes place after more than 24 hours of stimulation. The regulation of PDGF expression and activity by other factors in GO however needs further investigation.

Nevertheless, although the cause of the increased PDGF expression in GO orbital tissue still remains unknown, it is clear that PDGF-AA, PDGF-AB and PDGF-BB expression is increased in GO orbital tissue.

PDGF enhances the immunopathological effects of TSHR stimulating autoantibodies in Graves' ophthalmopathy

TSHR stimulating autoantibodies induce IL-6 and hyaluronan production by orbital fibroblasts.[28-29] Moreover, TSHR autoantibody levels and orbital TSHR expression both correlate positively with GO disease activity.[30-31] This suggests that TSHR stimulating autoantibodies contribute to the development and severity of GO through the activation of orbital fibroblasts.[32]

The studies presented in this thesis demonstrate for the first time that PDGF-AB and PDGF-BB enhance TSHR expression on orbital fibroblasts. This increases the responsiveness of orbital fibroblasts to the stimulatory activity of TSHR stimulating autoantibodies that are present in the immunoglobulin fraction of GD patients (GD-IgG, chapter 8). The activation of TSHR on orbital fibroblasts by TSHR stimulating autoantibodies stimulates the production of pro-inflammatory cytokines, including IL-6, IL-8, CCL2 and CCL5, by orbital fibroblasts. The cytokines induced very likely contribute to the pathophysiology of GO, as will be discussed in more detail in the next paragraph.

PDGF enhances orbital inflammation in Graves' ophthalmopathy

Orbital tissue from GO patients is characterized by an orbital infiltrate comprising T cells, B cells, monocytes, macrophages and mast cells (chapter 7).[1, 8, 33] Orbital fibroblasts can produce a broad array of cytokines and chemokines that control the recruitment and activation of these leukocytes. On the other hand, orbital fibroblasts themselves become activated by inflammatory mediators that are released by the infiltrated leukocytes. Therefore, in GO orbital fibroblasts and leukocytes together shape the orbital inflammatory environment. The early stage of GO is dominated by a T-helper 1 (Th1) cytokine profile, but switches to a Th2-dominated cytokine environment in later stages of GO.[1, 3]

GD-IgG stimulate cytokine/chemokine production by orbital fibroblasts, which is further enhanced by PDGF through upregulation of TSHR expression on orbital fibroblasts. PDGF, and especially the PDGF-AB and PDGF-BB isoforms, however also directly stimulate the production of a variety of inflammatory cytokines/chemokines, including IL-6, IL-8, CCL2, CCL5 and CCL7, by orbital fibroblasts (chapter 5). All these factors, except for CCL7, have previously been associated with GO and are likely to be involved in the regulation of orbital inflammation.[9, 30, 34-36]

IL-6 and IL-8 mRNA levels are increased in orbital tissue from GO patients and are expressed at even higher levels in active GO than in inactive GO.[30, 35] This indicates that they are especially relevant to the inflammatory process in GO. IL-8 is known as a strong neutrophil attractant that is associated with inflammatory conditions in general.[37-38] Neutrophils are however absent in GO orbital tissue. IL-8 may therefore regulate other processes in GO, such as chemotaxis and proliferation of endothelial cells and fibroblasts, recruitment of T cells and

B cells, and adhesion and subsequent tissue infiltration by monocytes.[39] This has however so far not been studied in GO. IL-6 has been recognized to increase TSHR expression by orbital fibroblasts that mature into adipocytes.[40-41] In parallel to the observed stimulation of TSHR expression by PDGF, this could indicate that IL-6 also makes orbital fibroblasts more prone to produce cytokines/chemokines when activated by TSHR stimulating autoantibodies. IL-6 furthermore recruits and activates B cells and stimulates plasma cell differentiation and immunoglobulin production[42], which is in line with the presence of B cells and IgG-producing plasma cells in GO orbital tissue.[33, 43-44]

CCL5 expression so far has not been studied in GO orbital tissue, but orbital fibroblasts have previously been found to produce CCL5 when activated by stimulatory autoantibodies directed at the IGF-1R in GO.[34, 45] CCL5 is well-known for its ability to attract T cells[38, 46] and may thus contribute to T-cell recruitment into orbital tissue from GO patients.

Although CCL2 may play a role in Th2 skewing (which occurs in late stages of GO)[3, 47], it is best known as a regulator of monocyte trafficking and macrophage activity.[48] Similarly, CCL5 and CCL7 also exert chemoattractive properties for monocytes.[38, 46] CCL2 mRNA expression is increased in GO orbital tissue and its expression levels correlate positively with macrophage numbers in the orbital tissue.[49] Therefore, CCL2, CCL5 and CCL7 can be expected to orchestrate orbital monocyte infiltration and their subsequent differentiation into macrophages in GO patients.[10-11] Monocytes and macrophages in the orbital tissue produce various pro-inflammatory mediators, such as IL-1β and TNF-α[35], through which they influence orbital fibroblast behaviour.[9] This resembles the strong activating effects that alveolar macrophages exert on lung fibroblasts and Kupfer cells on hepatic stellate cells in lung and liver fibrosis, respectively.[50-53] In line with such a function of monocytes and macrophages in the regulation of fibroblast activity, orbital monocytes and macrophages produce PDGF-A and PDGF-B chains, and dimeric isoforms of these PDGF chains potently stimulate orbital fibroblast effector functions (chapter 7).

CCL2 can also stimulate mast cell degranulation[38, 54], while CCL5 can regulate mast cell recruitment and activation.[38, 46 55] Increased numbers of mast cells have been found in fibrotic disorders of other organ systems such as the lungs, skin and liver.[56-59] Mast cells may produce and secrete many pro-inflammatory mediators, such as histamine and tryptase, and increased histamine and tryptase activity contributes to lung and skin fibrosis through fibroblast activation.[59-61] Mast cells numbers are increased in GO orbital tissue, where they predominantly locate adjacent to orbital fibroblasts and have features of degranulation.[8, 62] This suggests that mast cells can influence orbital fibroblast behaviour in GO through the production and release of specific mediators. Chapter 7 shows that mast cells in GO orbital tissue produce PDGF. Therefore, in GO, mast cells may facilitate cytokine and hyaluronan production and TSHR expression by orbital fibroblasts through the release of PDGF. In addition to this, the mast cell products histamine and tryptase also stimulate the production

of inflammatory factors by orbital fibroblasts (Van Steensel *et al*., manuscript in preparation). Altogether these data point at a role for mast cells in the activation of orbital fibroblasts in GO.

PDGF enhances tissue remodelling and expansion in Graves' ophthalmopathy

PDGF is well recognized for its role in wound healing and tissue fibrosis where it activates fibroblasts to proliferate and to produce ECM components.[51] The orbital tissue expansion in early/active GO includes at least orbital fibroblast proliferation and hyaluronan production.[1, 63] The studies in this thesis show that all PDGF isoforms activate these processes, and that PDGF-B containing PDGF isoforms are the most potent in doing so (chapter 4 and 7).

Another major component of orbital tissue expansion in GO which especially plays a role during inactive/late stages of GO, is the differentiation of orbital fibroblasts into adipocytes.[1, 32, 63-64] *In vitro* differentiation of orbital fibroblasts into adipocytes can be induced in serum-free medium supplemented with dexamethasone and isobutylmethylxanthine (IBMX).[64-66] Adipogenic differentiation of orbital fibroblasts is characterized by accumulation of lipid droplets and the expression of adipocyte-specific genes, such as peroxisome proliferator-activated receptor (PPAR)-γ.[66] Adipogenic differentiation of orbital fibroblasts is further enhanced when PPAR-γ agonists or IL-6 are added to the culture medium.[40, 65, 67] Preliminary studies reveal that adding PDGF-BB to serum-free adipogenesis-stimulating medium strongly increases lipid accumulation by orbital fibroblasts (Figure 1; Van Steensel *et al.,* unpublished observations). Remarkably, this effect of PDGF-BB is also observed in the absence of dexamethasone and IBMX, which are generally regarded as essential triggers for adipogenic differentiation.[64] Furthermore, this effect of PDGF-BB appeared to be specific for orbital fibroblasts, as dermal fibroblasts did not obtain adipocyte-specific features upon PDGF-BB stimulation under these culture conditions (Figure 1).

The mechanisms by which PDGF induces adipogenic differentiation of orbital fibroblasts have however not been unravelled so far. Contrasting effects of PDGF on adipogenesis have been reported in pre-adipocytes from different sources and under different culture conditions.[68-69] Possibly, PDGF inhibits apoptosis of differentiating pre-adipocytes and thereby stimulates the adipogenic process under serum-free conditions.[70-71] Furthermore, PDGF stimulates IL-6 production by orbital fibroblasts (chapter 5 and 7) and IL-6 does enhance adipogenic differentiation of orbital fibroblasts.[40] Therefore, further studies are required to elucidate the exact molecular process and pathways involved in PDGF-induced adipogenic differentiation of orbital fibroblasts.

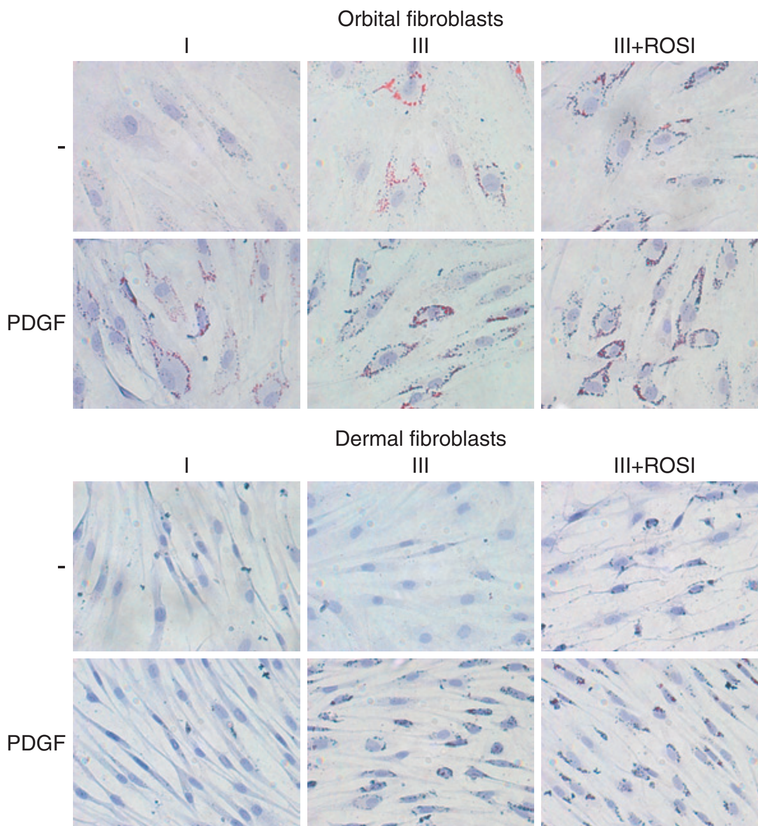


Figure 1 PDGF-BB-induced adipogenic differentiation of orbital fibroblasts. *Upper row*: No adipogenic differentiation of orbital fibroblasts is observed in serum-free medium (condition I) until prostacyclin and, for the first four days, dexamethasone and IBMX were added (condition III). The PPARγ agonist rositiglazone further enhances adipogenic differentiation of orbital fibroblasts (condition III+ROSI). *Second row*: Addition of PDGF-BB to the media clearly increased adipogenic differentiation of orbital fibroblasts in all culture conditions. *Third* and *fourth row*: No adipogenic differentiation was observed in dermal fibroblasts in the presence or absence of PDGF-BB. Fibroblasts were cultured for 10 days in the indicated culture media, stained with Oil-Red-O to visualize lipid droplets and counter-stained with hematoxylin/eosin.

Orbital fibroblasts display unique features that contribute to Graves' ophthalmopathy

Depending on the tissue they originate from and reside in, fibroblasts can express distinct phenotypic features and activity.[72-73] This is supported by the observation that orbital fibroblasts respond differently to PDGF stimulation than fibroblasts from other anatomical locations: 1) PDGF induces TSHR expression by orbital fibroblasts but not by dermal fibroblasts, and 2) PDGF induces cytokine/chemokine production by orbital fibroblasts that exceeded the production induced in lung or dermal fibroblasts (chapters 5 and 8). These findings are in line with previous notions that orbital fibroblasts respond more vigorously

to certain stimuli than fibroblasts from other anatomical locations.[74-79] Moreover, TGF-β_1 stimulation decreases c-Abl protein levels in orbital fibroblasts, while c-Abl levels in lung fibroblasts remain unaffected by TGF-β_1 stimulation (chapter 4). These unique features of orbital fibroblasts, together with the space-limited bony orbit in which they reside, may explain the orbital development of GO.

Differences in responses to certain stimuli have also been noted between orbital fibroblasts from GO patients and orbital fibroblasts from healthy controls.[45, 75, 80-83] The studies in this thesis did however not find such differences after PDGF or TGF-β_1 stimulation. This resembles studies by other groups that found no differences between orbital fibroblasts with regard to IL-1-stimulated hyaluronan production and adipogenic differentiation.[84-85] This stresses the significance of the increased expression of the PDGF-A and PDGF-B chains in GO orbital tissue, as that will determine the level of orbital fibroblast activity in GO patients, rather than intrinsic differences between orbital fibroblasts from GO patients and healthy controls.

In conclusion, orbital fibroblast-stimulating properties of many factors (including cytokines and growth factors) have been investigated in the pathophysiology of GO. Most of these factors stimulate some orbital fibroblast effector functions (e.g hyaluronan and cytokine production by IFN-γ), but on the other hand inhibit other orbital fibroblast functions (e.g. adipogenic differentiation by IFN-γ).[86 87-88] PDGF, and especially PDGF-B chain containing PDGF isoforms, however seem to be unique as they stimulate several major components of the pathophysiology of GO in which orbital fibroblast activity is involved, as it 1) enhances the potential of TSHR stimulating autoantibodies to stimulate cytokine/chemokine production by orbital fibroblasts by increasing TSHR expression, 2) directly stimulates cytokine/chemokine production by orbital fibroblasts, and 3) stimulates proliferation, hyaluronan production and adipogenic differentiation by orbital fibroblasts (Figure 2). This, together with the fact that PDGF is produced by monocytes, macrophages and mast cells in GO orbital tissue, turns PDGF into a master regulator of the intricate network of cells and mediators that is involved in orbital inflammation and tissue remodelling in GO.

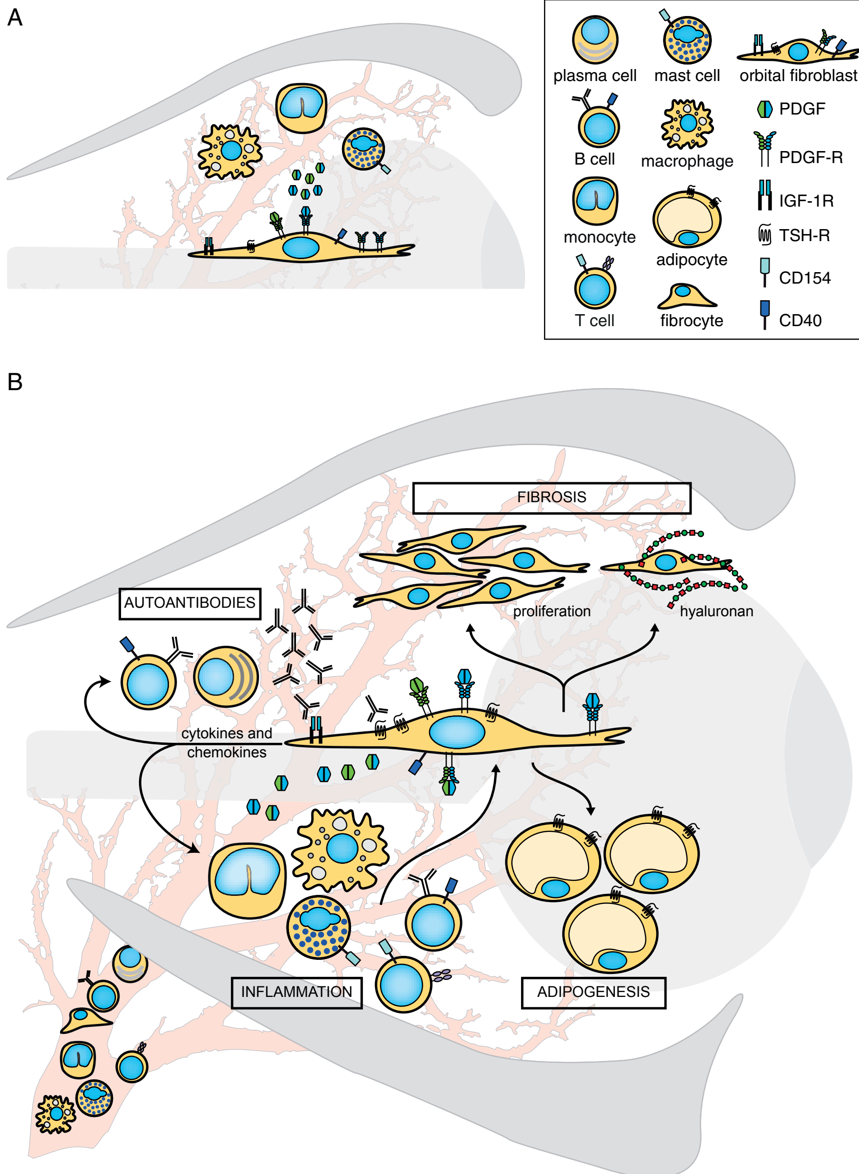


Figure 2 Involvement of PDGF in orbital fibroblast activation in the pathophysiology of Graves' ophthalmopathy. A: Monocytes, macrophages and mast cells produce PDGF-A and PDGF-B chains that activate PDGF-receptors on orbital fibroblasts. B: PDGF stimulation of orbital fibroblasts leads to 1) increased TSHR expression on orbital fibroblasts that can be activated by TSHR stimulating autoantibodies, 2) inflammation; the production of cytokines and chemokines by orbital fibroblasts that recruit and activate leukocytes into the orbital tissues, 3) fibrosis; increased proliferation and hyaluronan production by orbital fibroblasts, and 4) adipogenesis; increased differentiation of fibroblasts into adipocytes.

PDGF IN GRAVES' OPHTHALMOPATHY: ACTIONS BEYOND ORBITAL FIBROBLAST ACTIVATION?

The studies in this thesis clearly demonstrate that PDGF can exert strong activating effects on orbital fibroblasts. PDGF-receptors are however expressed by many different cell types, including endothelial cells, muscle cells, neuronal cells and pericytes.[7] Also immune cells such as T cells, macrophages and mast cells express PDGF-receptors.[7, 89-92] PDGF-BB stimulates IL-2 production and subsequent proliferation by T cells, while it represses IFN-γ, IL-4 and IL-5 secretion.[93] These effects of PDGF-BB on T cells can however be lost upon chronic activation of PDGF-Rβ.[93] Therefore, the precise effect of PDGF on T cells *in vivo*, also in GO, remains unclear so far.

During differentiation of monocytes into macrophages PDGF-Rα and PDGF-Rβ expression is upregulated.[89, 92, 94] In line with this, PDGF-AA and PDGF-BB both enhance monocyte maturation[89] and chemotaxis[89-91], while PDGF-BB also stimulates macrophage proliferation[92, 95]and decreases macrophage colony-stimulating factor (M-CSF) production.[95] This latter suggests a feedback loop by which PDGF limits the differentiation of monocytes into macrophages. Monocytes express lower levels of PDGF-Rα than PDGF-Rβ, which fits the fact that PDGF-AA less potently activates monocyte/macrophage differentiation and chemotaxis than PDGF-BB.[89, 91, 96] Interestingly, PDGF-AA is often only capable to activate cells in the presence of other inflammatory factors such as IL-1β or IFN-γ[90-91], suggesting that PDGF-AA especially has a role during inflammatory conditions. PDGF-AA however only weakly stimulated proliferation, hyaluronan production and cytokine production by orbital fibroblasts, while it did not affect TSHR expression by orbital fibroblasts (chapter 7). Possibly, PDGF-AA fulfills functions other than orbital fibroblast activation in GO, such as the regulaton of monocyte recruitment and differentiation in GO orbital tissue.[89-91] However, the role of PDGF isoforms in GO (besides activation of orbital fibroblasts), has not been studied so far and needs further studies.

INHIBITION OF PDGF ACTIVITY: AN ATTRACTIVE POSSIBILITY FOR THE TREATMENT OF GRAVES' OPHTHALMOPATHY?

GO is characterized by an initial active phase of inflammation that is dominated by Th1-driven cytokines. During this active phase orbital fibroblasts and leukocytes actively interact with each other, which results in orbital inflammation and edema. Eventually, this early inflammatory phase is skewed towards a Th2 cytokine-dominated environment that contributes to tissue remodelling events in the orbital tissue that finally determine residual tissue fibrosis.[1, 30, 36, 97-99] Concomitant with the resolution of active orbital inflammation, the strong leukocyte - orbital fibroblast interactions subside. The fibrotic tissue remodelling processes however seem to continue in an inflammation-independent manner, and may last

for months to years.[1, 100]

The main treatment for moderate-to-severe and sight-threatening GO consists of systemic corticosteroids and surgery. Decompression surgery is predominantly used to rehabilitate residual disease features, but may occasionally also be used when corticosteroids fail to sufficiently dampen the inflammatory process.[101] Corticosteroids are only effective in the initial inflammatory phase and, more importantly, may negatively influence the fibrotic process when inflammation has subsided.[101] Corticosteroids, such as dexamethasone, stimulate PDGF-B production by macrophages and enhance PDGF-Rα expression on fibroblasts, which augments fibroblast effector functions in lung fibrosis.[102-104] In contrast to the ambivalent effects that corticosteroids can have with regard to inflammation and fibrosis, the ideal therapy for GO should be effective regardless the stage of disease the patient is in. However, studies exploring novel medical treatment options for GO have so far concentrated on therapeutics directed at immune cells (e.g. rituximab) or mediators (e.g. TNF-α) that especially play a role during the active inflammatory phase of GO.[30, 105-106] A selection of potential therapeutic targets in the treatment of GO is given in Table 1.

Table 1. Potential therapeutic targets in Graves' ophthalmopathy

Target	Agent	Description
Inflammatory factor-directed treatments		
IL-1β	canakinumab	anti-IL-1β monoclonal antibody
IL-1R	anakinra	IL-1 receptor antagonist
IL-6	tocilizumab	anti-IL-6 monoclonal antibody
PDGF	imatinib mesylate, nilotinib, dasatinib	small molecule inhibitor of PDGF-R, c-Abl and c-Kit tyrosine kinases
TGF-β	lerdelimumab, GC1008	anti-TGF-β monoclonal antibodies
TNF-α	adalimumab, infliximab	anti-TNF monoclonal antibodies
TNF	etanercept	TNF receptor – IgG Fc fusion protein
Immune cell-directed treatments		
CD3	otelixizumab, teplizumab	anti-CD3 monoclonal antibodies
CD20	rituximab, ocrelizumab, ofatumumab	anti-CD20 monoclonal antibodies
CD28	abatacept	CTLA-4 – immunoglobulin fusion protein
CD154	toralizumab	anti-CD154 monoclonal antibody
Other treatments		
Oxygen free radicals	selenium	trace element
PPAR-γ	selective PPAR modulators	PPAR-γ antagonists
Somatostatin receptor	SOM230	synthethic somatostatin analogue
TSHR	NIDDK/CEB-52	low-molecular-weight TSHR antagonist

Table modified from Bahn *et al. 2010*

PDGF-driven orbital fibroblast activation can be expected to be involved in all stages of GO. This makes inhibition of PDGF-induced orbital fibroblast activation the most attractive therapeutic approach identified in GO thus far. The studies in this thesis show that the PDGF receptor tyrosine kinase inhibitor imatinib mesylate efficiently blocks PDGF-induced TSHR expression, proliferation, hyaluronan production, cytokine production, and adipogenic differentiation of orbital fibroblasts.[107-108] The potential of imatinib mesylate to interfere with pathological processes in GO is further supported by the efficient inhibition of IL-6 and hyaluronan production by imatinib mesylate in GO whole orbital tissue cultures (chapter 6). Therefore, imatinib mesylate or comparable molecules such as nilotinib (AMN107), have the capacity to interfere with a number of the major immunopathological processes during all stages of GO.

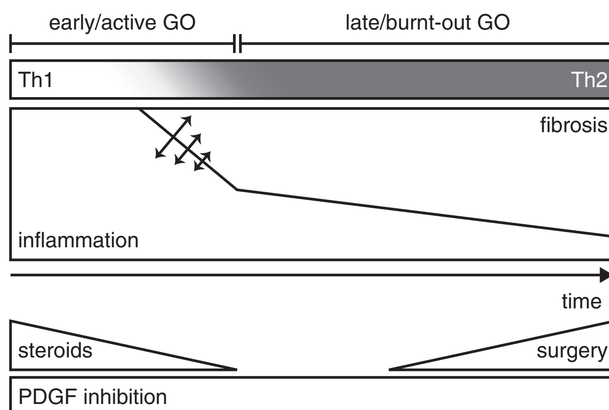


Figure 3 A hypothetical scheme of the pathophysiology and treatment of Graves' ophthalmopathy (GO). Early GO is characterized by a Th1-dominated inflammatory environment which leads to massive orbital tissue inflammation and edema. In time, this Th1 environment is skewed towards a Th2-dominated environment in which inflammation subsides, but fibrotic tissue remodelling continues. Current mainstream treatment of GO consists of corticosteroids and surgery, of which the first is especially effective in treating orbital inflammation. Surgery may be effective in early/active stages of GO, but is predominantly used for rehabilitation of GO patients. Increased PDGF activity contributes to all stages of GO and inhibition of PDGF activity may therefore be effective in all stages of GO.

PDGF receptor tyrosine kinase inhibitors, such as imatinib mesylate, are already in clinical use and promising results in the treatment of fibro-proliferative disorders from lungs, skin and liver have been described.[109-111] These studies indicate that these therapeutics may have the capacity to reverse established fibrosis.[112] Specific side effects that are unwanted in GO patients, such as peri-orbital edema, have however been reported in patients treated with imatinib mesylate.[113-115] Nilotinib is a second generation tyrosine kinase inhibitor that also blocks the tyrosine kinase activity of the c-Abl, PDGF-R and c-Kit molecules.[116] Side effects of nilotinib occur dose-dependently and are generally manageable with dose interruptions or

reductions[113, 116-118] Severe side effects leading to discontinuation of nilotinib treatment as well as peri-orbital edema (<1% of patients) only occur at daily dosages higher than 600 mg. [113, 116-118] Thyroid dysfunction ((transient) hypo/hyperthyroidism) has occasionally been observed in patients treated with nilotinib.[119] Considering its limited range of severe side effects, especially with regard to peri-orbital edema, nilotinib may be a more attractive choice to block PDGF-receptor activity in GO than imatinib mesylate. Inhibition with PDGF neutralizing antibodies would however be another approach as PDGF-BB neutralizing antibodies consistently, and equally effective as imatinib mesylate, reduced IL-6 and hyaluronan production by whole orbital tissues from GO patients (chapter 7). PDGF neutralizing antibodies are however thus far not clinically available.

CONCLUDING REMARKS

The studies in this thesis identify monocyte, macrophage and mast cell-derived PDGF (especially the PDGF-AB and PDGF-BB isoforms) as master regulators of several major pathophysiological processes in GO. All PDGF isoforms stimulate proliferation, hyaluronan production and cytokine/chemokine production by orbital fibroblasts, while PDGF-AB and PDGF-BB also increase TSHR expression and adipogenic differentiation by orbital fibroblasts. As PDGF is increased in all disease stages of GO, it can be considered to represent a true key factor in the development and perpetuation of GO, and thus an attractive therapeutic target. Following the pre-clinical studies presented here, clinical studies need to further investigate the potential of PDGF-targeted therapies in GO.

REFERENCES

1. Bahn RS. *Graves' ophthalmopathy*. N Engl J Med 2010;362:726-738.
2. van Steensel L, Dik WA. *The orbital fibroblast: a key player and target for therapy in graves' ophthalmopathy*. Orbit 2010;29:202-206.
3. Eckstein AK, Johnson KT, et al. *Current insights into the pathogenesis of Graves' orbitopathy*. Horm Metab Res 2009;41:456-464.
4. Dik WA, McAnulty RJ, et al. *Short course dexamethasone treatment following injury inhibits bleomycin induced fibrosis in rats*. Thorax 2003;58:765-771.
5. Nagaoka I, Trapnell BC, et al. *Upregulation of platelet-derived growth factor-A and -B gene expression in alveolar macrophages of individuals with idiopathic pulmonary fibrosis*. J Clin Invest 1990;85:2023-2027.
6. Holdsworth SR, Summers SA. *Role of mast cells in progressive renal diseases*. J Am Soc Nephrol 2008;19:2254-2261.
7. Heldin CH, Westermark B. *Mechanism of action and in vivo role of platelet-derived growth factor*. Physiol Rev 1999;79:1283-1316.
8. Boschi A, Daumerie C, et al. *Quantification of cells expressing the thyrotropin receptor in extraocular muscles in thyroid associated orbitopathy*. Br J Ophthalmol 2005;89:724-729.
9. Chen MH, Liao SL, et al. *Role of macrophage infiltration in the orbital fat of patients with Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2008;69:332-337.
10. Quadbeck B, Stucke M, et al. *Dysregulation of TNF/TNFR superfamily members: a systemic link between intra- and extrathyroidal manifestations in Graves' disease*. Scand J Immunol 2006;64:523-530.
11. Eckstein AK, Quadbeck B, et al. *Thyroid associated ophthalmopathy: evidence for CD4(+) gammadelta T cells; de novo differentiation of RFD7(+) macrophages, but not of RFD1(+) dendritic cells; and loss of gammadelta and alphabeta T cell receptor expression*. Br J Ophthalmol 2004;88:803-808.
12. Bonner JC, Badgett A, et al. *Inhibition of platelet-derived growth factor-BB-induced fibroblast proliferation by plasmin-activated alpha 2-macroglobulin is mediated via an alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein-dependent mechanism*. J Biol Chem 1995;270:6389-6395.
13. Lei H, Kazlauskas A. *Growth factors outside of the platelet-derived growth factor (PDGF) family employ reactive oxygen species/Src family kinases to activate PDGF receptor alpha and thereby promote proliferation and survival of cells*. J Biol Chem 2009;284:6329-6336.
14. Russo K, Ragone R, et al. *Platelet-derived growth factor-BB and basic fibroblast growth factor directly interact in vitro with high affinity*. J Biol Chem 2002;277:1284-1291.
15. Wang X, Waldeck H, et al. *The effects of TGF-alpha, IL-1beta and PDGF on fibroblast adhesion to ECM-derived matrix and KGF gene expression*. Biomaterials 2010;31:2542-2548.
16. Ng F, Boucher S, et al. *PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages*. Blood 2008;112:295-307.
17. Kordes C, Brookmann S, et al. *Differential and synergistic effects of platelet-derived growth factor-BB and transforming growth factor-beta1 on activated pancreatic stellate cells*. Pancreas 2005;31:156-167.
18. Kunz D, Walker G, et al. *Platelet-derived growth factor and fibroblast growth factor differentially regulate interleukin 1beta- and cAMP-induced nitric oxide synthase expression in rat renal mesangial cells*. J Clin Invest 1997;100:2800-2809.
19. Lindroos PM, Coin PG, et al. *Interleukin 1 beta (IL-1 beta) and the IL-1 beta-alpha 2-macroglobulin complex upregulate the platelet-derived growth factor alpha-receptor on rat pulmonary fibroblasts*. Am J Respir Cell Mol Biol 1995;13:455-465.
20. Kolb M, Margetts PJ, et al. *Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis*. J Clin Invest 2001;107:1529-1536.
21. Raines EW, Dower SK, et al. *Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA*. Science 1989;243:393-396.
22. Suzuki H, Shibano K, et al. *Interferon-gamma modulates messenger RNA levels of c-sis (PDGF-B chain), PDGF-A chain, and IL-1 beta genes in human vascular endothelial cells*. Am J Pathol 1989;134:35-43.

23. Shaw RJ, Benedict SH, et al. *Pathogenesis of pulmonary fibrosis in interstitial lung disease. Alveolar macrophage PDGF(B) gene activation and up-regulation by interferon gamma*. Am Rev Respir Dis 1991;143:167-173.
24. Liu JY, Brass DM, et al. *TNF-alpha receptor knockout mice are protected from the fibroproliferative effects of inhaled asbestos fibers*. Am J Pathol 1998;153:1839-1847.
25. Hajjar KA, Hajjar DP, et al. *Tumor necrosis factor-mediated release of platelet-derived growth factor from cultured endothelial cells*. J Exp Med 1987;166:235-245.
26. Daniel TO, Gibbs VC, et al. *Agents that increase cAMP accumulation block endothelial c-sis induction by thrombin and transforming growth factor-beta*. J Biol Chem 1987;262:11893-11896.
27. Bonner JC. *Regulation of PDGF and its receptors in fibrotic diseases*. Cytokine Growth Factor Rev 2004;15:255-273.
28. Zhang L, Bowen T, et al. *Thyrotropin receptor activation increases hyaluronan production in preadipocyte fibroblasts: contributory role in hyaluronan accumulation in thyroid dysfunction*. J Biol Chem 2009;284:26447-26455.
29. Kumar S, Schiefer R, et al. *A stimulatory thyrotropin receptor antibody (M22) and thyrotropin increase interleukin-6 expression and secretion in Graves' orbital preadipocyte fibroblasts*. Thyroid 2010;20:59-65.
30. Wakelkamp IM, Bakker O, et al. *TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients*. Clin Endocrinol (Oxf) 2003;58:280-287.
31. Gerding MN, van der Meer JW, et al. *Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2000;52:267-271.
32. Wiersinga WM. *Autoimmunity in Graves' Ophthalmopathy: The Result of an Unfortunate Marriage Between TSH Receptors and IGF-1 Receptors?* J Clin Endocrinol Metab 2011.
33. Kahaly G, Hansen C, et al. *Immunohistochemical staining of retrobulbar adipose tissue in Graves' ophthalmopathy*. Clin Immunol Immunopathol 1994;73:53-62.
34. Pritchard J, Han R, et al. *Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway*. J Immunol 2003;170:6348-6354.
35. Kumar S, Bahn RS. *Relative overexpression of macrophage-derived cytokines in orbital adipose tissue from patients with graves' ophthalmopathy*. J Clin Endocrinol Metab 2003;88:4246-4250.
36. Hiromatsu Y, Yang D, et al. *Cytokine profiles in eye muscle tissue and orbital fat tissue from patients with thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 2000;85:1194-1199.
37. Kobayashi Y. *The role of chemokines in neutrophil biology*. Front Biosci 2008;13:2400-2407.
38. Castellani ML, De Lutiis MA, et al. *Impact of RANTES, MCP-1 and IL-8 in mast cells*. J Biol Regul Homeost Agents 2010;24:1-6.
39. Mukaida N. *Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases*. Am J Physiol Lung Cell Mol Physiol 2003;284:L566-577.
40. Jyonouchi SC, Valyasevi RW, et al. *Interleukin-6 stimulates thyrotropin receptor expression in human orbital preadipocyte fibroblasts from patients with Graves' ophthalmopathy*. Thyroid 2001;11:929-934.
41. Bahn RS. *Thyrotropin receptor expression in orbital adipose/connective tissues from patients with thyroid-associated ophthalmopathy*. Thyroid 2002;12:193-195.
42. Hirano T. *Interleukin 6 and its receptor: ten years later*. Int Rev Immunol 1998;16:249-284.
43. Pappa A, Calder V, et al. *Analysis of extraocular muscle-infiltrating T cells in thyroid-associated ophthalmopathy (TAO)*. Clin Exp Immunol 1997;109:362-369.
44. McLachlan SM, Prummel MF, et al. *Immunoglobulin A in Graves' orbital tissue: deoxyribonucleic acid amplification by polymerase chain reaction*. J Endocrinol Invest 1994;17:247-252.
45. Pritchard J, Horst N, et al. *Igs from patients with Graves' disease induce the expression of T cell chemoattractants in their fibroblasts*. J Immunol 2002;168:942-950.
46. Schall TJ, Bacon K, et al. *Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES*. Nature 1990;347:669-671.
47. Deshmane SL, Kremlev S, et al. *Monocyte chemoattractant protein-1 (MCP-1): an overview*. J Interferon Cytokine Res 2009;29:313-326.

48. Proost P, Wuyts A, et al. *Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1*. J Leukoc Biol 1996;59:67-74.
49. Chen MH, Chen MH, et al. *Role of macrophage infiltration in the orbital fat of patients with Graves' ophthalmopathy*. Clin Endocrinol (Oxf) 2008;69:332-337.
50. Wynn TA. *Cellular and molecular mechanisms of fibrosis*. J Pathol 2008;214:199-210.
51. Mutsaers SE, Bishop JE, et al. *Mechanisms of tissue repair: from wound healing to fibrosis*. Int J Biochem Cell Biol 1997;29:5-17.
52. Heymann F, Trautwein C, et al. *Monocytes and macrophages as cellular targets in liver fibrosis*. Inflamm Allergy Drug Targets 2009;8:307-318.
53. Baffy G. *Kupffer cells in non-alcoholic fatty liver disease: the emerging view*. J Hepatol 2009;51:212-223.
54. Conti P, Boucher W, et al. *Monocyte chemotactic protein-1 provokes mast cell aggregation and [3H]5HT release*. Immunology 1995;86:434-440.
55. Zhang H, Yang H, et al. *Induction of IL-13 production and upregulated expression of protease activated receptor-1 by RANTES in a mast cell line*. Cytokine 2011;53:231-238.
56. Franceschini B, Ceva-Grimaldi G, et al. *The complex functions of mast cells in chronic human liver diseases*. Dig Dis Sci 2006;51:2248-2256.
57. Gruber BL. *Mast cells in the pathogenesis of fibrosis*. Curr Rheumatol Rep 2003;5:147-153.
58. Garbuzenko E, Nagler A, et al. *Human mast cells stimulate fibroblast proliferation, collagen synthesis and lattice contraction: a direct role for mast cells in skin fibrosis*. Clin Exp Allergy 2002;32:237-246.
59. Walls AF, Bennett AR, et al. *Mast cell tryptase and histamine concentrations in bronchoalveolar lavage fluid from patients with interstitial lung disease*. Clin Sci (Lond) 1991;81:183-188.
60. Lundequist A, Pejler G. *Biological implications of preformed mast cell mediators*. Cell Mol Life Sci 2011;68:965-975.
61. Jutel M, Akdis M, et al. *Histamine, histamine receptors and their role in immune pathology*. Clin Exp Allergy 2009;39:1786-1800.
62. Wegelius O, Asboe-Hansen G, et al. *Retrobulbar connective tissue changes in malignant exophthalmos*. Acta Endocrinol (Copenh) 1957;25:452-456.
63. Eckstein A, Mann K, et al. *[Role of TSH receptor autoantibodies for the diagnosis of Graves' disease and for the prediction of the course of hyperthyroidism and ophthalmopathy. Recommendations of the Thyroid Section of the German Society of Endocrinology]Bedeutung der TSH-Rezeptor-Antikorper fur die Diagnose des Morbus Basedow sowie die Prognoseabschatzung der Schilddrusenuberfunktion und der endokrinen Orbitopathie. Stellungnahme der Sektion Schilddruse der DGE*. Med Klin (Munich) 2009;104:343-348.
64. Sorisky A, Pardasani D, et al. *Evidence of adipocyte differentiation in human orbital fibroblasts in primary culture*. J Clin Endocrinol Metab 1996;81:3428-3431.
65. Valyasevi RW, Erickson DZ, et al. *Differentiation of human orbital preadipocyte fibroblasts induces expression of functional thyrotropin receptor*. J Clin Endocrinol Metab 1999;84:2557-2562.
66. Bell A, Gagnon A, et al. *Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts*. Am J Physiol Cell Physiol 2000;279:C335-340.
67. Valyasevi RW, Harteneck DA, et al. *Stimulation of adipogenesis, peroxisome proliferator-activated receptor-gamma (PPARgamma), and thyrotropin receptor by PPARgamma agonist in human orbital preadipocyte fibroblasts*. J Clin Endocrinol Metab 2002;87:2352-2358.
68. Artemenko Y, Gagnon A, et al. *Anti-adipogenic effect of PDGF is reversed by PKC inhibition*. J Cell Physiol 2005;204:646-653.
69. Krieger-Brauer HI, Kather H. *Antagonistic effects of different members of the fibroblast and platelet-derived growth factor families on adipose conversion and NADPH-dependent H2O2 generation in 3T3 L1-cells*. Biochem J 1995;307 (Pt 2):549-556.
70. Molgat AS, Gagnon A, et al. *Preadipocyte apoptosis is prevented by macrophage-conditioned medium in a PDGF-dependent manner*. Am J Physiol Cell Physiol 2009;296:C757-765.
71. Bachmeier M, Loffler G. *Influence of growth factors on growth and differentiation of 3T3-L1 preadipocytes in serum-free conditions*. Eur J Cell Biol 1995;68:323-329.

72. Chang HY, Chi JT, et al. *Diversity, topographic differentiation, and positional memory in human fibroblasts*. Proc Natl Acad Sci U S A 2002;99:12877-12882.
73. Kazim M, Goldberg RA, et al. *Insights into the pathogenesis of thyroid-associated orbitopathy: evolving rationale for therapy*. Arch Ophthalmol 2002;120:380-386.
74. Cao HJ, Wang HS, et al. *Activation of human orbital fibroblasts through CD40 engagement results in a dramatic induction of hyaluronan synthesis and prostaglandin endoperoxide H synthase-2 expression. Insights into potential pathogenic mechanisms of thyroid-associated ophthalmopathy*. J Biol Chem 1998;273:29615-29625.
75. Hwang CJ, Afifiyan N, et al. *Orbital fibroblasts from patients with thyroid-associated ophthalmopathy overexpress CD40: CD154 hyperinduces IL-6, IL-8, and MCP-1*. Invest Ophthalmol Vis Sci 2009;50:2262-2268.
76. Young DA, Evans CH, et al. *Leukoregulin induction of protein expression in human orbital fibroblasts: evidence for anatomical site-restricted cytokine-target cell interactions*. Proc Natl Acad Sci U S A 1998;95:8904-8909.
77. Kaback LA, Smith TJ. *Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy*. J Clin Endocrinol Metab 1999;84:4079-4084.
78. Metcalfe RA, Weetman AP. *Stimulation of extraocular muscle fibroblasts by cytokines and hypoxia: possible role in thyroid-associated ophthalmopathy*. Clin Endocrinol (Oxf) 1994;40:67-72.
79. Han R, Tsui S, et al. *Up-regulation of prostaglandin E2 synthesis by interleukin-1beta in human orbital fibroblasts involves coordinate induction of prostaglandin-endoperoxide H synthase-2 and glutathione-dependent prostaglandin E2 synthase expression*. J Biol Chem 2002;277:16355-16364.
80. Muhlberg T, Heberling HJ, et al. *Detection and modulation of interleukin-1 receptor antagonist messenger ribonucleic acid and immunoreactivity in Graves' orbital fibroblasts*. Invest Ophthalmol Vis Sci 1997;38:1018-1028.
81. Muhlberg T, Joba W, et al. *Interleukin-1 receptor antagonist ribonucleic acid and protein expression by cultured Graves' and normal orbital fibroblasts is differentially modulated by dexamethasone and irradiation*. J Clin Endocrinol Metab 2000;85:734-742.
82. Khoo TK, Coenen MJ, et al. *Evidence for enhanced Thy-1 (CD90) expression in orbital fibroblasts of patients with Graves' ophthalmopathy*. Thyroid 2008;18:1291-1296.
83. Heufelder AE, Bahn RS. *Modulation of Graves' orbital fibroblast proliferation by cytokines and glucocorticoid receptor agonists*. Invest Ophthalmol Vis Sci 1994;35:120-127.
84. Korducki JM, Loftus SJ, et al. *Stimulation of glycosaminoglycan production in cultured human retroocular fibroblasts*. Invest Ophthalmol Vis Sci 1992;33:2037-2042.
85. Cawood TJ, Moriarty P, et al. *The effects of tumour necrosis factor-alpha and interleukin1 on an in vitro model of thyroid-associated ophthalmopathy; contrasting effects on adipogenesis*. Eur J Endocrinol 2006;155:395-403.
86. Weetman AP. *Determinants of autoimmune thyroid disease*. Nat Immunol 2001;2:769-770.
87. Valyasevi RW, Jyonouchi SC, et al. *Effect of tumor necrosis factor-alpha, interferon-gamma, and transforming growth factor-beta on adipogenesis and expression of thyrotropin receptor in human orbital preadipocyte fibroblasts*. J Clin Endocrinol Metab 2001;86:903-908.
88. Yan SX, Wang Y. *Inhibitory effects of Triptolide on interferon-gamma-induced human leucocyte antigen-DR, intercellular adhesion molecule-1, CD40 expression on retro-ocular fibroblasts derived from patients with Graves' ophthalmopathy*. Clin Experiment Ophthalmol 2006;34:265-271.
89. Savikko J, von Willebrand E. *Coexpression of platelet-derived growth factors AA and BB and their receptors during monocytic differentiation*. Transplant Proc 2001;33:2307-2308.
90. Shure D, Senior RM, et al. *PDGF AA homodimers are potent chemoattractants for fibroblasts and neutrophils, and for monocytes activated by lymphocytes or cytokines*. Biochem Biophys Res Commun 1992;186:1510-1514.
91. Krettek A, Ostergren-Lunden G, et al. *Expression of PDGF receptors and ligand-induced migration of partially differentiated human monocyte-derived macrophages. Influence of IFN-gamma and TGF-beta*. Atherosclerosis 2001;156:267-275.
92. de Parseval N, Fichelson S, et al. *Expression of functional beta-platelet-derived growth factor receptors on hematopoietic cell lines*. Cytokine 1993;5:8-15.

93. Daynes RA, Dowell T, et al. *Platelet-derived growth factor is a potent biologic response modifier of T cells*. J Exp Med 1991;174:1323-1333.

94. Pantazis P, Kharbanda S, et al. *Coexpression of the genes for platelet-derived growth factor B-chain receptor and macrophage colony-stimulating factor 1 receptor during monocytic differentiation*. Proc Natl Acad Sci U S A 1991;88:2481-2485.

95. Inaba T, Shimano H, et al. *Expression of platelet-derived growth factor beta receptor on human monocyte-derived macrophages and effects of platelet-derived growth factor BB dimer on the cellular function*. J Biol Chem 1993;268:24353-24360.

96. Siegbahn A, Hammacher A, et al. *Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes*. J Clin Invest 1990;85:916-920.

97. Aniszewski JP, Valyasevi RW, et al. *Relationship between disease duration and predominant orbital T cell subset in Graves' ophthalmopathy*. J Clin Endocrinol Metab 2000;85:776-780.

98. Forster G, Otto E, et al. *Analysis of orbital T cells in thyroid-associated ophthalmopathy*. Clin Exp Immunol 1998;112:427-434.

99. McLachlan SM, Prummel MF, et al. *Cell-mediated or humoral immunity in Graves' ophthalmopathy? Profiles of T-cell cytokines amplified by polymerase chain reaction from orbital tissue*. J Clin Endocrinol Metab 1994;78:1070-1074.

100. Cawood T, Moriarty P, et al. *Recent developments in thyroid eye disease*. BMJ 2004;329:385-390.

101. Bartalena L, Baldeschi L, et al. *Consensus statement of the European Group on Graves' orbitopathy (EUGOGO) on management of GO*. Eur J Endocrinol 2008;158:273-285.

102. Brenner RE, Felger D, et al. *Effects of dexamethasone on proliferation, chemotaxis, collagen I, and fibronectin-metabolism of human fetal lung fibroblasts*. Pediatr Pulmonol 2001;32:1-7.

103. Dik WA, Versnel MA, et al. *Dexamethasone treatment does not inhibit fibroproliferation in chronic lung disease of prematurity*. Eur Respir J 2003;21:842-847.

104. Warshamana GS, Martinez S, et al. *Dexamethasone activates expression of the PDGF-alpha receptor and induces lung fibroblast proliferation*. Am J Physiol 1998;274:L499-507.

105. Paridaens D, van den Bosch WA, et al. *The effect of etanercept on Graves' ophthalmopathy: a pilot study*. Eye (Lond) 2005;19:1286-1289.

106. Hegedus L, Smith TJ, et al. *Targeted biological therapies for Graves' disease and thyroid-associated ophthalmopathy. Focus on B-cell depletion with Rituximab*. Clin Endocrinol (Oxf) 2011;74:1-8.

107. van Steensel L, Paridaens D, et al. *Platelet-derived growth factor-BB: a stimulus for cytokine production by orbital fibroblasts in Graves' Ophthalmopathy*. Invest Ophthalmol Vis Sci 2009.

108. van Steensel L, Paridaens D, et al. *Imatinib mesylate and AMN107 inhibit PDGF-signaling in orbital fibroblasts: a potential treatment for Graves' ophthalmopathy*. Invest Ophthalmol Vis Sci 2009;50:3091-3098.

109. van Daele PL, Dik WA, et al. *Is imatinib mesylate a promising drug in systemic sclerosis?* Arthritis Rheum 2008;58:2549-2552.

110. Rhee CK, Lee SH, et al. *Effect of Nilotinib on Bleomycin-Induced Acute Lung Injury and Pulmonary Fibrosis in Mice*. Respiration 2011.

111. Shaker ME, Zalata KR, et al. *Comparison of imatinib, nilotinib and silymarin in the treatment of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis*. Toxicol Appl Pharmacol 2011;252:165-175.

112. Akhmetshina A, Dees C, et al. *Dual inhibition of c-abl and PDGF receptor signaling by dasatinib and nilotinib for the treatment of dermal fibrosis*. FASEB J 2008;22:2214-2222.

113. Saglio G, Kim DW, et al. *Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia*. N Engl J Med 2010;362:2251-2259.

114. McClelland CM, Harocopos GJ, et al. *Periorbital edema secondary to imatinib mesylate*. Clin Ophthalmol 2010;4:427-431.

115. de Groot JW, Zonnenberg BA, et al. *Imatinib induces hypothyroidism in patients receiving levothyroxine*. Clin Pharmacol Ther 2005;78:433-438.

116. Kantarjian H, Giles F, et al. *Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL*. N Engl J Med 2006;354:2542-2551.

117. Kantarjian HM, Giles F, et al. *Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance*. Blood 2007;110:3540-3546.

118. Koren-Michowitz M, le Coutre P, et al. *Activity and tolerability of nilotinib: a retrospective multicenter analysis of chronic myeloid leukemia patients who are imatinib resistant or intolerant*. Cancer 2010;116:4564-4572.

119. Kim TD, Schwarz M, et al. *Thyroid dysfunction caused by second-generation tyrosine kinase inhibitors in Philadelphia chromosome-positive chronic myeloid leukemia*. Thyroid 2010;20:1209-1214.

Chapter 10

Summary

Populair-wetenschappelijke samenvatting

SUMMARY

Graves' disease (GD) is an autoimmune disease that is characterized by the presence of autoantibodies that stimulate the thyroid stimulating hormone receptor (TSHR) on thyrocytes, which causes hyperthyroidism. Graves' ophthalmopathy (GO) is the orbital manifestation of GD and is caused by orbital tissue expansion within the limited bony orbit. Clinical signs of GO comprise proptosis, upper eyelid retraction, edema, erythema of the peri-orbital tissues and conjunctivae, and strabismus. The (immuno)pathophysiological processes that lead to GO has three main components: 1) autoimmunity, 2) inflammation, and 3) tissue remodelling. Orbital fibroblasts are centrally involved in all these main (immuno)pathophysiological components of GO.

1) Orbital fibroblasts and autoimmunity: TSHR autoantibody levels and orbital TSHR expression correlate with GO disease activity. Orbital fibroblasts express increased levels of TSHR and can thus be targeted by TSHR stimulating autoantibodies. Orbital fibroblasts that are activated by TSHR stimulating antibodies produce cytokines that shape the inflammatory environment in GO. Moreover, TSHR activation enhances synthesis of the extracellular matrix (ECM) component hyaluronan by orbital fibroblasts. Thus, TSHR stimulating autoantibodies drive activation of orbital fibroblasts in GO, which leads to recruitment and activation of leukocytes and to ECM deposition within the orbital tissue.

2) Orbital fibroblasts and inflammation: Especially during the early phase of GO there is an active inflammatory process within the orbit. This inflammatory process is characterized by cytokine/chemokine production that drive the recruitment and activation of immune cells such as T cells, B cells, monocytes, macrophages and mast cells. The cytokines/chemokines however also stimulate orbital fibroblast activity. Activated orbital fibroblasts themselves also produce cytokines/chemokines and thereby regulate the recruitment and activation of leukocytes into the orbital tissue. Orbital fibroblasts play thus an important role in shaping the orbital inflammatory environment in GO.

3) Orbital fibroblasts and tissue remodelling: Orbital tissue expansion and remodelling within the limited bony orbit is an important determinant of the clinical signs of GO. Orbital fibroblasts are centrally involved in this. Firstly, orbital fibroblasts are very sensitive to mitogenic stimuli. Secondly, orbital fibroblasts are the main producers of ECM, of which hyaluronan is the major component in orbital tissues and is produced in excess in GO. Hyaluronan has strong water-binding capacity, which clearly contributes to orbital tissue expansion and edema in GO. Finally, orbital fibroblasts can easily differentiate into (large) adipocytes. Together, the processes of proliferation, hyaluronan synthesis and adipogenesis determine the amount of orbital tissue expansion in GO.

Orbital fibroblasts are unique in their exaggerated responses when activated by certain stimuli (e.g. IL-1β) compared to fibroblasts from other anatomical locations. Orbital

fibroblasts do however also share features with fibroblasts from other organ systems in which inflammation and tissue remodelling processes lead to fibrotic changes. The platelet-derived growth factor (PDGF) family is critically involved in the activation of fibroblasts in healing responses, but also in pathological conditions such as fibro-proliferative disorders of the lungs and skin. Because GO shares many features (inflammation, enhanced fibroblast proliferation and ECM synthesis) with such fibro-proliferative disorders of other organ systems, PDGF can be expected to contribute to GO development.

A prerequisite for mediators to regulate orbital fibroblast activity in GO is that they should be present in orbital tissue from GO patients. The studies in this thesis show that the PDGF-A and PDGF-B chains, but not the PDGF-C and PDGF-D chains nor PDGF-Rα and PDGF-Rβ, are increased in orbital tissue from GO patients. Remarkably, the PDGF-A and PDGF-B chain are equally increased in GO patients with active or burnt-out disease, suggesting involvement throughout the whole disease course. Monocytes, macrophages and mast cells were indentified as producers of PDGF in the orbital tissues, while T cells and B cells did not. Increased expression of the PDGF-A and PDGF-B chains in GO orbital tissue will lead to increased formation of the biologically active PDGF-AA, PDGF-AB and PDGF-BB isoforms. Of these isoforms especially PDGF-AB and PDGF-BB appeared to increase orbital fibroblast activity.

PDGF-AB and PDGF-BB potently increased TSHR expression on orbital fibroblasts, while no such effect was observed on dermal fibroblasts. More importantly, the PDGF-induced elevation of TSHR expression increased the capacity of orbital fibroblasts to produce cytokines/chemokines (i.e. IL-6, IL-8, CCL2 and CCL5, but not CCL7) upon stimulation with TSH or immunoglobulins from GD patients (GD-IgG). Blocking studies with a TSHR-inhibitory antibody or a cAMP-signalling inhibitor confirmed that the effect of GD-IgG was mediated through TSHR activation, most likely by TSHR stimulating autoantibodies. This indicates that TSHR stimulating autoantibody-induced production of cytokines/chemokines is involved in leukocyte recruitment and activation in the orbital tissue from GO patients. This process is augmented by PDGF, suggesting that specific PDGF isoforms enhance the immuno-pathogenic activity of THSR stimulating autoantibodies in GO.

It was also found that PDGF isoforms stimulate the production of various cytokines and chemokines (i.e. IL-6, IL-8, CCL2, CCL5 and CCL7) by orbital fibroblasts in a nuclear factor-κB dependent way. The combination of these cytokines/chemokines enable recruitment and activation of B cells, T cells, monocytes, macrophages and mast cells that are typically present in orbital tissue from GO patients. PDGF isoforms play thus a role in the inflammatory process in GO by stimulating cytokine/chemokine production by orbital fibroblasts.

Specific PDGF isoforms also stimulated orbital fibroblast proliferation and hyaluronan

production. The latter was associated with increased expression of especially the hyaluronan synthases (HAS) 1 and 2. TGF-β_1, which was also found at increased levels in GO orbital tissue, stimulated hyaluronan production by orbital fibroblasts, but not orbital fibroblast proliferation.

Treatment of GO predominantly depends on corticosteroid treatment or orbital decompression surgery. In the last two decades medical treatment of GO has not improved much, although promising results have been obtained with B-cell and TNF-directed therapies. Considering the orbital fibroblast activating properties of PDGF-isoforms, the therapeutic potential of targeting PDGF was investigated using imatinib mesylate, a PDGF receptor tyrosine kinase inhibitor. Imatinib mesylate efficiently blocked PDGF-receptor phosphorylation in orbital fibroblasts and reduced PDGF-induced proliferation, hyaluronan production, cytokine/chemokine production and TSHR expression by orbital fibroblasts.

Although these data indicate that targeting the PDGF system can be a promising approach in the treatment of GO it should be realized that these data were obtained in an *in vitro* cell culture system with a single mediator. Such a model clearly lacks the complexity of the altered orbital tissue environment from GO patients. Therefore, the therapeutic potential of imatinib mesylate was also tested in a whole orbital tissue culture system, which more adequately reflects the complex pathophysiological conditions in GO orbital tissue. This pre-clinical *ex vivo* approach revealed that imatinib mesylate efficiently inhibited the production of IL-6 and hyaluronan by GO orbital tissues. Moreover, the inhibition of hyaluronan correlated positively and significantly with patient-specific orbital PDGF-B mRNA expression. Based on these findings, clinical trials are warranted that investigate neutralization of PDGF activity in GO patients. For this purpose the tyrosine kinase inhibitor nilotinib would be preferred above imatinib mesylate, as the former is equally effective in preventing PDGF-receptor activation but has considerable less side effects.

In conclusion, the studies in this thesis demonstrate that PDGF-A and PDGF-B chains are increased in the orbital tissue of GO patients. Especially PDGF-B containing PDGF isoforms potently activate orbital fibroblasts, leading to increased orbital fibroblast proliferation, hyaluronan production, cytokine production and TSHR expression on orbital fibroblasts. PDGF-B chain containing PDGF isoforms thus influence the autoimmune, the inflammatory and tissue remodelling components of GO and can thus be regarded as master regulators of the (immuno)pathopysiology of GO and suitable targets for the treatment of GO.

POPULAIR-WETENSCHAPPELIJKE SAMENVATTING

De ziekte van Graves wordt gekarakteriseerd door een overmaat aan schildklierhormoon. Dit wordt veroorzaakt door stimulerende autoantistoffen tegen de schildklier-stimulerend hormoon receptor (TSHR), waardoor de schildklier wordt gestimuleerd tot het produceren van meer schildklierhormoon. In 25-50% van de Graves' patiënten manifesteren zich ook problemen aan de ogen. Dit ziektebeeld wordt Graves' ophthalmopathie (GO) genoemd.

GO kan zich uiten door oedeem (vocht) van de oogleden, een toename van orbitaalvet (oogkasvet) en een verdikking van de oogspieren. Het ziektebeloop van GO kan heel verschillend zijn, maar in het algemeen is er een eerste fase van actieve ontsteking die gevolgd wordt door een lange periode waarin de ontsteking verdwijnt maar waarin juist fibrosering (overmatig bindweefsel vorming) een rol speelt. Dit alles leidt tot toename van het weefsel in de oogkas en vervolgens uitpuiling van de ogen (proptose). In ernstige gevallen kan dit resulteren in schade aan de oogzenuw of het hoornvlies.

Bij de meeste patiënten verloopt de oogziekte mild en dooft deze na verloop van tijd uit. Ongeveer 5% van de patiënten kent een ernstig verloop waarvoor systemische behandeling met corticosteroïden noodzakelijk is. Deze therapie is echter alleen toepasbaar in het stadium van actieve ontsteking en niet altijd succesvol, wat soms chirurgisch ingrijpen nodig maakt. Voor het herstel van resterende ziekteverschijnselen blijkt chirurgisch ingrijpen ook vaak nog nodig. Medische behandelingsmogelijkheden van GO zijn beperkt, mede door een gebrek aan inzicht in de (moleculaire) mechanismen die het ontstaan en het beloop van GO bepalen.

Geactiveerde fibroblasten (bindweefselcellen) in de oogkas vervullen een sleutelrol in het ontstaan en de ernst van GO. Daarnaast zijn allerlei ontstekingscellen aanwezig in het oogkasweefsel van GO patiënten, waaronder T cellen, B cellen, monocyten, macrofagen en mestcellen. Het afweersysteem speelt dus ook een belangrijke rol in het ziekteproces van GO. Belangrijke processen die leiden tot de ontwikkeling van GO en waarbij activiteit van fibroblasten in de oogkas een belangrijke rol speelt zijn:

- Ontsteking: fibroblasten in de oogkas worden geactiveerd door allerlei ontstekingsfactoren (zoals cytokinen, groeifactoren en autoantistoffen (bijv. tegen de TSHR)) die door ontstekingscellen geproduceerd worden. Geactiveerde fibroblasten in de oogkas produceren zelf echter ook allerlei cytokinen. Deze cytokinen zorgen voor het aantrekken en activeren van ontstekingscellen in het oogkasweefsel en onderhouden daarmee de ontstekingsreactie in de oogkas.
- Fibrosering: GO wordt gekenmerkt door een toename en andere opbouw van het weefsel in de oogkas. Dit wordt onder andere veroorzaakt door versterkte proliferatie (celdeling) van fibroblasten in de oogkas. Daarnaast produceren fibroblasten uit de oogkas verhoogde hoeveelheden bindweefselmoleculen. Hyaluronan, het belangrijkste bindweefselmolecuul in GO, draagt door zijn unieke capaciteit om water te binden in belangrijke mate bij aan

de ontwikkeling van oedeem (vocht) achter en rondom de ogen.

Kennis van factoren die een belangrijke rol spelen in de activatie van fibroblasten in de oogkas is essentieel om meer begrip te krijgen van het ziekteproces in GO. Dergelijke kennis kan potentieel ook leiden tot nieuwe aangrijpingspunten voor de behandeling van GO. Platelet-derived growth factor (PDGF) is een familie van groeifactoren waarvan bekend is dat ze een belangrijke rol speelt in verschillende aandoeningen, zoals systemische sclerose en longfibrose, die gekenmerkt worden door overmatige bindweefselvorming.

De studies in dit proefschrift tonen aan dat PDGF-A en PDGF-B moleculen verhoogd aanwezig zijn in oogkasweefsel van GO patiënten, zowel in patiënten met actieve als ook met uitgebluste GO. Ontstekingscellen zoals monocyten, macrofagen en mestcellen blijken medeverantwoordelijk voor de productie van PDGF in de oogkas van GO patiënten. PDGF-A en PDGF-B kunnen leiden tot biologisch actieve PDGF-AA, PDGF-AB en PDGF-BB moleculen. Van deze drie verschillende PDGF moleculen bleek PDGF-BB en daarna PDGF-AB de krachtigste activator van fibroblasten van de oogkas te zijn, terwijl PDGF-AA nauwelijks leidde tot activatie van fibroblasten in de oogkas.

Dit proefschrift toont verder aan dat:

- Stimulatie met PDGF leidt tot meer TSHR op fibroblasten van de oogkas, waardoor deze gevoeliger worden voor TSHR stimulerende autoantistoffen. Dit resulteert in een hogere productie van allerlei cytokinen door deze fibroblasten.
- Stimulatie met PDGF activeert de fibroblasten van de oogkas ook direct, waardoor de orbitale fibroblasten verschillende ontstekingsfactoren gaat produceren, waaronder de zogenoemde IL-6, IL-8, CCL2, CCL5 en CCL7. Deze groep van ontstekingsfactoren is in staat om een variëteit aan ontstekingscellen te activeren en naar het oogkasweefsel te trekken.
- Stimulatie met PDGF leidt ook tot verhoogde celdeling en productie van hyaluronan door fibroblasten van de oogkas.

Nieuwe therapeutische mogelijkheden zijn gewenst voor de behandeling van GO patiënten. Daarom werd ook onderzocht of de effecten van PDGF geremd konden worden door gebruik te maken van een specifieke PDGF remmer zoals imatinib mesylaat.

Imatinib mesylaat bleek in staat om de door PDGF gestimuleerde TSHR expressie en productie van ontstekingsfactoren door fibroblasten van de oogkas te remmen. Ook remde het het de toegenomen celdeling en productie van hyaluronan door fibroblasten uit de oogkas. Het ziekteproces in de oogkas van GO patiënten is echter complex en omvat veel meer factoren en cellen dan alleen PDGF en fibroblasten. Imatinib mesylaat bleek ook in kweken van gehele weefsels uit de oogkas van GO patiënten de productie van IL-6 en hyaluronan productie te remmen.

Samenvattend toont dit proefschrift aan dat de PDGF-A en PDGF-B moleculen verhoogd zijn het oogkasweefsel van GO patiënten. PDGF (vooral PDGF-BB en PDGF-AB) stimuleert TSHR expressie op fibroblasten uit de oogkas, verhoogt de productie van ontstekingsfactoren en hyaluronan door fibroblasten uit de oogkas en zorgt ook voor een versnelde celdeling van deze fibroblasten. PDGF speelt dus een centrale rol in GO door de stimulatie van zowel ontstekings- als fibroseringsprocessen in de oogkas. Het remmen van het PDGF systeem lijkt een veelbelovende optie voor de behandeling van GO patiënten. Klinische studies met patiënten zullen moeten uitwijzen of deze tactiek inderdaad effectief is in GO patiënten.

Chapter 11

Abbreviations
Dankwoord
Curriculum Vitae
Publications
PhD portfolio

ABREVIATIONS

ABC	avidin-biotin complex
AEC	3-amino-9-ethyl-carbazole
AP	alkaline phosphatase
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAS	clinical activity score
CD	cluster of differentiation
CCL	chemokine (C-C-motif) ligand
CTLA4	cytotoxic T-lymphocyte antigen 4
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DMEM	Dulbecco’s Modified Eagle’s Medium
ECM	extracellular matrix
ELAM	endothelial-leukocyte adhesion molecule 1 (E-selectin)
ELISA	enzyme-linked immunosorbent assay
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
fT4	free thyroxine
FasL	Fas ligand
FCS	fetal calf serum
FOXP3	forkhead box P3
GD	Graves’ disease
GO	Graves’ ophthalmopathy
GM-CSF	granulocyte macrophage colony-stimulating factor
GR	glucocorticoid receptor
HLA	humal leukocyte antigen
HRP	horseradish peroxidase
HAS	hyaluronan synthase
HFL-1	human fetal lung fibroblast-1
IBMX	isobutylmethylxanthine
ICAM	intercellular adhesion molecule
IFN	interferon
IGF-1	insulin-like growth factor-1
IGF-1R	insulin-like growth factor-1 receptor
IL	interleukin
IL-6R	interleukin-6 receptor
IL-1Ra	interleukin-1 receptor antagonist
Ig	immunoglobulin

LFA	lymphocyte function associated protein-1
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
M-CSF	macrophage colony-stimulating factor
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinase
MHC	major histocompatibility complex
NF-κB	nuclear factor-kappa B
OD	optical density
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGF-R	platelet-derived growth factor receptor
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PI3	Phosphatidylinositol 3
PPAR	peroxisome proliferator-activated receptor
PTGHS-2	prostaglandin-endoperoxide synthase-2
PTPN22	protein tyrosine phosphatase, non receptor type 22
RANTES	regulated upon activation, normal T cell expressed, and secreted
RQ-PCR	real-time quantitative – polymerase chain reaction
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
sst	somatostatin receptor subtype
TCR	T cell receptor
Tg	thyroglobulin
TGF	transforming growth factor
Th	T-helper
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
Treg	T regulatory cells
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
TPO	thyroid peroxidase
uPA	urokinase plasminogen activator
VLA	very late antigen
VCAM	vascular cell adhesion molecule

DANKWOORD

De studies in dit proefschrift zijn slechts tot stand gekomen dankzij de hulp en inzet van veel mensen. Ik heb niet de illusie dat ik jullie allen zal kunnen benoemen en zal me beperken tot het afzonderlijk benoemen van slechts enkele mensen. De vele anderen die hebben bijgedragen echter niet minder bedankt.

Prof. van Hagen, beste Martin. Je allereerste bemoeienis met mijn werk stamt al uit 2006 toen ik de resultaten van mijn afstudeeronderzoek met je kwam bespreken. Je was gelijk enthousiast en dat is de afgelopen jaren ook zo gebleven. Dank voor al je raad, wijze adviezen over het dokter-zijn en de vele goede en gezellige congressen.

Prof. Hooijkaas, beste Herbert. De afgelopen jaren heb je mijn werk van de zijlijn nauwlettend gevolgd en heb je alle ontwikkelingsmogelijkheden geboden die er waren. Hartelijk dank voor al je adviezen. Ik heb het gewaardeerd dat je altijd bereikbaar was voor vragen, ondanks je eigen drukte.

Dr. Dik, beste Wim. In 2007 kreeg je mij, een totaal onbekende student, op je bordje geschoven. Je enthousiaste, nauwgezette en resultaatgerichte begeleiding is denk ik de basis geweest van de totstandkoming van dit proefschrift. De congressen in Fort Lauderdale en in Londen hebben zeker bijgedragen aan de ontwikkeling van een goede verstandhouding en samenwerking. Hartelijk dank voor al je inspanningen.

Dr. Paridaens, beste Dion. Het spijt me dat ik me niet tot de oogheelkunde aangetrokken voel, maar anders had ik onze samenwerking graag voortgezet. Hartelijk dank voor je altijd positieve en enthousiaste inbreng en je bereidheid om elke keer weer patiëntenmateriaal in te sturen.

Deze afgelopen jaren zouden niet zo snel zijn gegaan zonder de gezelligheid van het IRD lab. Kim en Jeroen, fijn dat jullie mijn paranimfen willen zijn, maar vooral hartelijk dank voor de gezelligheid, wetenschappelijk en minder wetenschappelijke discussies, de gezamenlijke sponsoring van Haribo en nog meer. Tante Conny, je was een onmisbare 'moeder' voor het lab: je gezelligheid, organisatie, adviezen en hulp had ik niet willen missen. Nicole, ik ben blij dat het 'bot' zijn snel over was, zonder dat ik je cursus 'haar stylen' echt nodig heb gehad. Gemma en Benjamin: zonder jullie hulp en expertise waren de moleculaire en histochemische proeven nooit zo vlot verlopen. Jan Willem: ik hoop op eenzelfde goede samenwerking later in de kliniek. Er zijn nog veel anderen die hebben bijgedragen: Marten, Jaap, Salim, Dagmar, Jacolien, Kees…

Alle andere collega's van de afdeling immunologie: hartelijk dank voor alle hulp, gezelligheid, snoepjes en borrels. Deelnemers aan de ophthalmo-immuno besprekingen: dank voor de wetenschappelijke discussies en adviezen en succes met het voortzetten van alle onderzoekslijnen. Sandra: hartelijk dank voor al je werk aan mijn figuren. Ik wil niet weten hoe vaak ik bij jou langs ben geweest met wijzigingen. Maar op de figuren kunnen we trots zijn!

Familie, vrienden en bekenden: hartelijk dank voor jullie interesse in mijn werk met 'die diertjes'.
Anneke: ik ben blij dat ik met jou getrouwd ben en ben dankbaar voor je gezelligheid, begrip en steun. Dankzij jou (en later ook Niek) was het thuiskomen ook een bijkomen.

Tot slot dank ik de Heere God die mij de gaven, het inzicht en de vaardigheden gaf om dit promotieonderzoek te kunnen doen en het ook voorspoedig liet verlopen. Hem alleen zij de eer!

CURRICULUM VITAE

Leendert van Steensel was born on May the 5th of 1984 in Abakaliki, Nigeria. He attended secondary school at the Wartburg College in Rotterdam, from which he graduated *cum laude* in 2002. Hereafter, he started Medical School at the Erasmus University Rotterdam. He graduated from Medical School in September 2009.

In 2006 he did a research internship entitled '*IGF-1 receptor antibodies in Graves' ophthalmopathy'* at the Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands (supervisor prof. dr. H.A. Drexhage) and at the Los Angeles Biomedical Research Institute, Harbor-UCLA Medical Center, Los Angeles, USA (supervisor prof. dr. T.J. Smith). He proceeded this work in 2007 as a PhD-student at the Department of Immunology, Erasmus MC, University Medical Center, Rotterdam on a project entitled: *Graves' ophthalmopathy: a comprehensive role for platelet-derived growth factors* (supervisors: dr. W.A. Dik, dr. D. Paridaens, prof. dr. H. Hooijkaas and prof. dr. P.M. van Hagen).

In January 2012 he started as surgical resident at the Ikazia hospital in Rotterdam (dr. T. den Hoed).

PUBLICATIONS

L. van Steensel, D. Paridaens, B. Schrijver, G.M. Dingjan, P.L.A. van Daele, P.M. van Hagen, W.A. van den Bosch, H.A. Drexhage, H. Hooijkaas and W.A. Dik
Imatinib mesylate and AMN107 inhibit PDGF-signalling in orbital fibroblasts; a potential treatment for Graves' ophthalmopathy
Invest Ophthalmol Vis Sci. 2009;50:3091-3098.

L. van Steensel, D. Paridaens, G.M. Dingjan, P.L.A. van Daele, P.M. van Hagen, R.W.A.M. Kuijpers, W.A. van den Bosch, H.A. Drexhage, H. Hooijkaas and W.A. Dik
Platelet-derived growth factor-BB: a stimulus for cytokine production by orbital fibroblasts in Graves' ophthalmopathy
Invest Ophthalmol Vis Sci. 2010 Feb;51(2):1002-7.

L. van Steensel, W.A. Dik
The orbital fibroblast: a key player and target for therapies in Graves' ophthalmopathy
Orbit, 2010 29(4), 202–206.

L. van Steensel, P.M. van Hagen, D. Paridaens, W.A. van den Bosch, R.W.A.M. Kuijpers, H.A. Drexhage, H. Hooijkaas and W.A. Dik
Whole orbital tissue cultures identifies imatinib mesylate and adalimumab as potential therapeutics for Graves' ophthalmopathy
Br J Ophthalmol. 2011 May; 95(5):735-8.

L. van Steensel, D. Paridaens, M. van Meurs, P.M. van Hagen, W.A. van den Bosch, R.W.A.M. Kuijpers, H.A. Drexhage, H. Hooijkaas and W.A. Dik
Orbit-infiltrating mast cells, monocytes and macrophages produce PDGF isoforms that orchestrate orbital fibroblast activation in Graves' ophthalmopathy
J Clin Endocrinol Metab. 2012, in press

L. van Steensel, H. Hooijkaas, D. Paridaens, W.A. van den Bosch, R.W.A.M. Kuijpers, H.A. Drexhage, P.M. van Hagen and W.A. Dik
PDGF enhances orbital fibroblast responses to TSHR stimulating autoantibodies in Graves' ophthalmopathy patients
Submitted for publication

L. van Steensel, D. Paridaens, B. Schrijver, W.A. van den Bosch, R.W.A.M. Kuijpers, H.A. Drexhage, P.M. van Hagen and W.A. Dik
Mast cell factors in Graves' ophthalmopathy: tryptase stimulates orbital fibroblast cytokine and hyaluronan production
Manuscript in preparation

PHD PORTFOLIO

Name PhD student:	L. van Steensel	PhD period:	2009 - 2012
Erasmus MC Department:	Immunology	Promotores:	Prof. Dr. P.M. van Hagen
Postgraduate School:	Molecular Medicine		Prof. Dr. H. Hooijkaas
		Copromotores:	Dr. W.A. Dik
			Dr. D. Paridaens

PhD training	Year	Workload (ECTS)
Courses		
- Academic Writing in English for PhD students	2008	1.6
- Molecular Immunology	2008	3.0
- Classical Methods for data-analysis	2009	5.9
- NIBI Management voor promovendi en post-docs	2010	1.0
- Basic and translational endocrinology	2011	2.0
Seminars and workshops		
- Seminars Immunology	2007-2011	2.0
- Journal Club	2007-2011	2.0
Presentations		
- National conferences	2009-2010	4.0
- International conferences	2008-2011	6.0

Teaching	Year	Workload (ECTS)
Supervising practicals		
- Basic histology for medical students	2008-2009	4.0
- Immunology cases for medical students	2009-2011	4.0
- Bachelor thesis	2008	2.0