NEW INSIGHTS IN THE PATHOGENESIS OF GRAVES' OPHTHALMOPATHY:

POTENTIALS FOR TARGETED THERAPY

NIEUWE INZICHTEN IN DE PATHOGENESE VAN GRAVES' OPHTHALMOPATHIE :

MOGELIJKHEDEN VOOR GERICHTE THERAPIE

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Adapted from:

Current perspectives on the role of orbital fibroblasts in the pathogenesis of Graves' ophthalmopathy

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Platelet-derived growth factor: a key factor in the pathogenesis of Graves' ophthalmopathy and potential target for treatment

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Graves' disease and Graves' ophthalmopathy

Graves' disease (GD; Graves' hyperthyroidism) is one of the most common autoimmune disorders and accounts for the majority of cases of hyperthyroidism. Hyperthyroidism is a pathological syndrome in which tissue is exposed to excessive amounts of thyroid hormone, causing typical symptoms as nervousness or anxiety, weight loss, palpitations, heat intolerability and fatigue. Hyperthyroidism in GD is caused by specific autoantibodies that stimulate the thyrotropin receptor (TSH-receptor; TSHR), thereby mimicking the effect of pituitary thyroid stimulating hormone (TSH) [1].

Graves' ophthalmopathy (GO), also referred to as thyroid eye disease, is an extra-thyroidal complication that develops in ~25-50% of patients with GD and is characterized by inflammation and extensive remodeling of the soft tissues surrounding the eyes [2]. Most patients exhibit extraocular muscle and adipose/connective tissue volume increase, while in some patients either extraocular muscle enlargement or adipose/connective tissue expansion may predominate [2]. Fibroblast and adipocyte numbers are increased in extraocular muscle and adipose/connective tissue from GO patients, leading to collagen and glycosaminoglycan accumulation between the muscle fibers and within the adipose/connective tissue [3]. Clinical symptoms of GO result from the increased orbital tissue volume within the non-compliant space-limited bony orbit and comprise of upper eyelid retraction, edema, erythema of the periorbital tissues and conjunctivae, and proptosis. Keratitis can occur in case of severe and prolonged proptosis, while optic neuropathy can result from optic nerve compression [2, 3].

Early active GO is characterized by infiltration of the extraocular muscles and adipose/connective tissue with mononuclear cells, primarily CD4+ T-lymphocytes, some CD8+ T-lymphocytes, monocytes, macrophages, B-lymphocytes and plasma cells [2, 4-8]. Mast cells are more abundant in the late fibrotic disease phase [3, 9, 10]. These inflammatory cells activate orbital fibroblasts via the secretion of inflammatory mediators (e.g. cytokines) or by direct cellular interaction [2]. Moreover, orbital fibroblasts in GO may be activated by stimulatory autoantibodies directed against the TSHR and the insulin-like growth factor-1 receptor (IGF-1R) [2, 11]. The activated orbital fibroblasts increase their proliferative activity, produce inflammatory mediators, differentiate into adipocytes and myofibroblasts and produce excess amounts of extracellular matrix (ECM) components. Thereby, orbital fibroblasts fulfill central roles in orbital inflammation and tissue remodeling in GO. This activation, combined with several unique properties and heterogeneity within the orbital fibroblast pool, has led to the concept that orbital fibroblasts represent the central cell type in the pathogenesis of GO. Important effector functions and characteristics of orbital fibroblasts that contribute to the pathogenesis of GO will be further discussed.

Orbital fibroblasts contribute to orbital inflammation

The inflammatory environment within GO orbital tissue is determined by soluble and cellular components and strongly influences orbital fibroblast behaviour. In early active GO, T-helper 1 (Th_1)-lymphocytes dominate and Th_1-like cytokines (including a.o. interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α) that facilitate cell mediated immunity are abundantly present. Although less evident, Th_2-lymphocytes and associated cytokines (including IL-4 and IL-10) may dominate the later disease stage characterized by tissue remodeling and fibrosis (late GO), fitting the current paradigm that Th_2-like cytokine responses predominate in chronic inflammation and fibrosis [2, 12-16]. Other T-helper cell subsets that have been indicated in auto-immune disease and fibrosis are Th_{17} and Th_{22} [17-21]. However, so far, involvement of Th_{17} and Th_{22} cells in GO has not been examined, although an association between specific IL-23 receptor polymorphisms and GO was suggested; IL-23 drives Th17 pathogenicity and is a primary inducer of IL-22 [22]. There are however some indications that Th_{17} and Th_{22} cells are involved in GD as increased frequencies of Th_{17} and Th_{22} cells in peripheral blood from GD patients have been described, but studies on this are not conclusive [23-25]. Other inflammatory cell types, including monocytes, macrophages and mast cells also contribute to the increased orbital cytokine/growth factor levels in GO [10, 26] although the contribution of mast cells and their contents to GO remain poorly studied to date.

The effects of several cytokines and growth factors elevated in GO orbital tissue on orbital fibroblast inflammatory activity have been examined. IFN-γ stimulates the production of chemokine (C-C motif) ligand (CCL)2, a chemotactic factor for monocytes, as well as T-lymphocyte chemoattractants such as chemokine (C-X-C motif) ligand (CXCL)9, CXCL10 and CXCL11, which is synergistically enhanced by TNF-α [27-29]. Cytokines and growth factors such as IL-1β, TNF-α and platelet-derived growth factor (PDGF)-AA, PDGF-AB and PDGF-BB also stimulate orbital fibroblasts to produce cytokines/chemokines like CCL2, CCL5, CCL7, IL-6, IL-8, and IL-16 that are collectively involved in recruitment and activation of monocytes, T-lymphocytes, B-lymphocytes and mast cells [10, 27, 30-33]. Moreover, IL-1β and leukoregulin stimulate prostaglandin E_2 (PGE_2) production by orbital fibroblasts [34, 35]. PGE_2 stimulates B-lymphocyte maturation, activates mast cells and induces Th_2 skewing, but also stimulates IL-6 production by orbital fibroblasts [36-39].

Leukocyte infiltration and activation in tissue not only depends on local chemokine gradients, but also requires expression of adhesion and co-stimulatory molecules on leukocytes, endothelial cells and tissue resident cells. Intercellular adhesion molecule (ICAM)-1 expression on orbital fibroblasts is upregulated by IL-1α, IL-1β, IFN-γ, TNF-α [40-42]. The co-stimulatory molecule CD40, highly expressed by orbital fibroblasts from GO patients, is further upregulated by IFN-γ stimulation [31]. CD40-

CD154 ligation is involved in physical interactions between orbital fibroblasts and T-lymphocytes in GO and enhances ICAM-1 expression as well as cytokine and prostaglandin production (e.g. CCL2, IL-1α, IL-6, IL-8, PGE_2) by orbital fibroblasts [31, 43-45].

Collectively, these data illustrate that orbital fibroblasts, through the production of inflammatory molecules, are involved in regulating the orbital inflammatory process in GO where they orchestrate leukocyte recruitment and activation.

Orbital fibroblasts contribute to orbital tissue expansion

Proliferation, extracellular matrix production (especially hyaluronan) and differentiation of orbital fibroblasts into adipocytes and myofibroblasts are important determinants of orbital tissue volume expansion and fibrosis in GO [2, 3] and will be discussed.

Orbital fibroblast proliferation

Fibroblast proliferation is an important contributor to tissue remodeling and fibrotic responses [46]. The basal proliferative activity of GO orbital fibroblasts was found to be higher than that from normal orbital fibroblasts [47]. In addition, cellular interactions such as CD40-CD154 ligation between T-lymphocytes and orbital fibroblasts, but also various cytokines and growth factors, including IL-4, insulin-like growth factor (IGF)-1, PDGF and transforming growth factor (TGF)-β more strongly increase the proliferation rate of GO orbital fibroblasts than that of control orbital fibroblasts [47, 48]. Still, studies on this are not always consistent, as it has also been described that PDGF-BB stimulates proliferation of GO and control orbital fibroblasts equally and that TGF-β has no effect on orbital fibroblast proliferation [49]. PDGF-BB was found to be a stronger mitogen for orbital fibroblasts than PDGF-AB, which in turn is more potent than PDGF-AA [10]. The picture that emerges is that GO orbital fibroblasts are extremely sensitive to mitogenic factors and that exaggerated proliferation by these cells contributes to orbital tissue expansion and fibrosis in GO.

Hyaluronan production by orbital fibroblasts

GO orbital tissue contains increased amounts of non-sulfated glycosaminoglycans (especially hyaluronan) as well as collagen, which are produced by orbital fibroblasts [3]. Hyaluronan is the ECM component mostly contributing to orbital

tissue expansion in GO. Hyaluronan is estimated to occupy ~75000 times the volume of that of an equivalent weight of collagen, which is mainly related to its massive water binding capacity [3]. Hyaluronan synthesis is regulated by cell membrane expressed hyaluronan synthases (HASs), of which three different isoforms exist, HAS1, HAS2, and HAS3 [50]. Of these, HAS2 is considered to represent the major HAS isoform involved in hyaluronan synthesis by orbital fibroblasts in GO [51, 52].

Inflammatory mediators such as leukoregulin, IL-1, TNF-α, IFN-γ, TGF-β, IGF-1, PDGF, prostaglandins and cellular interactions with immune cells enhance hyaluronan production by orbital fibroblasts [10, 42, 49, 51, 53-59]. Cytokines may act synergistically on hyaluronan production by orbital fibroblasts. For instance IL-4 and IFN-γ have been described to augment the effect of IL-1β on hyaluronan production by orbital fibroblasts [34]. Still, the contribution of specific cytokines and their interactions with other cytokines in the pathogenesis of GO is complex and incompletely understood, as illustrated by the divergent effects of IL-4 and IFN-γ on IL-1β-induced hyaluronan and PGE_2 production by orbital fibroblasts [34].

Hyaluronan accumulation depends on the balance between synthesis and degradation. Recently orbital fibroblasts were found to produce three different hyaluronidase isoforms [60]. And although enhanced hyaluronan synthesis rather than diminished breakdown appears to be the main mechanism of accumulation in GO [60], the interaction between hyaluronan synthesis and degradation in GO orbital tissue is still incompletely understood.

Adipogenic and myofibroblastic differentiation potential of orbital fibroblasts is distinguished by Thy1 expression

Functional and phenotypic heterogeneity exists within the orbital fibroblast pool with regard to their capacity to differentiate into adipocytes. This is confined to at least two different orbital fibroblast subpopulations, Thy1(CD90)$^+$ and Thy1$^-$ orbital fibroblasts [61-63].

Thy1$^-$ orbital fibroblasts exhibit high capacity to differentiate into adipocytes [61, 63-66]. Inflammatory mediators including IL-1β, IL-6 and PGD_2 enhance adipogenesis by orbital fibroblasts [42, 58, 67]. Remarkably, Th_1 cytokines such as IFN-γ and TNF-α inhibit adipogenic differentiation by orbital fibroblasts, while IL-1α and IL-4 do not affect these processes [42, 67]. This is consistent with a role for Th_1-related cytokines in the early active inflammatory phase of GO, rather than the late tissue remodeling phase of the disease. Moreover, physical interaction between orbital fibroblasts and autologous T-lymphocytes drives adipogenic differentiation of orbital fibroblasts in a prostaglandin

dependent manner [68]. When cultured under pathological pressure in a three-dimensional collagen matrix Thy1$^-$ orbital fibroblasts differentiate into adipocytes. This implies that increased mechanical pressure encountered by orbital fibroblasts within the space-limited noncompliant orbit may provide pro-adipogenic signals in GO [69]. Cigarette smoking is the strongest modifiable risk factor for developing GO and cigarette smoke extract promotes adipogenic differentiation by orbital fibroblasts [70], although it is unclear how this relates to Thy1 expression.

PPAR-γ is an adipocyte predominant nuclear receptor that functions as transcription factor and regulates glucose and lipid homeostasis [71]. Activation of PPAR-γ with rosiglitazone enhances adipogenesis by orbital fibroblasts [65]. Thiazolidinediones as rosiglitazone or piogliatozone are used as treatment for type-2 diabetes. Remarkably, GO patients treated with these drugs for type-2 diabetes may encounter orbital deterioration due to PPAR-γ activation and adipose tissue expansion [72]. On the other hand, PPAR-γ agonists may inhibit orbital inflammation and hyaluronan accumulation [57, 73]. PPAR-γ may thus represent an important regulatory factor in GO and well balanced PPAR-γ activity may be beneficial in GO [73].

Thy1$^+$ orbital fibroblasts have low adipocyte differentiation potential but exhibit high capacity to differentiate into α-smooth muscle actin expressing myofibroblasts, for instance when cultured in the presence of the Th_2-related growth factor TGF-β [66, 74]. Myofibroblasts are the main cell type responsible for contraction and collagen accumulation in fibrotic tissue [75]. These observations are thus consistent with a role of Th_2-related cytokines in the tissue remodeling/fibrotic phase of late inactive GO where Thy1$^+$ orbital fibroblast derived myofibroblasts contribute to fibrosis of the orbital tissues [63].

Remarkably, the majority of the fibroblast pool from the adipose/connective orbital tissue consists of Thy1$^+$ fibroblasts, while ~30-40% of the fibroblasts are Thy1$^-$. In contrast, fibroblasts from the extra-ocular muscles uniformly express Thy1 [63]. So far there is no clear explanation what controls this heterogeneity. There is, however, evidence that supports cross-talk between Thy1$^+$ and Thy1$^-$ orbital fibroblast populations. Culture medium from Thy1$^+$ orbital fibroblasts was found to inhibit adipocytic differentiation by Thy1$^-$ orbital fibroblasts, indicating secretion of anti-adipogenic factors by Thy1$^+$ orbital fibroblasts [74]. It has been suggested that the autoimmune inflammation in GO disrupts the ability of Thy1$^-$ orbital fibroblasts to respond appropriately to the anti-adipogenic signal produced by the Thy1$^+$ orbital fibroblasts, which facilitates adipogenesis in GO [74]. It should however be noted that isolation and culture of Thy1$^+$ and Thy1$^-$ orbital fibroblast populations can be troublesome as both purified Thy1$^+$ and Thy1$^-$ orbital fibroblast populations may rapidly revert into the original mixed phenotype fibroblast pool [69]. Nevertheless, differences in

the relative proportion of $Thy1^+$ and $Thy1^-$ orbital fibroblast populations between GO patients and their degree of exposure to specific stimuli, such as TGF-β, may be involved in observed differences in adipose tissue and extraocular muscle involvement in GO patients [2, 63].

Orbital fibroblasts as target for TSHR and IGF-1R autoantibodies

TSHR is the autoantigen responsible for hyperthyroidism in GD. The close clinical association between GD and eye disease has led to the shared (auto)antigen hypothesis, which is supported by the positive correlation between TSHR autoantibody titer and activity and severity of GO in GD patients [76-78]. In addition, TSHR is expressed in orbital tissue, which is even higher in GO orbital tissue. This expression is confined to orbital fibroblasts, which seems to be a rather unique feature for orbital fibroblasts, since fibroblasts from other anatomical sites mostly do not express TSHR [9, 15, 79-83]. The differentiation of orbital fibroblasts into adipocytes is associated with increased TSHR expression, which is currently considered as main route of enhanced orbital TSHR expression in GO [2, 67, 84]. PDGF-AB and PDGF-BB were found to rapidly increase TSHR expression on orbital fibroblasts [83], but the relation with adipogenesis is unclear so far. In contrast, TGF-β reduces TSHR expression without affecting adipogenesis [84, 85]. These data illustrate that the level of *in vivo* TSHR expression by orbital fibroblasts/adipocytes in GO is most likely determined by the interplay between the various cytokines/growth factors present within the orbital tissue.

Although the observations described above favor a role for TSHR stimulatory autoantibodies and TSHR in GO pathogenesis only few studies examined the effect of TSHR activation on orbital fibroblasts. Activation of orbital fibroblasts by TSH, TSHR specific stimulatory antibodies, or GD-IgG induced cAMP signaling, phosphoinositide 3-kinase (PI3K) signaling and the production of cytokines (e.g. CCL2, CCL5, IL-6, IL-8), ICAM-1 and hyaluronan [40, 41, 52, 83, 86-88]. Furthermore, TSHR activation acts pro-adipogenic on orbital fibroblasts [86, 89]. Importantly, PDGF-enhanced TSHR expression in orbital fibroblasts was found to augment the capacity of GD-IgG to stimulate cytokine and hyaluronan production by orbital fibroblasts [83]. This points at a direct link between TSHR expression levels in orbital fibroblasts and the pathogenicity of the TSHR stimulatory autoantibodies in GO. TSHR expression has also been found in pretibial fibroblasts from GD patients where it may thus contribute to pretibial myxedema, another (less frequent) extra-thyroidal complication of GD that is also characterized by increased hyaluronan deposition [2, 3, 82, 90, 91].

The IGF-1R is expressed at high level by orbital fibroblasts from GO patients [92]. Stimulatory autoantibodies against IGF-1R have been suggested to contribute to GO by

stimulating the production of the T-lymphocyte chemoattractants IL-16 and CCL5 as well as hyaluronan by orbital fibroblasts [92, 93]. Adversely, a recent study does not support the hypothesis that IGF-1R autoantibodies contribute to GO pathogenesis, as a similar prevalence of IGF-1R autoantibody positivity was found in GO patients and healthy controls. Moreover, in this study the IGF-1R autoantibodies did not activate IGF-1R signaling but exerted an inhibitory activity on IGF-1R signaling [94]. Furthermore it was found that an IGF-1R blocking antibody inhibits M22 (a monoclonal TSHR stimulatory antibody)-induced hyaluronan production by orbital fibroblasts, although this may be related to a physical and functional association between TSHR and IGF-1R [95, 96]. Therefore, further studies that examine the significance of IGF-1R autoantibodies and the pathogenic role they play in orbital fibroblast activation in GO are still required.

Orbital fibroblasts display unique biological responses

Depending on the anatomical location fibroblasts display characteristic transcriptional patterns, indicating that fibroblasts of different anatomical origin represent distinctly differentiated cell types [97]. In addition to their unique anatomical location, orbital fibroblasts are from neuro-ectodermal origin while most other tissue fibroblasts are from mesenchymal origin [98]. Moreover, orbital fibroblasts display clear morphological differences with fibroblasts from other anatomical regions [99]. This implicates that orbital fibroblasts likely display characteristic features and several studies demonstrated that orbital fibroblasts respond differently to stimulation than fibroblasts from other anatomical regions. For instance, activation with IL-1β, IFN-γ, TNF-α, leukoregulin, PDGF-BB, or CD40-CD154 ligation results in significantly higher cytokine/chemokine, prostaglandin, plasminogen-activator inhibitor type-1 and hyaluronan production by orbital fibroblasts compared to other types of fibroblasts [30, 31, 33, 43, 51, 55, 100, 101]. In contrast, skin fibroblasts have been reported to produce significantly more CCL7 upon PDGF-BB stimulation than orbital fibroblasts [33]. Moreover, PDGF-AB and PDGF-BB enhance TSHR expression on orbital fibroblasts, while they do not in skin fibroblasts [83]. Also, orbital fibroblasts generally produce higher amounts of hyaluronan upon activation than fibroblasts from other anatomical regions [55, 102]. Altered regulation of cell signaling pathways between orbital fibroblasts and other fibroblasts may be involved in these different responses, for instance different regulation of NF-κB and TGF-β_1 signaling have been proposed in orbital fibroblasts [33, 49].

Orbital fibroblasts from GO patients have repeatedly been reported to exhibit different features compared to orbital fibroblasts from healthy controls. For instance, higher expression levels of CD40, Thy1 and IGF-1R have been described on orbital fibroblasts from GO patients [31, 92, 103]. Moreover, GO orbital fibroblasts have been reported to display increased proliferative activity under basal conditions or when

stimulated with certain cytokines/growth factors [47]. GO orbital fibroblasts also produced markedly less IL-1 receptor antagonist (IL-1RA) upon stimulation with various cytokines (e.g. IL-1α, IFN-γ, TNF-α, TGF-β) than normal orbital fibroblasts [104], suggestive of disturbed anti-inflammatory responses. Finally, GO orbital fibroblasts, unlike control orbital fibroblasts, have been found to spontaneously differentiate into adipocytes when cultured in a three-dimensional collagen matrix [69].

Chronic inflammation and fibrosis can lead to the emergence of epigenetically altered fibroblasts that display a phenotype with DNA methylation aberrancies and increased histone deacetylase activity that promotes inflammation and pathologic tissue remodeling [105-108]. Although not examined so far, occurrence of epigenetic alterations in orbital fibroblasts from GO patients may contribute to observed differences with healthy control orbital fibroblasts.

Orbital fibrocyte recruitment contributes to GO

In tissue repair processes fibroblasts can originate from local proliferation, recruitment from surrounding undamaged tissue, or through de-differentation processes referred to as epithelial/endothelial mesenchymal transition. Furthermore, at sites of tissue inflammation/healing fibroblast-like cells can derive from recruitment and differentiation of circulating fibrocytes [109]. Fibrocytes are bone-marrow derived mesenchymal cells that circulate as peripheral blood mononuclear cells and express a.o. CD34, CD45, chemokine receptors such as chemokine (C-C motif) receptor (CCR)3, CCR5, CCR7 and chemokine (C-X-C motif) receptor (CXCR)4, as well as extracellular matrix molecules like type-I, type-III, type-IV collagen and fibronectin [110]. Fibrocytes rapidly infiltrate sites of tissue damage where they participate in inflammation, healing and tissue remodeling, but they are also involved in fibrosis [110]. Increased fibrocyte numbers have been detected in the fibrotic tissue as well as peripheral blood from patients with fibrotic conditions [109, 110].

Increased numbers of circulating fibrocytes have been reported in peripheral blood from GO patients [111]. In GO circulating fibrocytes infiltrate the orbital tissue where they differentiate into $CD34^+$ orbital fibroblasts, whereas orbital tissue from healthy individuals predominantly contains $CD34^-$ orbital fibroblasts [111]. Orbital fibroblast cultures from GO patients contained fibrocyte resembling cells (CD34 and collagen type-I positive) that spontaneously differentiated into adipocytes [111]. How this relates to the previously noted association between Thy1 negativity and the capacity of orbital fibroblasts to differentiate into adipocytes is unclear so far, but it may very well contribute to the earlier discussed heterogeneity with regard to adipocyte differentiation in orbital fibroblast cultures.

The pathways and molecules involved in fibrocyte migration into orbital tissue in GO are largely unknown, but increased production of specific chemokines within the orbital tissue is most likely involved. A major role has been identified for the CXCL12/CXCR4 axis in fibrocyte recruitment into tissue [112]. Although to date no data are available on CXCL12 production in GO several other chemokines involved in fibrocyte recruitment, such as CCL2, CCL5 and CCL7 [113] are produced by orbital fibroblasts, for instance upon stimulation with IFN-γ, IL-1β, TNF-α or PDGF-BB, factors that are abundantly present in orbital tissue from GO patients [10, 15, 26, 27, 33]. Fibrocytes also express the PDGF-Rα and PDGF-Rβ chains, and the PDGF-BB/PDGF-R-β axis was recently identified as being critical for fibrocyte migration into fibrotic lungs [114]. However, whether these or other ways of orbital fibrocyte recruitment are involved in GO needs to be determined.

Fibrocytes express marginal amounts of IGF-1R but high level of TSHR [111]. Although the IGF-1R and TSHR expression levels were similar between circulating fibrocytes from GO and healthy controls, the fraction of circulating $TSHR^+$ fibrocytes was increased in GO, which did not relate to disease activity or smoking history [111, 115]. TSH and the TSHR-activating antibody M22 stimulate CCL2, CCL3, CCL4, CCL5, CXCL10, granulocyte colony-stimulating factor (G-CSF), IL-6, IL-8, IL-12 and TNF-α production by fibrocytes [111, 115]. Fibrocytes do express substantially higher levels of TSHR and CD40 than orbital fibroblasts and produce high levels of cytokines in response to CD154 [111, 115, 116]. Fibrocytes also produce significantly more IL-6 upon TSHR activation than orbital fibroblasts, and this response is even more vigorous in GD fibrocytes [117]. Possibly this is related to the increased fraction of circulating $TSHR^+$ fibrocytes observed in GD patients [115].

Besides TSHR, fibrocytes were found to express the thyroid proteins thyroglobulin (Tg) and thyroid peroxidase (TPO), which are also targets for autoantibody generation in GD [118]. Fibrocytes thus potentially represent a source that can contribute to extra-thyroidal accumulation of thyroid proteins, for instance in orbital tissue as has been observed for Tg in GO [119, 120]. This raises the possibility that, besides TSHR, other thyroid antigens expressed by fibrocytes have a role as autoantigen in the orbital tissue from GO patients [118, 119].

Circulating fibrocytes produce higher levels of sIL-1RA than orbital fibroblasts from GO patients [121]. However, when $CD34^+$ and $CD34^-$ orbital fibroblast populations were sorted from parental (mixed) GO orbital fibroblast populations and subsequently stimulated with IL-1β, it appeared that the $CD34^+$ orbital fibroblasts exhibited greater capacity to produce sIL-1RA than the $CD34^-$ fibroblasts and the parental fibroblast population [121]. Therefore it has been suggested that $CD34^+$ orbital fibroblasts revert to fibrocytes when cultured in the absence of $CD34^-$ orbital fibroblasts. Consequently, the

authors proposed that fibrocytes that transit into $CD34^+$ orbital fibroblasts encounter signals from the native $CD34^-$ orbital fibroblast population leading to a dramatic reduction in sIL1-RA production capacity by the $CD34^+$ orbital fibroblasts and thus diminished capacity to oppose IL-1α and IL-1β activity within the orbit [121]. Although attractive as a model, the currently available data are insufficient to support this relationship between $CD34^+$ fibrocytes, $CD34^+$ orbital fibroblasts, $CD34^-$ orbital fibroblasts and sIL-1RA and additional studies are thus required.

Fibrocytes express the HLA-class II molecules HLA-DP and HLA-DQ at high level, and HLA-DQ, the co-stimulatory molecule CD86 and adhesion molecules CD11a, CD54 and CD58 are expressed at a level similar to that of monocytes, while the co-stimulatory molecule CD80 is weakly expressed [122]. In line with this, fibrocytes potently activate CD4+ T-lymphocytes in an antigen dependent manner, suggesting that fibrocytes may also be involved in initiation of antigen-specific immunity [122]. Whether fibrocytes fulfill such a role in the (auto)immune pathogenesis of GO remains unclear.

The scant data so far available imply that fibrocytes expressing a.o. CD34, CD40 and thyroid autoantigens, including TSHR, infiltrate orbital tissue from GO patients where they differentiate into $CD34^+$ orbital fibroblasts thereby contributing to orbital fibroblast heterogeneity. The $CD34^+$ orbital fibroblasts can be activated by GO associated autoantibodies and other inflammatory factors to contribute to inflammation and adipose tissue expansion. However, additional studies that aim at unraveling the exact contribution of fibrocytes to the pathogenesis of GO are clearly required.

Although the disease initiating trigger is unknown so far, our understanding of the pathogenetic processes involved in GO has hugely increased during the last decades. In our current concept of the disease exaggerated orbital fibroblast activity is placed at the center, where these cells play a crucial role in the initiation and maintenance of the inflammatory response as well as in orbital tissue expansion and remodeling through proliferation, differentiation into adipocytes and myofibroblasts and enhanced ECM production. The unique hyper-responsive phenotype of orbital fibroblasts along with heterogeneity within the orbital fibroblast pool (e.g. $Thy1^+/Thy1^-$, fibrocytes/$CD34^+$ orbital fibroblasts, but possibly also subpopulations with mesenchymal stem cell properties [123, 124]) and the inflammatory milieu within the noncompliant space-limited bony orbit may very well underlie the orbital manifestations and disease course of GO. The contribution of orbital fibroblasts to the pathogenesis of GO is summarized in Figure 1 and Table 1.

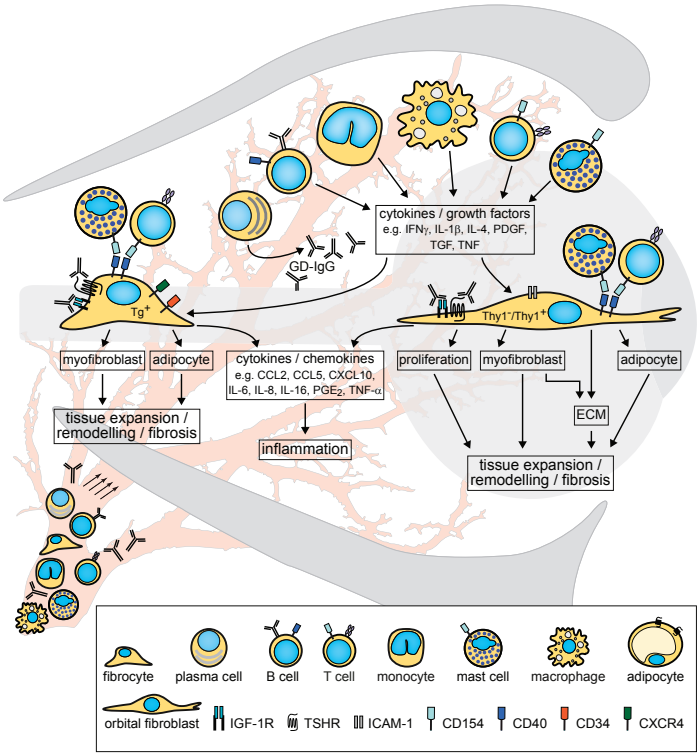

Figure 1. The immunopathobiology of GO.

Immune cells and fibrocytes are recruited into the orbital tissue. Fibrocytes differentiate into $CD34^+$ orbital fibroblasts that express IGF-1R, TSHR and other thyroid antigens, including thyroglobulin (Tg). Together with the $Thy1^-$ and $Thy1^+$ orbital fibroblasts they constitute the heterogeneous orbital fibroblast pool. The infiltrated immune cells interact with the (activation-prone) orbital fibroblasts either via cell-cell interactions, involving molecules such as CD40 and CD154, or via secreted factors, including cytokines, chemokines, growth factors, and stimulatory autoantibodies (GD-IgG) directed against TSHR and IGF-1R. This leads to activation of the orbital fibroblasts which in turn contributes to orbital inflammation via the production of cytokines and chemokines and subsequent recruitment and activation of immune cells. Furthermore the activated orbital fibroblasts display increased proliferative activity, differentiate into adipocytes (especially $Thy1^-$ orbital fibroblasts as well as $CD34^+$ orbital fibroblasts) or myofibroblasts (especially $Thy1^+$ orbital fibroblasts as well as $CD34^+$ orbital fibroblasts) and produce increased amounts of extracellular matrix (ECM). Altogether these processes cause pathologic remodeling and expansion of the orbital tissue within the noncompliant space-limited bony orbit which contributes to the clinical features of GO.

Table 1. Responses of orbital fibroblasts to factors involved in GO

stimulus	Effect on orbital fibroblasts							
	inflammatory mediator production	Adhesion molecule expression	Co-stimulatory molecule expression	Proliferation	Hyaluronan production	TSHR expression	Adipogenesis	Myofibroblast differentiation
Inflammatory mediators/ growth factors								
IL-1α		↑: ICAM-1		↑			-	
IL-1β	↑: IL-6, IL-8, IL-16, CCL2, PGE_2	↑: ICAM-1			↑		↑	
IL-4				↑	↑		-	
IL-6				-		↑	↑	
IFN-γ	↑: CCL2, CXCL9, CXCL10,CXCL11	↑: ICAM-1	↑: CD40		↑	↓	↓	
IGF-1				↑	↑			
Leukoregulin	↑: PGE_2				↑			
PDGF-AA	↑: IL-6			↑	↑	↓		
PDGF-AB	↑: IL-6			↑	↑	↑		
PDGF-BB	↑: IL-6, IL-8, CCL2, CCL5, CCL7			↑	↑	↑		
PGD_2					↑		↑	
PGE_2	↑: IL-6							
TGF-β				↑, -	↑	↓	-	↑
TNF-α	↑: IL-6, IL-8	↑: ICAM-1			↑	↓	↓	
Cellular interaction								
T cells	↑: IL-1α, IL-6, IL-8, CCL2, PGE_2	↑: ICAM-1		↑	↑		↑	
Mast cells	↑: PGE_2				↑			
Autoantibodies								
TSHR	↑: IL-6, IL-8, CCL2, CCL3, CCL4, CCL5, CXCL10, G-CSF, TNF-α	↑: ICAM-1			↑		↑	
IGF-1R	↑: IL-16, CCL5				↑			
Other factors								
Pressure							↑	↑
Smoking							↑	

↑ represents inducing effect, ↓ represents inhibitory effect, - represents no effect.

Platelet-derived growth factor; an important factor in GO that may represent an attractive therapeutic target?

From data discussed above it appears that PDGF isoforms, especially PDGF-AB and PDGF-BB, represent important growth factors in the activation of orbital fibroblasts in GO and thus the regulation of several major pathophysiological process in GO. Inhibition of PDGF activity may thus be considered as therapeutic strategy in GO and therefore the PDGF/PDGF receptor system is discussed in more detail.

PDGF is a family of growth stimulating polypeptides that exerts broad functions in health and disease [125]. There are four different PDGF genes that encode the peptide chains PDGF-A, PDGF-B, PDGF-C and PDGF-D [125]. Disulfide bridging between PDGF chains results in the formation of the homodimeric molecules PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD or the heterodimeric PDGF-AB molecule [125]. The pro-peptide chains of PDGF-A and PDGF-B dimerize intracellularly and have to be activated before secretion by removal of their N-terminal ends [125]. PDGF-CC and PDGF-DD are secreted as latent molecules that contain CUB domains at their N-terminal ends [125]. Activation of these PDGF isoforms occurs after proteolytic removal of the CUB domains by proteases such as plasmin and tissue plasminogen activator [125].

PDGF dimers exert their biologic actions via activation of specific receptors consisting of two PDGF receptor (PDGF-R) chains (αα, αβ or ββ chains). The PDGF-A and PDGF-C chains are ligands for PDGF-Rα, the PDGF-D chain is a ligand for PDGF-Rβ, while the PDGF-B chain can bind both to PDGF-Rα and PDGF-Rβ, but with a higher affinity for PDGF-Rβ [125]. PDGF-R chains consist of an extracellular and an intracellular part. The extracellular part contains five immunoglobulin-like domains while the intracellular part consists of split kinase domains (Figure 2A). Depending on the PDGF ligand that binds PDGF-R chains dimerize in either one of three dimeric forms; αα, αβ or ββ (Figure 2A). The PDGF-receptor belongs to the tyrosine kinase receptor family and PDGF binding is followed by autophosphorylation of crucial tyrosine residues within the receptor chains (Figure 2B) with subsequent activation of downstream signaling molecules such as RAS-MAPK, PI3K and PLC-γ [125, 126].

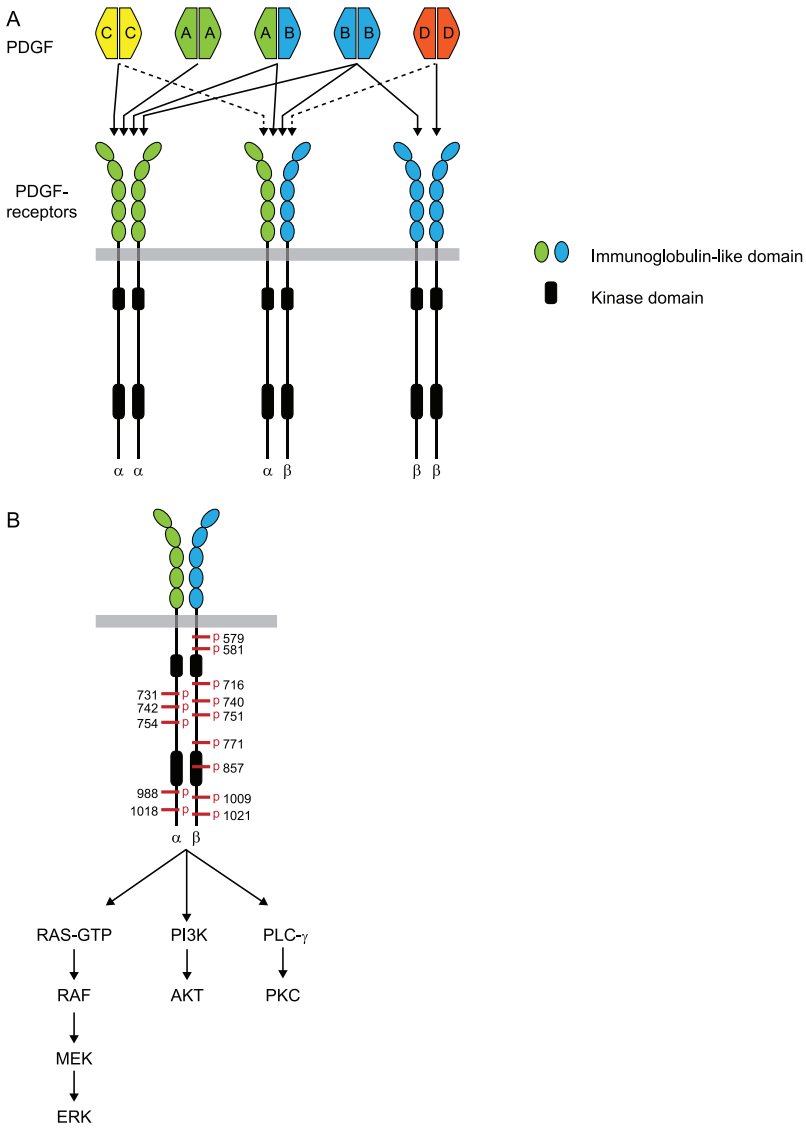

Figure 2. PDGF and PDGF-receptor.

A. PDGF-A and PDGF-C chains are ligands for PDGF-Rα, PDGF-D chain is a ligand for PDGF-Rβ, while the PDGF-B chain can bind both to PDGF-Rα and PDGF-Rβ. Dotted lines indicate weak interactions or conflicting results [125]. **B.** Autophosphorylation of crucial tyrosine residues within the receptor chain results in the activation of downstream signaling molecules.

In normal physiology, PDGF signaling fulfills important roles in organogenesis, organ/tissue homeostasis and wound healing processes. For instance, PDGF-signaling is involved in alveogenesis, villus morphogenesis, spermatogenesis, nephrogenesis, angiogenesis, glomerulogenesis, tooth morphogenesis and development of dermis and lens [125]. Also in wound healing different PDGF isoforms play an important role as they recruit and activate neutrophils, macrophages and fibroblasts, thereby facilitating the tissue remodeling process [127]. However, sustained or elevated PDGF production and signaling is associated with many different diseases including cancers, vasculopathy and fibrosis [125, 128]. A general characteristic of tissue fibrosis is excessive fibroblast activity with resultant hyperproliferation and extracellular matrix production by these cells, processes highly stimulated by PDGF isoforms and all contributing to GO as well. Data described before indicate that in GO PDGF-AA, but especially PDGF-AB and PDGF-BB, stimulate proliferation, hyaluronan and cytokine/chemokine production and TSHR expression by orbital fibroblasts (Figure 3). This, along with the elevated expression of PDGF-A and PDGF-B chains in orbital tissue during all GO disease stages, indicates that inhibition of PDGF signaling may represent as an attractive way for treatment of GO. However this requires further investigation into effects of PDGF on other aspects of orbital fibroblast activation. This holds especially true for adipogenesis in GO, which is a major contributor to orbital tissue expansion while opposite effects of PDGF on adipogenesis of fibroblasts and pre-adipocytes from different anatomical sites have been described [129, 130].

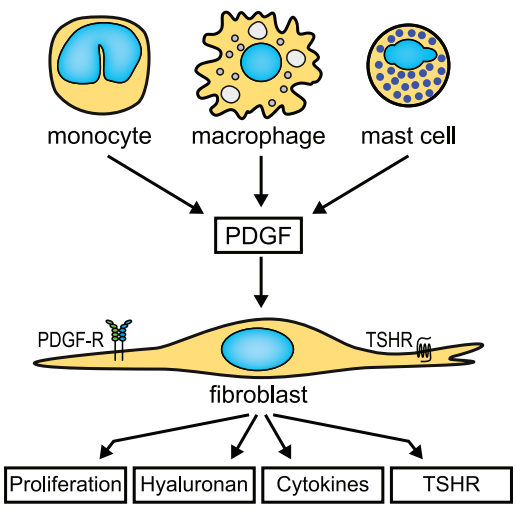

Figure 3. Role of PDGF signaling in GO.

Monocytes, macrophages and mast cells produce PDGF-A and PDGF-B chains in orbital tissue from GO, resulting in the formation of PDGF-AA, PDGF-AB and PDGF-BB

dimeric molecules. These PDGF-isoforms stimulate proliferation, cytokine and hyaluronan production by orbital fibroblasts while PDGF-AB and PDGF-BB also enhance TSHR expression on orbital fibroblasts. In general PDGF-BB is the PDGF-isoform exhibiting the most potent effect on orbital fibroblasts, while PDGF-AA is the weakest.

Treatment of Graves' ophthalmopathy; is inhibition of PDGF activation a possibility?

Currently, the most effective well-tolerated treatment for active moderate-to-severe and sight-threatening GO is (high dose) corticosteroids, while radiotherapy or orbital decompression surgery are considered when patients fail to respond to corticosteroids or for rehabilitating purposes [131, 132]. Effectiveness of corticosteroid treatment relies mainly on the activity of the disease, with a high success rate when introduced in the initial active inflammatory phase of the disease [131, 132]. However, corticosteroid treatment may negatively influence the tissue remodeling or fibrotic phase when inflammation has subsided [132]. 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 [133-135]. In contrast to the ambivalent effects that corticosteroids can have with regard to inflammation, tissue remodeling and fibrosis, the ideal therapy for GO should be effective regardless of the stage of disease. However, so far novel medical treatment options for GO have mainly concentrated on therapeutics directed at immune cells (e.g. B-lymphocytes; rituximab) or mediators (e.g. TNF-α; etanercept) that mainly are involved in the active inflammatory phase of GO [136, 137].

PDGF targeting seems an attractive therapeutic option in GO, as PDGF-driven orbital fibroblast activation most likely occurs in all stages of GO (Figure 4). Several approaches to interfere with PDGF-signaling in GO can be thought of : 1) neutralization of PDGF-molecules, for instance with specific neutralizing antibodies or soluble receptor molecules, 2) blockage of the PDGF-receptor chains with neutralizing antibodies or dominant negative ligands and 3) inhibition of PDGF-receptor signaling by using tyrosine-kinase inhibitors that prevent receptor autophosphorylation upon ligand binding (Figure 5) [125].

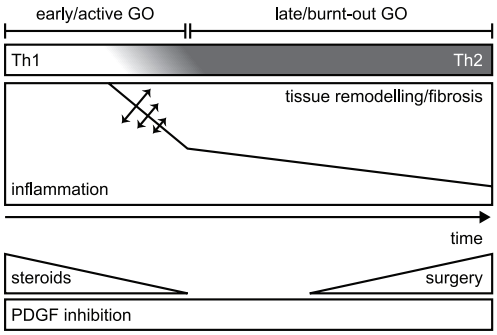

Figure 4. A hypothetical scheme of the pathophysiology and treatment of GO.

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 remodeling continues. Current mainstream treatment of GO consists of corticosteroids and surgery, of which the corticosteroids have a relatively a high success rate when introduced in the active inflammatory phase of the disease, but may negatively influence the tissue remodeling or fibrotic phase when inflammation has subsided. 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.

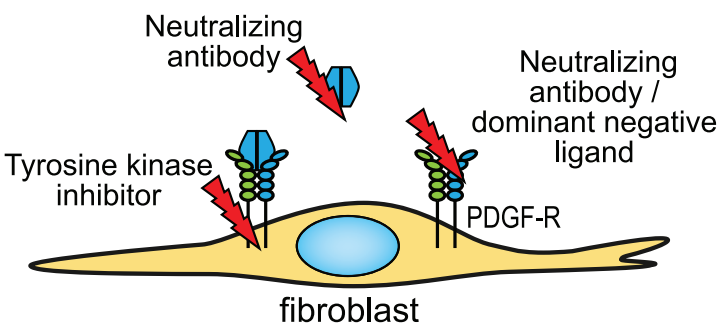

Figure 5. Approaches to target PDGF signaling.

The PDGF system can be blocked by targeting the PDGF molecule, for instance with a neutralizing antibody, or by targeting the PDGF-Receptor, for instance with a neutralizing antibody or a tyrosine kinase inhibitor with specificity for the PDGF-Receptor.

Although neutralizing antibodies directed towards PDGF isoforms or PDGF-receptors are currently not available for clinical use, such an approach might be of benefit in the treatment of GO as it was found that a neutralizing antibody directed towards PDGF-BB reduced IL-6 and hyaluronan secretion by orbital tissue from GO patients in a newly developed orbital tissue culture approach [10]. Remarkably, in this culture system inhibition of PDGF-AA with a neutralizing antibody was hardly effective, underlining the importance of PDGF-B chain containing PDGF isoforms in the pathophysiology of GO.

Several tyrosine kinase inhibitors (TKIs) that exhibit specificity for the PDGF-receptor, amongst which imatinib mesylate and nilotinib, are widely applied to treat BCR-ABL positive chronic myeloid leukemia (CML) as the tyrosine kinase ABL is a target for these TKIs as well [138]. In addition, imatinib mesylate has been used successfully to treat gastrointestinal tumors and mastocytosis by targeting c-Kit kinase activity [139, 140]. Imatinib mesylate and nilotinib were both found to prevent PDGF-induced TSHR expression, proliferation, cytokine and hyaluronan production by orbital fibroblasts from GO patients [10, 33, 49, 83]. Moreover, imatinib mesylate attenuated IL-6 and hyaluronan secretion by cultured GO orbital tissue, while the TNF-α neutralizing agent adalimumab only reduced IL-6 secretion [141]. Although these data point at the attractiveness of TKI usage in the treatment of GO, imatinib mesylate and nilotinib were found to cause serious side effects such as peri-orbital edema, peripheral arterial occlusive disease and cerebrovascular events in CML treatment [142]. Moreover, it was recently shown that imatinib mesylate can stimulate adipogenesis by orbital fibroblasts [69], although this was at high imatinib mesylate concentration. Based on the described adverse effects, imatinib mesylate and nilotinib are not directly regarded as candidate TKIs for a clinical study in GO, at least not when applied in the same dose as used for CML treatment. Therefore studies into other TKIs that inhibit PDGF-R activity in (GO) orbital fibroblasts are warranted. For instance, dasatinib which is associated with less severe side effects than imatinib mesylate [143, 144]. Interestingly, recent studies demonstrated efficacy of nintedanib (a TKI with high specificity for PDGF-R but also FGF and VEGF receptors) in the treatment of idiopathic pulmonary fibrosis, a form of pulmonary fibrosis that involves elevated PDGF, FGF and VEGF activity and for which no suitable treatment was available to date [145, 146]. Although FGF and VEGF have been suggested to contribute to GO their effects on orbital fibroblast activity are poorly studied to date [147-150]. Therefore further studies into the orbital fibroblast activating effects of FGF and VEGF, along that of PDGF, and the effect of nintedanib on this are warranted [147, 149].

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Chapter 2

Aim of the thesis

Orbital fibroblasts are considered as the central cell type in the pathophysiology of Graves' ophthalmopathy (GO) as they display excessive proliferation, production of inflammatory mediators and hyaluronan and differentiate into adipocytes and pro-fibrotic myofibroblasts. There is data suggesting that platelet-derived growth factor-BB (PDGF-BB) is an important mediator driving orbital fibroblast activation in GO, and consequently PDGF-BB, its receptors and downstream signaling molecules may represent attractive targets for therapy. In addition there is preliminary data that suggests that mast cells can contribute to orbital fibroblast activation in GO, but which mast cell-derived factors are involved in this is far from clear.

The aims of this thesis are:

a) to examine the effect of PDGF-BB, either alone or in conjuction with basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) on several aspects of orbital fibroblast activity involved in GO
b) to determine wheter this fibroblast activation can be blocked by different clinically available tyrosine kinase inhibitors and
c) to examine the effect of the mast cell mediator histamine on orbital fibroblast activity.

Chapter 3

The tyrosine kinase inhibitor dasatinib effectively block PDGF-induced orbital fibroblast activation

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Abstract

Background

Graves' ophthalmopathy (GO) remains hard to treat. Excessive orbital fibroblast activation by platelet-derived growth factor (PDGF)-BB contributes to GO. The tyrosine kinase inhibitors (TKIs) imatinib mesylate and dasatinib both target PDGF-receptor tyrosine kinase activity, albeit with a different potency. We compared the efficacy of these TKIs on PDGF-BB-induced proliferation, and on cytokine and hyaluronan production by orbital fibroblasts. Also the capacity of dasatinib to suppress GO-associated gene expression in orbital tissue was examined.

Methods

Orbital fibroblasts from four GO patients and five control subjects were used. The efficacy of the two TKIs was tested by: 1) pre-incubating orbital fibroblasts overnight with different TKI concentrations, followed by 24 h stimulation with PDGF-BB, 2) adding TKI and PDGF-BB simultaneously to the orbital fibroblasts in 24 h cultures. Proliferation was assessed by colorimetric assay. Hyaluronan and cytokine production were measured by ELISA. Furthermore, orbital tissue was obtained from a patient with active GO, and the effect of dasatinib on the expression levels of HAS2-, CCL2-, IL6-, and IL8- mRNA expression was examined by real-time quantitative PCR.

Results

Pre-incubation of orbital fibroblasts with imatinib mesylate or dasatinib resulted in significant and dose-dependent inhibition of PDGF-BB-induced orbital fibroblast proliferation, and hyaluronan and cytokine production. Dasatinib exhibited these effects at far lower concentrations. The same results were observed in the setting where TKI and PDGF-BB treatments were commenced simultaneously. In orbital tissue from active GO, dasatinib significantly suppressed HAS2-, CCL2-, IL6- and IL8-mRNA levels.

Conclusion

Dasatinib may be a promising alternative to high-dose steroids in the treatment of GO.

Introduction

Graves' disease (GD) is one of the most common autoimmune disorders and accounts for ~80% of the total cases of hyperthyroidism in the Western world and iodine-sufficient regions. Clinical manifestations of GD result from stimulatory autoantibodies directed against the thyroid stimulating hormone receptor (TSHR). These autoantibodies target the thyroid gland where they stimulate thyroid hormone production [1]. Between 25-50 % of GD patients develop some degree of Graves' ophthalmopathy (GO), which is characterized by orbital tissue inflammation and expansion [2]. Increased orbital fibroblast proliferation, and enhanced production of extracellular matrix (ECM) components (especially hyaluronan) and cytokines by these cells constitute key events in the pathophysiology of GO and contribute to clinical manifestations such as chemosis, edema, proptosis and ocular motility dysfunction [3]. GO often constitutes a considerable physical and mental burden. Unfortunately, however, no clear improvement in treatment has been achieved during the last decades, and it still mostly consists of anti-inflammatory therapy with corticosteroids [4] or orbital decompression surgery. However, this treatment is often a high burden for patients, due to accompanying side-effects of corticosteroid treatment. Moreover, a significant number of patients fail to respond to these therapies. Although biologicals such as etanercept [5] or infliximab [6], that specifically neutralize TNF-α, or rituximab [7], that depletes B-cells, initially showed promising results in GO, other treatment options for GO are eagerly needed, as experience with these compounds in clinical practice in GO is limited and results are contradictory [7, 8].

Platelet-derived growth factor (PDGF) is important in normal wound healing, and increased levels or activity of PDGF have been shown to be involved in pulmonary, liver, dermal and cardiac fibrosis, in which it primarily acts as a mitogen for fibroblasts with a myofibroblast phenotype [9]. Previously we identified PDGF-BB and PDGF-AB as important contributors to GO as well. The level of these growth factors is increased in orbital tissue from GO patients. There they potently stimulate proliferation and production of cytokines such as CCL2, IL-6 and IL-8 as well as hyaluronan by orbital fibroblasts [10-12]. PDGF isoforms thus stimulate several key pathogenic pathways in GO. Therefore, they represent attractive therapeutic targets for the treatment of GO.

Imatinib mesylate and nilotinib are small molecule tyrosine kinase inhibitors (TKI) that block c-Abl kinase activity. They are used to inhibit the constitutive Abl kinase activity of the BCR-ABL fusion protein in chronic myeloid leukemia [13]. In addition, imatinib mesylate and nilotinib inhibit PDGF receptor (PDGF-R) tyrosine kinase activity and thereby can prevent PDGF-induced PDGF-R autophosphorylation and signaling, also in orbital fibroblasts [11]. Recently, we demonstrated that imatinib mesylate and nilotinib block PDGF-BB and PDGF-AB induced proliferation, hyaluronan and cytokine

production by orbital fibroblasts [10-12]. Moreover, we found that imatinib mesylate reduced IL-6 and hyaluronan production in whole orbital tissue cultures from GO patients, which correlated with the PDGF levels present in the tissue [14]. This further supports our notion that suppression of the PDGF-signaling cascade represents an attractive therapeutic target in GO. However, treatment of chronic myeloid leukemia with imatinib mesylate or nilotinib is associated with serious side effects such as peri-orbital edema, peripheral arterial occlusive disease and cerebrovascular events [15]. Imatinib mesylate and nilotinib may therefore not represent the preferable type of medication for GO, at least not when given in dosages comparable to those used to treat chronic myeloid leukemia. Nevertheless, considering the attractiveness of targeting the PDGF pathway in GO, PDGF-R targeting with TKI other than imatinib mesylate or nilotinib might constitute potential therapeutic options in GO.

Dasatinib is a TKI which is structurally distinct from imatinib mesylate and nilotinib. Although dasatinib is a less specific TKI than imatinib mesylate and nilotinib, it displays a considerably higher inhibitory potency (pIC50) for the PDGF receptor, both PDGF-Rα and PDGF-Rβ chain, than imatinib mesylate and nilotinib [13]. Dasatinib is currently approved as second-line therapy for treatment of chronic myeloid leukemia, with a more beneficial outcome and fewer side effects than imatinib mesylate [16, 17]. Dasatinib has also been tested for treatment of bleomycin-induced dermal fibrosis in mice. It was found to decrease skin thickness, myofibroblast numbers and collagen production in this model in which PDGF signaling is highly active [18]. Moreover, dasatinib reduced the production of the ECM components fibronectin and collagen by skin fibroblasts from a systemic sclerosis patient [18]. Interestingly, dasatinib suppressed nucleic acid-induced interferon production by plasmacytoid dendritic cells from patients with auto-immune diseases such as systemic lupus erythematosus and psoriasis at much lower concentrations than imatinib mesylate did [19]. The level of biochemical activity between different TKI may thus clearly differ within a specific cell type. Dasatinib might thus be effective at a lower dosage for treatment of auto-immune disease than that required for treatment of chronic myeloid leukemia, suggesting that less severe side effects can be expected as well. Therefore, the aim of this study was to determine the effect of wide concentration ranges of imatinib mesylate and dasatinib on PDGF-BB–induced proliferation, cytokine and hyaluronan production by orbital fibroblasts to provide an *in vitro* basis for TKI-based treatment of GO patients.

Materials and Methods

Cell culture and reagents

Orbital fibroblasts were obtained from orbital tissue of four patients with GO at an inactive stage of disease who underwent orbital decompression surgery and from five controls without thyroid or inflammatory disease and undergoing orbital surgery for other reasons, as described previously [11]. Briefly, the tissues were cut into small pieces and put in culture with Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics (penicillin and streptomycin; Cambrex BioWhittaker, Verviers, Belgium). The tissues were cultured at 37°C and 5% CO_2 until monolayers of orbital fibroblasts were obtained. Orbital fibroblasts were serially passaged and used for experiments between the 4th and 12th passage.

All patients were euthyroid at the time of orbital surgery and had not received corticosteroid or other immunosuppressive treatment for at least three months prior to surgery. All tissues were obtained at the Rotterdam Eye Hospital after informed consent and in accordance with the principles of the Declaration of Helsinki and with approval by the institutional review board at the Erasmus MC, University Medical Center (Rotterdam, The Netherlands). Orbital fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin: Cambrex BioWhittaker, Verviers, Belgium) at 37°C and 5% CO_2. Recombinant human PDGF-BB was obtained from R&D Systems (Abingdon, UK). Tyrosine kinase inhibitors, imatinib mesylate (S1026; molecular weight 589.71 and dasatinib (S1021; molecular weight 488.01), were purchased from Selleckchem (Houston, TX). The imatinib mesylate and dasatinib concentrations used were not toxic to the orbital fibroblasts, based on lactate dehydrogenase (LDH) release and microscopic appearance (concentration range: imatinib mesylate 2.5 µg/ml (4.2 µM)- 0.04 µg/ml (0.07 µM), dasatinib 2.5 µg/ml (5.0 µM)- 0.04 µg/ml (0.08 µM)). IL-6 and IL-8 ELISA were obtained from Invitrogen (Frederick, MD) and CCL2 and hyaluronan ELISA from R&D Systems.

Orbital fibroblast proliferation assay

Orbital fibroblasts were seeded at 6.0 x 10^3 cells/well in 96-well plates in DMEM 1% FCS and allowed to adhere overnight. In order to examine the maximal pharmacologic effect of imatinib mesylate and dasatinib, the orbital fibroblasts were pre-incubated overnight with a broad concentration range of imatinib mesylate or dasatinib, and thereafter stimulated with PDGF-BB (50 ng/ml). In another setting, imatinib mesylate or dasatinib were added to the orbital fibroblasts at the time of PDGF-BB (50 ng/ml) stimulation. Six replicates were performed per condition, and proliferation was

Table 1 Real-time quantitative PCR primer-probe combination

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5' FAM - 3' TAMRA)
IL6	TAGCCGCCCCACACAGA	GTGCCTCTTTGCTGCTTTCAC	AGCCACTCACCTCTTCAGAACGAATTGACA
IL8	GGCCGTGGCTCTCTTGG	GGGTGGAAAGGTTTGGAGTATGT	TGTGTGAAGGTGCAGTTTTGCCAAGGA
HAS2	AATGGGGTGGAAAAAGAGAAGTC	CAACCATGGGATCTTCTTCTAAAAC	TCCACACTTCGTCCCAGTGCTCTGA
ABL	TGGAGATAACATCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT

assessed after 24 hours by colorimetric assay based on the uptake and subsequent release of methylene blue dye. Proliferation was calculated as percentage above control by comparing stimulated to unstimulated conditions as described before [11].

Hyaluronan, CCL2, IL-6 and IL-8 production by orbital fibroblasts

Orbital fibroblasts were seeded at 5.0 x 10^4 cells/well into 12-well plates in DMEM 10% FCS and allowed to grow until fully confluent monolayers were established. Then, the cultured orbital fibroblast monolayers were put overnight in DMEM 1% FCS. The effect of imatinib mesylate and dasatinib on PDGF-BB-induced hyaluronan, CCL2, IL-6 and IL-8 production was studied, again both for TKI pre-incubation and simultaneous treatment with TKI and PDGF-BB. Supernatant was collected after 24 hours of stimulation with PDGF-BB (50 ng/ml) and the amount of hyaluronan, CCL2, IL-6 and IL-8 was determined by ELISA according to the manufacturer's protocol.

***HAS2*, *CCL2*, *IL6* and *IL8* mRNA expression by whole orbital tissue in culture**

Orbital tissue was obtained from a patient who underwent emergency orbital decompression surgery because of active GO (clinical activity score: 3/7) despite intravenous corticosteroid treatment. Orbital tissue was divided into two parts. One part was cultured overnight in the presence of dasatinib while the other part was cultured in the absence of dasatinib in DMEM 1% FCS. For this proof of principle experiment examining the effectiveness of dasatinib on active GO tissue, dasatinib was used in a concentration of 2.5 µg/ml. RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit according to manufacturer's protocol (Sigma-Aldrich, St Louis, MO) and was reverse transcribed into cDNA [11]. *HAS2* (Hyaluronan synthase 2), *CCL2*, *IL6* and *IL8* mRNA expression levels were determined in triplicate by real-time quantitative PCR (7900 PCR system; Applied Biosystems, Foster City, CA) and the analyzed gene transcripts were normalized to the control gene *ABL. CCL2* mRNA level was determined with commercially available primer-probe assay (TaqMan Gene Expression Assays Hs00234140_m1, Life technologies, Foster City, CA). Other primer-probe combinations used are listed in table 1 [11, 12].

Statistical analysis

Differences between unstimulated or stimulated orbital fibroblasts with PDGF-BB, and with or without TKI were analyzed using the Mann-Whitney *U* test. Differences in gene expression level in orbital tissue with and without dasatinib incubation were analyzed using the paired t-test. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

Results

Dasatinib inhibits PDGF-BB-induced orbital fibroblast proliferation more effectively than imatinib mesylate

PDGF-BB stimulated orbital fibroblasts proliferation (figure 1A), which is in line with our previous observations [11, 12]. No difference in PDGF-BB-induced proliferation was observed between orbital fibroblasts obtained from GO and healthy controls (data not shown), again in line with our previous observations [11, 12]. Pre-incubation with imatinib mesylate dose-dependently reduced PDGF-BB-induced orbital fibroblast proliferation (figure 1A). This inhibitory effect of imatinib mesylate reached statistical significance from a concentration of 0.16 µg/ml and higher (figure 1A). Dasatinib blocked PDGF-BB-induced orbital fibroblast proliferation much more effective than imatinib mesylate, already reaching statistical significance at a concentration of 0.01 µg/ml (figure 1B). We found no effect of the concentrations of imatinib mesylate and dasatinib used on basal orbital fibroblast proliferation (data not shown).

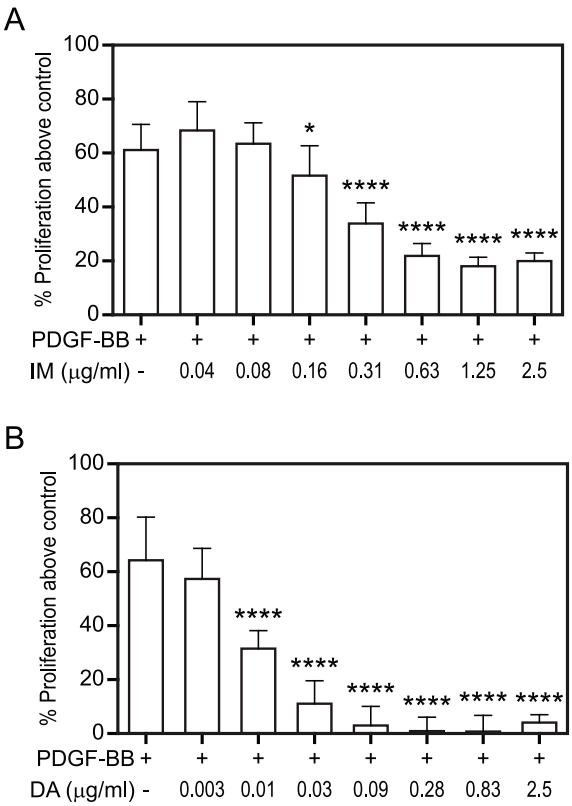

Fig. 1 Control ($n = 5$) and GO ($n = 4$) orbital fibroblasts were pre-incubated with either imatinib mesylate or dasatinib for 16 h, then stimulated with 50 ng/ml PDGF-BB. Twofold serial dilution was performed with imatinib mesylate, whereas threefold serial dilution was done with the highly potent dasatinib. After 24 h, proliferation was detected by methylene blue staining. Imatinib mesylate (**a**) significantly inhibits PDGF-BB-induced orbital proliferation from a concentration of 0.16 µg/ml and higher. Dasatinib (**b**) inhibits PDGF-BB-induced orbital proliferation significantly from a concentration as low as 0.01 µg/ml. Results are presented as the mean value from nine different orbital fibroblast cultures, with error bars representing SEM. **** and * represent a *p*-value of <0.0001 and <0.05, respectively, compared to PDGF-BB stimulation. IM, imatinib mesylate: DA, dasatinib: GO, Graves' ophthalmopathy; PDGF-BB, platelet-derived growth factor-BB

Dasatinib inhibits PDGF-BB-induced hyaluronan production by orbital fibroblasts more effectively than imatinib mesylate

PDGF-BB induced hyaluronan production by orbital fibroblasts, in line with our previous observations [12]. No difference in PDGF-BB-induced hyaluronan production was observed between orbital fibroblasts obtained from GO and healthy controls (data not shown), as we observed previously [11, 12]. Pre-incubation with imatinib mesylate inhibited PDGF-BB-induced hyaluronan production by orbital fibroblasts in a dose-dependent manner, reaching statistical significance from a concentration of 0.16 µg/ml and higher (figure 2A). Pre-incubation with dasatinib inhibited hyaluronan production much more effective than imatinib mesylate, with statistical significance at a concentration of 0.04 µg/ml (figure 2B). The concentrations of imatinib mesylate and dasatinib used did not inhibit basal hyaluronan production by orbital fibroblasts (data not shown).

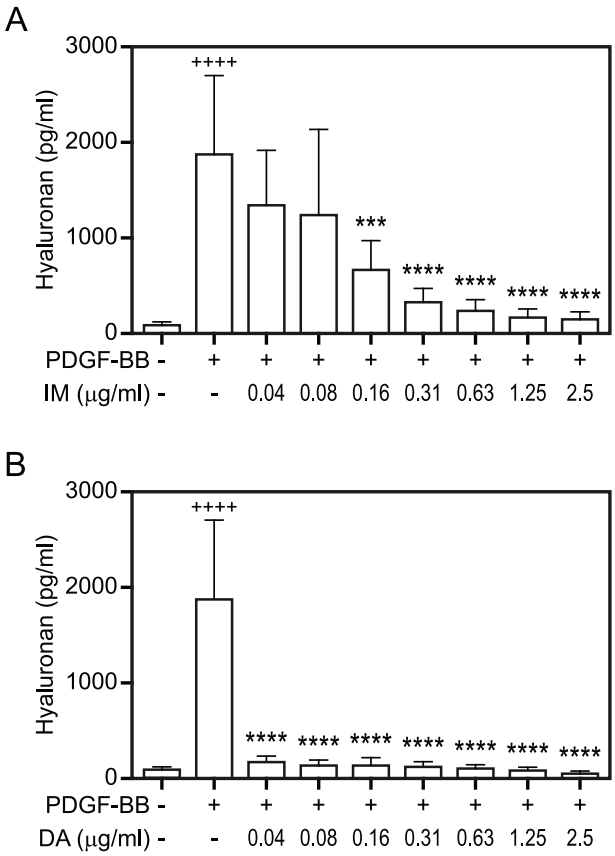

Fig. 2 Control ($n = 5$) and GO ($n = 4$) orbital fibroblasts were pre-incubated with either imatinib mesylate or dasatinib for 16 h, and then stimulated with 50 ng/ml PDGF-BB. Twofold serial dilution was performed both with imatinib mesylate and dasatinib. After 24 h, supernatant was collected, and hyaluronan levels were measured by ELISA. Imatinib mesylate (**a**) significantly reduces PDGF-BB-induced hyaluronan production from a concentration of 0.16 μg/ml and higher. Dasatinib (**b**) significantly inhibits PDGF-BB-induced hyaluronan production from a concentration of 0.04 μg/ml and higher. Results are presented as the mean value from nine different orbital fibroblast cultures, with error bars representing SEM. $^{++++}$ indicates a p-value of <0.0001 compared to the unstimulated condition, and **** and *** represent a p-value of <0.0001 and <0.001, respectively, compared to PDGF-BB stimulation. IM, imatinib mesylate: DA, dasatinib; GO, Graves' ophthalmopathy; PDGF-BB, platelet-derived growth factor-BB

Dasatinib inhibits PDGF-BB-induced CCL2, IL-6, and IL-8 production by orbital fibroblasts more effectively than imatinib mesylate

PDGF-BB induced CCL2, IL-6, and IL-8 production to comparable levels by orbital fibroblasts from GO patients and healthy controls, similar to our previous observation [10, 12]. Pre-incubation with imatinib mesylate significantly reduced PDGF-BB-induced cytokine production by orbital fibroblasts in a dose-dependent manner from a concentration of 0.04 µg/ml for IL-6, 0.31 µg/ml for IL-8 and 0.16 µg/ml for CCL2 (figure 3). Pre-incubation with dasatinib showed more effective inhibition of PDGF-BB-induced CCL2, IL-6 and IL-8 production by orbital fibroblasts than imatinib mesylate, from a concentration of 0.04 µg/ml for all three cytokines (figure 3). The concentrations of imatinib mesylate and dasatinib used did not inhibit basal production of cytokines by orbital fibroblasts (data not shown).

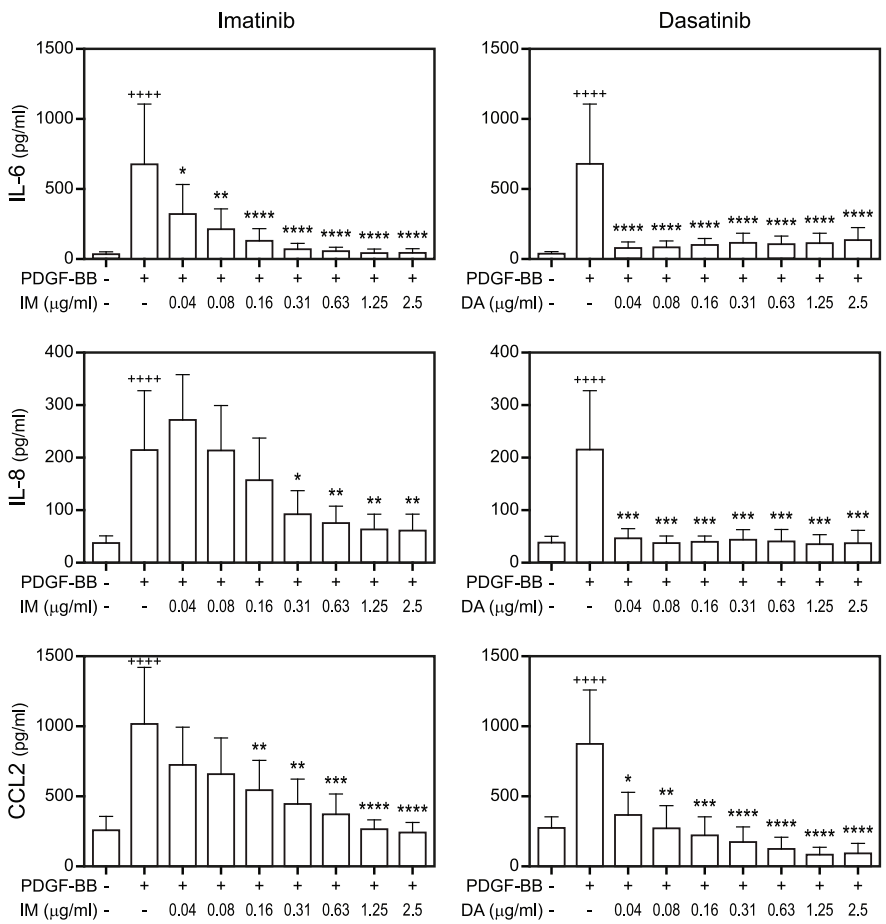

Fig. 3 Control ($n = 5$) and GO ($n = 4$) orbital fibroblasts were pre-incubated with either imatinib mesylate or dasatinib for 16 h, and then stimulated with 50 ng/ml PDGF-BB. Twofold serial dilution was performed both with imatinib mesylate and dasatinib. After 24 h, supernatant was collected, and IL-6, IL-8, and CCL2 levels were measured by ELISA. Imatinib mesylate (**a**) and dasatinib (**b**) significantly reduce PDGF-BB-induced IL-6, IL-8, and CCL2 production by orbital fibroblasts, with dasatinib being effective at lower concentrations. Results are presented as the mean value from nine different orbital fibroblasts, with error bars representing the SEM. $^{++++}$ indicates a *p*-value of <0.0001 compared to the unstimulated condition, and ****, ***, **, and * represent *p*-value of <0.0001, <0.001, <0.01, and <0.05, respectively, compared to PDGF-BB stimulation. IM, imatinib mesylate; DA, dasatinib; GO, Graves' ophthalmopathy; PDGF-BB, platelet-derived growth factor-BB

Dasatinib exhibits a stronger effect than imatinib mesylate in blocking PDFG-BB-induced orbital fibroblast activities when TKI and PDGF-BB are administered simultaneously

We further investigated the effect of imatinib mesylate and dasatinib without pre-incubation step. Hereto two concentrations were used, the highest (2.5 µg/ml) and the lowest (0.04 µg/ml) concentrations tested in the previous studies. Imatinib mesylate only inhibited PDGF-BB-induced orbital fibroblast proliferation (figure 4A), hyaluronan (figure 4B), IL-6 (figure 4C) and CCL2 (figure 4E) production at a concentration of 2.5 µg/ml, while the inhibitory effect on IL-8 production was not significant. Dasatinib suppressed PDGF-BB-induced orbital fibroblast proliferation (figure 4A), hyaluronan (figure 4B), IL-6 (figure 4C), and CCL2 (figure 4E) production at both the concentration of 2.5 and 0.04 µg/ml, while PDGF-BB-induced IL-8 production was only significantly inhibited with the concentration of 2.5 µg/ml (figure 4D).

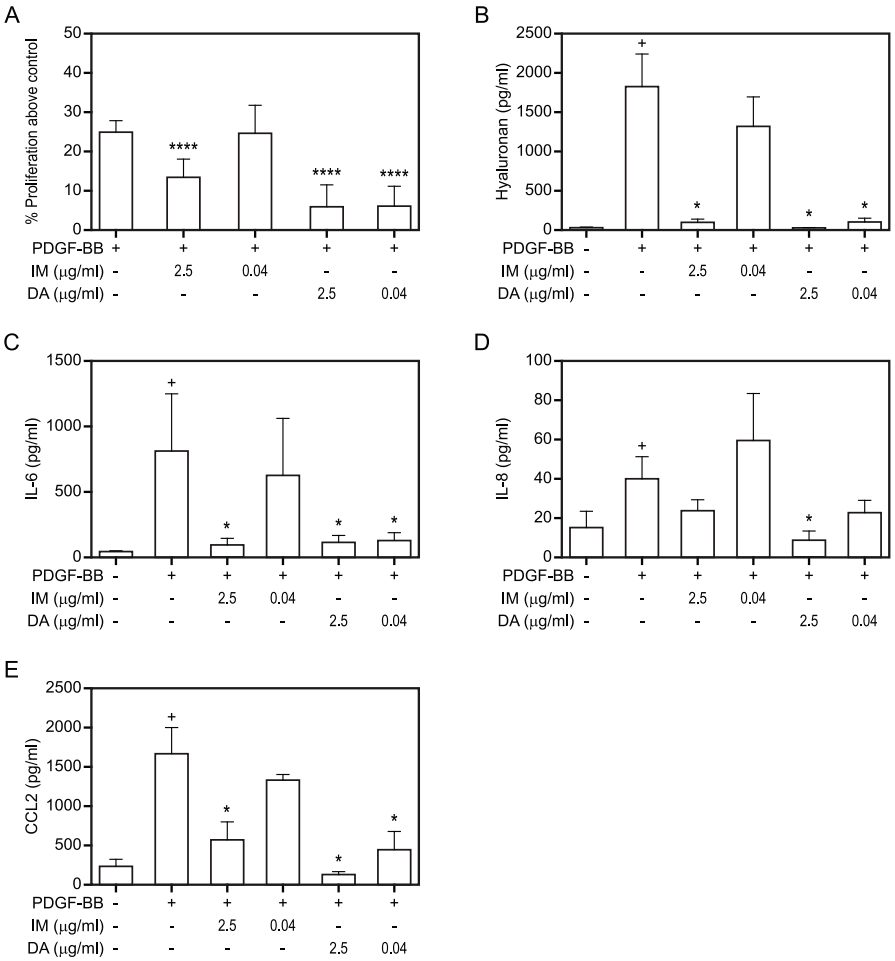

Fig. 4 Control ($n = 2$) and GO ($n = 2$) orbital fibroblasts were stimulated with 50 ng/ml PDGF-BB simultaneously with either imatinib mesylate or dasatinib for 24 h before assessing proliferation (**a**) and production of hyaluronan (**b**), IL-6 (**c**), IL-8 (**d**), and CCL2 (**e**). Dasatinib suppresses PDGF-BB-induced orbital fibroblast proliferation and production of hyaluronan, IL-6, and CCL2 more effectively than imatinib mesylate. Results are presented as the mean value from four different orbital fibroblast cultures, with error bars representing the SEM. $^{+}$ indicates a p-value of <0.05 compared to the unstimulated condition, and **** and * represent a p-value of <0.0001 and <0.05, respectively, compared to PDGF-BB stimulation. IM, imatinib mesylate; DA, dasatinib; GO; Graves' ophthalmopathy; PDGF-BB, platelet- derived growth factor-BB

Dasatinib reduces HAS2, CCL2, IL6, and IL8 mRNA expression levels in orbital tissue from active GO

Dasatinib significantly reduced the mRNA expression levels of the cytokine genes *CCL2*, *IL6* and *IL8* in orbital tissue from active GO by approximately 20 - 30 fold. Expression of the *HAS2* gene, which encodes the major hyaluronan synthase in GO [12], was also significantly reduced in the orbital tissue by approximately 2-fold by dasatinib (figure 5).

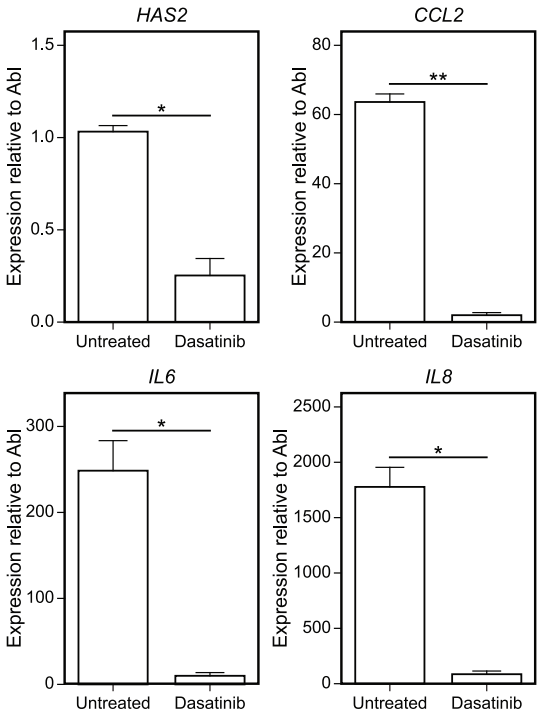

Fig. 5 Whole orbital tissue was obtained from a patient with active GO and separated into two parts. One part was cultured overnight in the presence of dasatinib, while the other part was cultured in the absence of dasatinib in DMEM 1 % FCS. For this proof of principle experiment, dasatinib was used in a concentration of 2.5 µg/ml. HAS2, CCL2, IL6, and IL8 mRNA expression levels were determined by real-time quantitative PCR. Dasatinib significantly suppressed HAS2, CCL2, IL6, and IL8 mRNA levels in the orbital tissue. Results are presented as the mean value from three independent real-time quantitative PCR reactions. Error bars represent SEM. * indicates a *p*-value of <0.05.

Discussion

GO is characterized by orbital tissue inflammation. Symptoms may range from very mild inflammation merely causing cosmetic complaints to severe inflammation of all orbital tissues that causes decreased ocular motility and visual acuity. Despite recent progress in the understanding of the pathogenesis of GO, medical treatment is as yet limited to the administration of corticosteroids. The outcome of corticosteroid treatment is often not satisfactory and holds side-effects. Novel therapeutic possibilities are eagerly awaited. In this study we demonstrate that the TKI dasatinib effectively blocks activation (proliferation, cytokine and hyaluronan production) of orbital fibroblasts by PDGF-BB, a growth factor that fulfils a central pathogenic role in GO [10, 11, 20], as well as cytokine and hyaluronan synthase gene expression in orbital tissue from active GO. In recent years we identified PDGF-AB and especially PDGF-BB, that are produced within the orbital tissue from GO patients by monocytes, macrophages and mast cells, as potent stimuli of inflammatory mediator (e.g. CCL2, IL-6 and IL-8) and hyaluronan production, as well as proliferation by orbital fibroblasts [10, 12]. Consequently, we proposed that specific inhibition of the action of PDGF-B chain containing isoforms could be a promising therapeutic approach for the treatment of GO [12]. Such treatment could be done with neutralizing antibodies which, to our knowledge, are currently not available for clinical application. PDGF dimers exert their effects via activation of two receptor chains: PDGF-Rα and PDGF-Rβ. Upon ligand binding, receptor chains dimerize and subsequently autophosphorylation of tyrosine residues within the cytoplasmic tails and signaling occur [9]. PDGF-BB can signal via αα, αβ and ββ receptor dimers, while PDGF-AB signals via αα and αβ dimers [9]. In previous studies we showed that the PDGF-R targeting TKI imatinib mesylate blocked PDGF-BB and PDGF-AB induced proliferation of orbital fibroblasts and decreased hyaluronan and cytokine production in both orbital fibroblasts and whole orbital tissue cultures of GO patients [10-12, 14]. Based on these findings we hypothesized that TKIs constitute promising agents to treat GO. Recently, however, Bournia et al. [21] reported on the safety and efficacy of imatinib mesylate in systemic sclerosis. They concluded that imatinib mesylate was not well tolerated by all patients while a large proportion of patients did not respond to therapy.

Therefore, we now studied the *in vitro* effects of the second-generation TKI dasatinib. We found that dasatinib, a TKI with high affinity for both the PDGF-Rα and the PDGF-Rβ chain, suppressed PDGF-BB-induced proliferation, hyaluronan production and CCL2, IL-6 and IL-8 production by orbital fibroblasts very effectively at low dosages. Moreover, our data show that dasatinib exhibits stronger anti-inflammatory, anti-proliferative and hyaluronan synthesis suppressing effects than imatinib mesylate. We found this superior effect of dasatinib in two different experimental strategies: a strategy in which orbital fibroblasts were exposed to the TKI

before PDGF-BB stimulation, and a more clinically relevant strategy, in which TKI treatment was commenced simultaneously with PDGF-BB exposure. Especially the results from the latter experimental strategy suggest that dasatinib is an attractive drug to target the over-active PDGF signaling that is associated with GO. For our current studies we used orbital fibroblasts obtained from GO at inactive stage of disease, while it is mostly patients with moderate-severe and active disease that are undergoing immunosuppressive therapy. Previously we demonstrated equal elevation of PDGF-BB levels in orbital tissue from GO patients with active and inactive disease [11]. Moreover, our previous studies revealed that orbital fibroblasts from GO patients with active or inactive disease responded equally to PDGF-BB with regard to proliferation, hyaluronan and cytokine production [10, 11]. In addition, the tyrosine kinase inhibitors imatinib mesylate and nilotinib blocked these effects of PDGF-BB equally effective in orbital fibroblasts obtained from active and inactive GO tissue. We therefore expect dasatinib to be effective on orbital fibroblasts from patients with active GO as well. In support of this, we demonstrated that dasatinib suppressed the mRNA expression levels of the cytokines CCL2, IL-6 and IL-8 as well as that of the hyaluronan synthase HAS2 in orbital tissue from a GO patient that underwent emergency decompression surgery because of active disease.

In a recent large randomized clinical trial in patients with CML, comparing dasatinib and imatinib mesylate, dasatinib was found to be well tolerated and with the exception of pleural effusion, all of the most common drug-related non-hematological adverse events were either lower with dasatinib or comparable between arms [22]. The most important non-hematological adverse effects include skin rash, nausea, vomiting, diarrhea, fatigue and headache [22, 23] Pleural effusion grade 1 and 2 occurs in approximately 7-10% of CML patients and led to discontinuation of dasatinib treatment in 1 to 3 % of patients [22, 23]. After discontinuation pleural effusion was found to be reversible in these studies. Hematological adverse events such as anemia, thrombocytopenia and neutropenia occur in about 15% of patients [23]. These side effects cannot be excluded to occur when used for GO treatment, but the incidences reported in cancer treatment are relatively low and the occasional occurrence of pleural effusion was found to be reversible upon discontinuation of the therapy. Importantly in this respect, our study clearly indicates that dasatinib effectively inhibits PDGF-induced orbital fibroblast activation already at low dose. Altogether, we presume that dasatinib might be safe for treatment of GO in lower doses as used in CML.

On the other hand, it should be taken into account that GO may show a self-limiting course. Therefore, the use of a compound like dasatinib, with potential side effects should be introduced in clinics with greatest precaution. We therefore feel that dasatinib might be of value in treating those GO patients with still active and progressive disease under currently available immunosuppressive treatment modalities.

In conclusion, our current data suggest that dasatinib might be an attractive drug to inhibit PDGF activity in GO. Its effectiveness at relative low dosages suggests that it can suppress both orbital tissue inflammation and expansion in patients with GO without causing serious side effects. We therefore suggest that dasatinib should be clinically evaluated as treatment option for GO.

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Chapter 4

Platelet-derived growth factor-BB enhances adipogenesis in orbital fibroblasts

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Abstract

Purpose

Platelet-derived growth factor (PDGF)–BB has been identified as important factor in pathogenesis of Graves' ophthalmopathy (GO). It stimulates proliferation, cytokine, and hyaluronan production, and thyrotropin receptor expression by orbital fibroblasts. Therefore, the PDGF-pathway has been proposed as a target for pharmacological intervention in GO. However, increased adipogenesis is another major pathological characteristic of GO and it is unknown whether this is affected by PDGF-BB. The aim of this study was to investigate the effect of PDGF-BB on adipocyte differentiation by orbital fibroblasts.

Methods

Orbital fibroblasts from five healthy controls and nine GO patients were collected. Adipogenesis was induced by culturing orbital fibroblasts in differentiation medium, either in the presence or absence of PDGF-BB. Adipogenesis was determined by Oil- Red-O staining, triglyceride measurement, and peroxisome proliferator-activated receptor (PPAR)–γ mRNA expression.

Results

Platelet-derived growth factor-BB significantly enhanced adipocyte differentiation by orbital fibroblasts (Oil-Red-O staining [$P < 0.0001$], triglyceride measurement [$P < 0.05$], and PPAR-γ mRNA expression [$P < 0.05$]). It enhanced IL-6 production early during differentiation, but the effect of PDGF-BB on adipogenesis was independent of autocrine IL-6 signaling as it was not abrogated by IL-6–receptor-α neutralizing antibody. The clinically applicable tyrosine kinase inhibitor dasatinib and tyrphostin AG1296, which both block PDGF receptor tyrosine kinase activity, inhibited PDGF-BB–enhanced adipogenesis ($P < 0.05$) in orbital fibroblasts. Moreover, dasatinib reduced PPAR-γ mRNA expression in cultured GO orbital tissue.

Conclusions

Platelet-derived growth factor–BB enhances adipogenesis in orbital fibroblasts, and, thus, may contribute to adipose tissue expansion in GO. Therefore, the PDGF-signaling cascade may represent a target of therapy to interfere with adipogenesis in GO.

Introduction

Graves' ophthalmopathy (GO) is an extrathyroidal complication of Graves' hyperthyroidism, and results from inflammation and expansion of the soft tissues surrounding the eyes. In most patients the adipose/connective tissue and extraocular muscle volumes increase, while in some patients either adipose/connective tissue expansion or extraocular muscle enlargement may predominate.[1] Activation of orbital fibroblasts by inflammatory cytokines and lipids, growth factors, and stimulatory autoantibodies against the thyrotropin receptor (TSHR), and possibly the insulin-like growth factor-1 receptor is the central hallmark of GO's pathogenesis.[1] The activated orbital fibroblasts produce cytokines and chemokines that are involved in the recruitment, activation, and differentiation of immune cells.[2,3] In addition, the orbital fibroblasts display increased proliferative activity, produce excess amounts of glycosaminoglycans (especially hyaluronan), and differentiate into mature adipocytes.[1] Together, these processes contribute to orbital tissue volume expansion within the noncompliant space-limited bony orbit, which causes typical clinical features, including upper eyelid retraction, edema, erythema of the periorbital tissues and conjunctivae, and proptosis.[1]

Previously we found that orbital tissue from GO patients contains increased levels of platelet-derived growth factor (PDGF)–BB, a growth factor that is important in developmental and healing processes, while excessive amounts are associated with diseases characterized by pathologic tissue remodeling.[1] Platelet-derived growth factor–BB appeared to be a potent stimulator of proliferation, and cytokine and hyaluronan production by orbital fibroblasts.[4–6] Moreover, PDGF-BB enhanced TSHR expression on orbital fibroblasts, which augmented the capacity of TSHR stimulatory autoantibodies from GO patients to stimulate the production of various cytokines by orbital fibroblasts.[7] These data implicate that PDGF-BB may represent an important pathologic growth factor in GO where it regulates orbital inflammation, proliferation of fibroblasts, and hyaluronan production. However, until now the effect of PDGF-BB on adipocyte differentiation by orbital fibroblasts, another major determinant of orbital tissue expansion in GO,[8,9] has remained unknown.

The present study was done to investigate the effect of PDGF-BB on adipocyte differentiation by orbital fibroblasts. We showed that PDGF-BB enhances adipocyte differentiation by orbital fibroblasts cultured in a proadipocytic culture environment independent of autocrine IL-6–receptor signaling. In addition, we showed that inhibition of the PDGF receptor with the tyrosine kinase inhibitor (TKI) dasatinib or tyrphostin AG1296 diminishes the effects of PDGF-BB on adipogenesis.

Materials and Methods

Cell Culture

Orbital tissue was obtained from nine euthyroid GO patients who underwent orbital decompression surgery at an inactive stage of disease. The patients had not received steroid or other immunosuppressive treatment for at least 3 months before surgery. Furthermore, orbital tissue was obtained from five controls without thyroid or inflammatory disease who underwent orbital surgery for other reasons. All orbital tissues were obtained at 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 from the orbital tissues as described previously.[4] Once fibroblast monolayers were obtained, cultures were passaged serially after gentle treatment with trypsin/EDTA, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin; Cambrex BioWhittaker, Verviers, Belgium).[4] Orbital fibroblasts used for experiments were between the fourth and 12th passage.

Adipocyte Differentiation

Orbital fibroblasts were seeded at 4.0×10^5 cells/well into 6-well plates in DMEM 10% FCS and allowed to adhere overnight. Adipocyte differentiation was induced as described previously.[8] Briefly, culture medium was changed into differentiation medium consisting of 1:1 serum-free DMEM/Ham's F12 (HyClone, Logan, UT, USA) supplemented with antibiotics, 33 µM biotin (Sigma-Aldrich Corp., St. Louis, MO, USA), 17 µM pantothenic acid (Sigma-Aldrich Corp.), 1 µM insulin (Sigma-Aldrich Corp.), 10 µg/mL transferrin (Merck, Darmstadt, Germany), 0.2 nM Triiodothyronine (T3; Sigma- Aldrich Corp.), 0.2 µM carbaprostaglandin (PGI_2; Cayman Chemical Company, Ann Arbor, MI, USA), and 10 µM rosiglitazone (Sigma-Aldrich Corp.) for 14 days. In the first 3 days the differentiation medium also was supplemented with 10 µM dexamethasone (Sigma-Aldrich Corp.) and 0.1 mM isobutylmethylxantine (IBMX; Sigma-Aldrich Corp.). The differentiation protocol was continued for 14 days, differentiation medium was refreshed every 3 to 4 days. Adipocyte differentiation was compared between orbital fibroblasts cultured with differentiation medium, and orbital fibroblasts cultured with medium devoid of PGI_2 and rosiglitazone (nondifferentiation medium).

The effect of PDGF-BB on adipocyte differentiation was compared between orbital fibroblasts cultured in differentiation medium in the presence or absence of

recombinant human PDGF-BB (50 ng/mL; R&D Systems, Abingdon, UK). Adipogenesis was assessed by Oil-Red-O staining, triglyceride measurement, and transcript measurement of the adipocyte predominant transcription factor peroxisome proliferator- activated receptor-γ (PPAR-γ). Concentration of IL-6 was determined by ELISA (Invitrogen, Frederick, MD, USA) in culture supernatant obtained following 3, 7, 10, and 14 days of differentiation.

Oil-Red-O Staining

Oil-Red-O was freshly prepared by mixing 6 mL 1% Oil-Red-O stock solution with 4 mL milli-Q and filtering through a 0.45-µm filter (Whatman, Dassel, Germany). The adipocyte differentiation cultures were washed twice with 1 mL PBS (pH 7.4) per well and stained by adding 1 mL Oil-Red-O solution for 10 minutes at room temperature. Subsequently, the culture plates were washed 4 times with distilled water to remove excess Oil-Red-O and visualized using an Axiovert 100 light microscope (Zeiss, Oberkochen, Germany), and photographed at ×200 magnification using an AxioCam MR5 (Zeiss).

For quantification of Oil-Red-O staining, the Oil-Red-O was eluted from the cells with 1 mL absolute isopropanol, 200 µL solution was transferred into a 96-well plate, and the optical density was measured with a spectrophotometer at 490 nm.

Triglyceride Measurement

Two control and two GO orbital fibroblast stains were selected randomly for triglyceride measurement. Briefly, after 14 days of adipocyte differentiation, the cells were washed with 1 mL cold PBS (4°C, pH 7.4), harvested by scraping in PBS, and further disrupted with a syringe. Triglyceride was measured using a Triglycerides FS kit (DiaSys, Holzheim, Germany) according to the manufacturer's protocol.

Real-Time Quantitative (RQ)–PCR Analysis

Messenger RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Corp.) and converted into cDNA as described previously.[4] Transcript level of PPAR-γ was determined by RQ-PCR (7900 PCR system; Applied Biosystems, Foster City, CA) and normalized to the control gene ABL.[4] Primer-probe combinations used are listed in the Table 1.

Table 1. Real-time quantitative PCR primer-probe combination

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5' FAM - 3' TAMRA)
ABL	TGGAGATAACATCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT
PPAR-γ	TGACAGCGACTTGGCAATATTT	TCTTCAATGGGCTTCACATTCA	CAAACCTGGGCGGTCTCCACTGAG

Poliferation Assay

Proliferation was examined in cultures from three control and three GO orbital fibroblast strains after the differentiation period of 14 days using Z2 Coulter Counter (Beckman Coulter, Brea, CA, USA). Proliferation was compared between the following culture conditions: nonadipocyte differentiation, adipocyte differentiation, and adipocyte differentiation in the presence of PDGF-BB.

IL-6 Receptor Neutralization

Two control and three GO orbital fibroblast strains were selected randomly and the fibroblasts were seeded into 6-well plates for adipocyte differentiation. Adipocyte differentiation was induced as described earlier in the presence or absence of PDGF- BB (50 ng/mL) or IL-6 (1.5 ng/mL; R&D Systems). A monoclonal mouse-anti-human IL-6-receptor-α neutralizing antibody (0.2 ng/mL; IgG1, MAB2227; R&D Systems) or mouse IgG_1 isotype control (0.2 ng/mL, MAB002; R&D Systems) was simultaneously added to the differentiation medium. The differentiation cultures were continued for 14 days and the medium, including the antibody, was refreshed every 3 to 4 days. Adipogenesis was determined by Oil-Red-O measurement.

The Effect of Dasatinib and Tyrphostin AG1296 on Adipocyte Differentiation

To demonstrate involvement of PDGF-receptor signaling in adipogenesis, two control and two GO orbital fibroblast strains were cultured in adipocyte differentiation medium with/without PDGF-BB (50 ng/mL), and either in the presence or absence of the clinically available TKI dasatinib (0.04 µg/mL [0.08 µM]; Selleckchem, Houston, TX, USA) that has high inhibitory potency for the PDGF receptor.[10] Adipogenesis was determined by Oil-Red-O staining. In an additional set of experiments with four control and four GO orbital fibroblast strains adipogenesis was quantified by measuring PPAR- γ mRNA expression levels. Furthermore, involvement of PDGF-receptor signaling was demonstrated using another specific inhibitor of PDGF-receptor kinase activity, the tyrphostin AG1296.[11,12] Hereto, three GO orbital fibroblast strains were cultured in adipocyte differentiation medium with/without PDGF-BB (50 ng/mL) and either in the presence or absence of AG1296 (10 and 100 µM; Merck). Adipogenesis was quantified by measuring PPAR-γ mRNA expression levels.

The Effect of Dasatinib on PPAR-γ mRNA Expression in Orbital Tissue From GO Patients

Orbital tissues were obtained from three GO patients who underwent orbital decompression surgery. Orbital tissue was divided into two parts and put in culture overnight in DMEM 1% FCS in the presence or absence of dasatinib (2.5 μg/mL) as described previously.[13] Messenger RNA was extracted, reversed transcribed into cDNA, and PPAR-γ mRNA expression level was determined.

Statistical Analysis

Data were analyzed using the paired Student's *t*-test. Correlation analysis was performed using Spearman's correlation test. A *P* value of <0.05 was considered statistically significant.

Results

Effect of PDGF-BB on Lipid Accumulation in Orbital Fibroblasts

Microscopic examination of Oil-Red-O stained orbital fibroblast cultures revealed that GO as well as control orbital fibroblasts cultured in differentiation medium clearly accumulated fat droplets when compared to culture in nondifferentiation medium (Fig. 1). Addition of PDGF-BB to the differentiation medium further enhanced fat droplet accumulation in the orbital fibroblast cultures when compared to differentiation medium alone (Fig. 1). To quantify the amount of lipid accumulation Oil-Red-O was eluted and the optical density of the eluate was measured. This showed a statistically significant ($P < 0.05$) increase of lipid accumulation in the orbital fibroblasts cultured in differentiation medium. Addition of PDGF-BB to the differentiation medium resulted in significantly ($P < 0.0001$) more Oil-Red-O accumulation when compared to differentiation medium alone (Fig. 2A). No differences in responses were observed between control and GO orbital fibroblasts (data not shown).

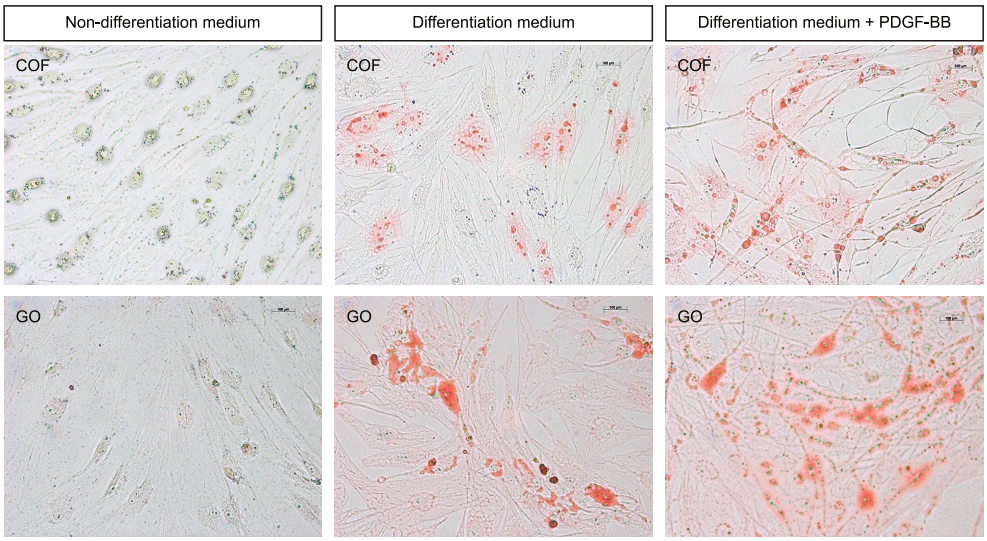

Figure 1 Oil-Red-O staining on orbital fibroblasts.

Control orbital fibroblasts (COF, upper panels) and GO orbital fibroblasts (GO, lower panels) were cultured in nondifferentiation medium (left column), adipocyte differentiation medium (middle column), and adipocyte differentiation medium in the presence of PDGF-BB (50 ng/mL, right column). Oil-Red-O staining was performed after 14 days of differentiation. Representative COF and GO orbital fibroblasts are depicted.

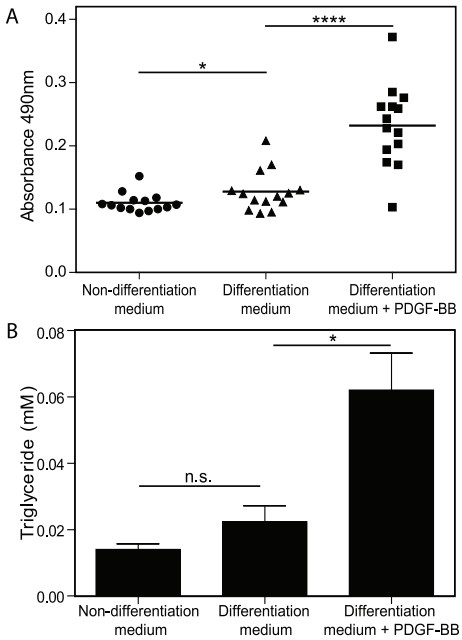

Figure 2 Quantification of adipogenesis.
(**A**) Oil-Red-O staining was quantified in eluate after 14 days of differentiation from orbital fibroblasts from controls (n = 5) and GO patients (n = 9) using a spectrophotometer at 490 nm. Each *dot* represents the orbital fibroblast strain from one individual and *horizontal bars* represent the mean values within a group. (**B**) Triglyceride accumulation was measured after 14 days of differentiation in orbital fibroblasts from controls (n = 2) and GO patients (n = 2) using a Triglycerides FS kit. Data are presented as the mean ± SEM. Data were analyzed using the paired Student's t-test. *P < 0.05 compared to nondifferentiation medium and ****P < 0.0001 compared to differentiation medium without PDGF-BB.

The adipogenesis enhancing effect of PDGF-BB was confirmed by triglyceride measurement, which showed significantly (P < 0.05) higher triglyceride accumulation in orbital fibroblasts cultured in differentiation medium containing PDGF-BB compared to orbital fibroblasts cultured in differentiation medium without PDGF-BB (Fig. 2B). Previously we identified PDGF-BB as a potent mitogen for orbital fibroblasts.[4,6] In our current experimental set-up, which has different culture conditions compared to our previous studies, PDGF-BB did not stimulate proliferation by the orbital fibroblasts during the 14 days of culture in adipogenesis promoting culture medium (data not shown). This precludes the possibility that the observed increases in Oil-Red-O uptake and triglyceride accumulation were related to increased cell replication.

Effect of PDGF-BB on PPAR-γ mRNA Expression by Orbital Fibroblasts

Culture of orbital fibroblasts in differentiation medium was associated with a significant (P< 0.0001) increase in PPAR-γ mRNA expression (Fig. 3A). Addition of PDGF-BB to the differentiation medium further enhanced the expression of PPAR-γ mRNA compared to differentiation medium alone (P < 0.05). Levels of PPAR-γ mRNA correlated positively and significantly with the Oil-Red-O measurement (r = 0.426, P = 0.005; Fig. 3B).

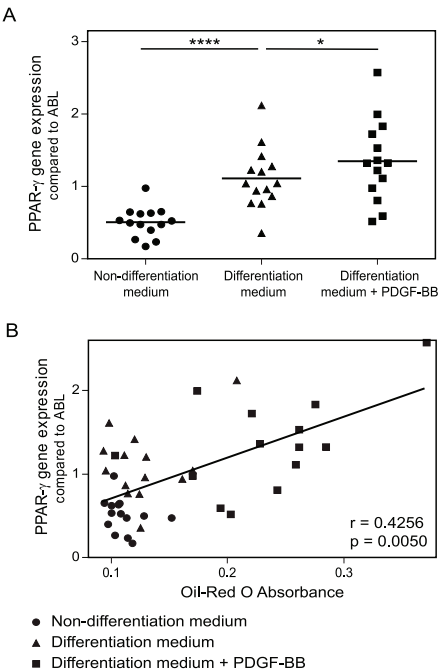

Figure 3 Expression of PPAR-γ mRNA in orbital fibroblasts upon adipocyte differentiation.

(**A**) Expression of PPAR-γ mRNA was determined by RQ-PCR in orbital fibroblasts from controls (n = 5) and GO patients (n = 9) after 14 days of adipocyte differentiation. Each *dot*represents the orbital fibroblast strain from one individual and *horizontal bars* represent the mean values within a group. Data were analyzed using the paired Student's t-test. *P < 0.05 and ****P < 0.0001, respectively. (**B**) Spearman's correlation analysis between Oil-Red-O staining and PPAR-γ mRNA expression including the following culture conditions: nonadipocyte differentiation, adipocyte differentiation, and adipocyte differentiation in the presence of PDGF- BB.

Effect of IL-6–Receptor Neutralization on PDGF-BB–Induced Adipogenesis

Platelet-derived growth factor–BB can stimulate orbital fibroblasts to produce IL-6, a cytokine previously associated with increased adipogenesis by orbital fibroblasts.[5,6,14] Therefore, we measured IL-6 secretion by the orbital fibroblasts in the culture medium. Orbital fibroblasts cultured for 3 days in differentiation medium secreted significantly ($P < 0.01$) higher levels of IL-6 compared to culture in nondifferentiation medium. The IL-6 levels were increased further ($P < 0.0001$ compared to differentiation medium) when PDGF-BB was added to the differentiation medium (Fig. 4A). The level of IL-6 secretion declined thereafter, with equal levels between the different culture conditions at days 7, 10, and 14 (Fig. 4A). The adipogenesis-enhancing effect of PDGF-BB, however, was not blocked by an IL-6-receptor neutralizing antibody (Fig. 4B). Interleukin-6–enhanced adipogenesis did not differ statistically from that of PDGF-BB– enhanced adipogenesis, but was significantly ($P < 0.01$) reduced by the IL-6 receptor neutralizing antibody (Fig. 4C).

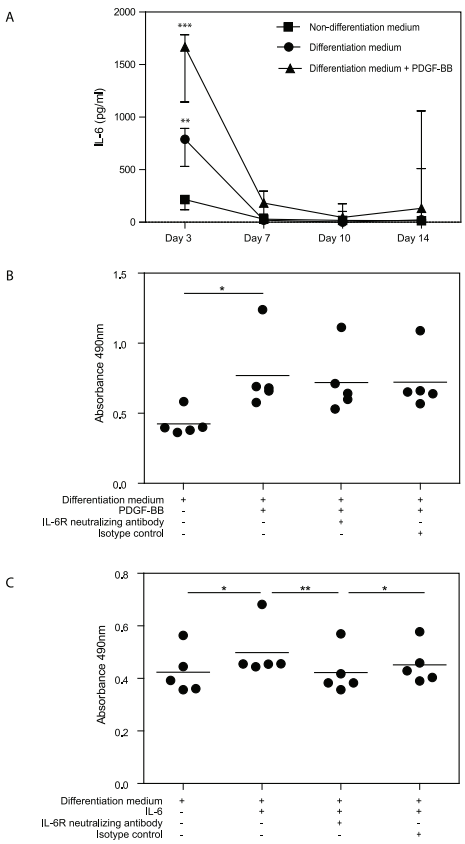

Figure 4 Production of IL-6 by orbital fibroblasts during adipocyte differentiation. (**A**) Concentration of IL-6 was determined by ELISA in culture supernatant obtained following 3, 7, 10, and 14 days of differentiation from controls (n = 2) and GO patients (n = 3) orbital fibroblasts. Data are presented as mean ± SEM, and analyzed using paired Student's t-test. $^{**}P < 0.01$ compared to nondifferentiation medium and $^{***}P < 0.001$ compared to differentiation medium without PDGF-BB. (**B**) Oil-Red-O staining was quantified in eluate from control (n = 2) and GO (n = 3) orbital fibroblasts after 14 days of adipocyte differentiation culture in the presence or absence of PDGF-BB without or in presence of a monoclonal mouse-anti-human IL-6-receptor-α neutralizing antibody or a mouse IgG1 isotype control (0.2 µg/mL). (**C**) Oil-Red-O staining was quantified in eluate from control (n = 2) and GO (n = 3) orbital fibroblasts after 14 days of adipocyte differentiation culture in the presence or absence of IL-6 (1.5 ng/mL) without or in presence of a monoclonal mouse-anti-human IL-6-receptor-α neutralizing antibody or a mouse IgG1 isotype control. Each *dot* represents the orbital fibroblast strain from one individual and

horizontal bars represent the mean values within a group. Data were analyzed using the paired Student's *t*-test. *$P < 0.05$ and **$P < 0.01$, respectively.

The Effect of Dasatinib and Tyrphostin AG1296 on PDGF-BB–Induced Adipogenesis by Orbital Fibroblasts

To confirm involvement of PDGF-receptor signaling we tested whether the clinically available TKI dasatinib (0.04 µg/mL) blocked the adipogenesis enhancing effect of PDGF-BB. Dasatinib significantly ($P < 0.05$) reduced PDGF-BB-induced Oil-Red-O accumulation up to the level achieved with differentiation medium alone (Fig. 5A). Dasatinib also reduced the PDGF-BB–induced increase in PPAR-γ mRNA up to the level achieved with differentiation medium alone, although this was not significant ($P = 0.09$, Fig. 5B). In addition, the specific PDGF receptor TKI AG1296 significantly ($P < 0.05$) inhibited PDGF-BB–induced PPAR-γ mRNA expression in the orbital fibroblasts in a dose-dependent manner (Fig. 5C).

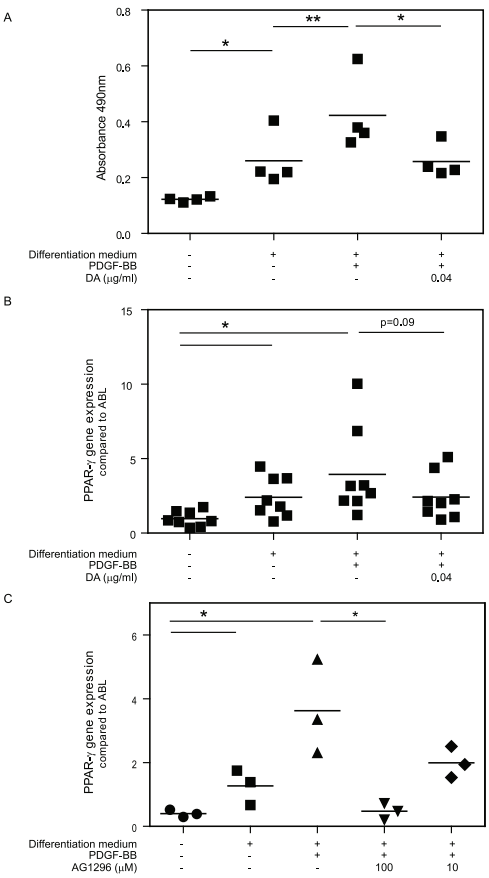

Figure 5 Effect of dasatinib on adipogenesis in orbital fibroblasts.

(**A**) Oil-Red-O staining was quantified in eluate from orbital fibroblasts (controls, *n* = 2; GO patients, *n* = 2) cultured for 14 days in differentiation medium with/without PDGF-BB and either in the presence or absence of the PDGF-receptor TKI dasatinib (0.04 µg/mL). (**B**) Expression of PPAR-γ mRNA was determined by RQ-PCR in orbital fibroblasts from controls (*n* = 4) and GO patients (*n* = 4) after 14 days of adipocyte differentiation with/without PDGF-BB and either in the presence or absence of dasatinib. (**C**) Expression of PPAR-γ mRNA was determined by RQ-PCR in orbital fibroblasts from GO patients (*n* = 3) after 14 days of adipocyte differentiation with/without PDGF-BB, and either in the presence or absence of tyrphostin AG1296 (10 and 100 µM) that inhibits PDGF receptor tyrosine kinase activity. Each *dot* represents the orbital fibroblast strain from one individual and *horizontal bars* represent the mean values within a group. Data were analyzed using the paired Student's *t*-test. **P* < 0.05 and ***P* < 0.01, respectively.

The Effect of Dasatinib on PPAR-γ mRNA Expression Orbital Tissue From GO Patients

To illustrate the potential use of dasatinib in targeting orbital adipogenesis in GO, orbital tissue specimens from three GO patients were cultured in the presence or absence of dasatinib. Dasatinib reduced the expression of PPAR-γ mRNA in all 3 orbital tissues compared to untreated tissues (Fig. 6).

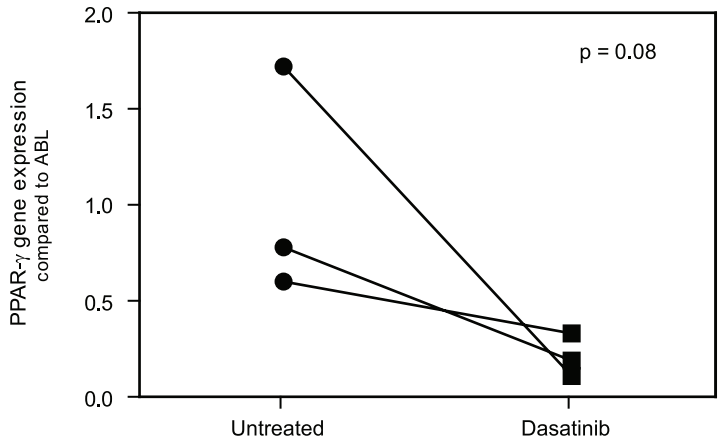

Figure 6 Effect of dasatinib on PPAR-γ mRNA expression in cultured orbital tissue from GO patients.
Orbital tissue from three GO patients was cultured in the presence or absence of dasatinib (2.5 μg/mL) overnight. Expression of PPAR-γ mRNA was determined by RQ-PCR. Data were analyzed using the paired Student's *t*-test.

Discussion

Orbital fibroblasts have the capacity to differentiate into adipocytes, and increased adipogenesis of orbital fibroblasts is a pathological characteristic and major determinant of orbital tissue volume expansion in GO.[8,9] Previously, we identified elevated orbital levels of PDGF-BB in GO,[4,6] and here we demonstrated that PDGF-BB enhances adipogenesis of orbital fibroblasts cultured in a proadipogenic environment.

In contrast to our findings, PDGF also has been reported to inhibit adipogenesis, for instance in human preadipocytes isolated from subcutaneous adipose tissue and human mesenchymal stem cells.[15,16] However, fibroblasts from different anatomical regions display characteristic and stable transcriptomes, indicating that they represent distinctly differentiated cell types.[17] In line with this, orbital fibroblasts exhibit unique features and respond differently upon stimulation with certain stimuli than fibroblasts from other anatomical regions.[4,5,18] This also holds true for PDGF-BB, which generally induces cytokine production more strongly in orbital fibroblasts than in other fibroblasts.[5] Moreover, in our current study we examined the effect of PDGF-BB on adipogenesis by three different methods: Oil-Red-O staining, intracellular triglyceride accumulation, and expression of PPAR-γ, an adipocyte predominant transcription factor of which increased expression is tightly linked to adipogenesis by orbital fibroblasts.[8,19] All three methods revealed consistent data and, therefore, indicate that PDGF-BB does enhance adipogenesis by orbital fibroblasts.

Oil-Red-O staining indicated that not all of the orbital fibroblasts accumulated fat droplets when cultured under adipocyte differentiation conditions, either in the absence or presence of PDGF-BB. Moreover, the extent of fat droplet accumulation varied among the orbital fibroblasts. These data fit with the previously noted heterogeneity within the orbital fibroblast pool with regard to adipocyte differentiation capacity.[9,20,21] Especially orbital fibroblasts that lack Thy-1 (CD90) expression were found to exhibit the capacity to differentiate into adipocytes, for instance upon stimulation with IL-1β or PGD_2.[22–24]Although not examined, it is plausible to assume that the adipogenesis-enhancing effect of PDGF-BB observed in our cultures was mediated via the Thy-1^- orbital fibroblast population. It has been suggested that the relative distribution between Thy-1^+ and Thy-1^-orbital fibroblasts within the orbital tissue is an important determinant of the extent of adipose tissue expansion in GO patients,[20] and our data strongly suggested that PDGF-BB exposure further contributes to this.

Previous studies revealed an association between IL-6 and adipogenesis in GO. Interleukin-6 serum levels were found to be increased in GO patients compared to Graves' disease patients without GO,[25] and orbital tissue IL-6 levels have been described to correlate positively with orbital adipose tissue expansion in GO.[26] Moreover, IL-6 enhances TSHR expression by orbital fibroblasts cultured in adipocyte differentiation medium, and enhanced adipogenesis is presumed to represent the main route of elevated TSHR expression by orbital fibroblasts in GO.[14,27] In our previous studies we found that PDGF-BB stimulated IL-6 production by orbital fibroblasts[5,28] and here we demonstrated that IL-6 promotes adipogenesis of orbital fibroblasts. Nevertheless, the adipogenesis-enhancing effect of PDGF-BB was not prevented by IL-6–receptor blockade, indicating that pathways other than induction of autocrine IL-6 signaling also are involved in the adipogenesis-enhancing effect of PDGF-BB. Platelet-derived growth factor signaling results in activation of kinase activity of the signaling molecule c-ABL.[29,30] Recently c-ABL activity was found to control the expression and activity of PPAR-γ and appeared to be indispensable for adipocyte differentiation by murine 3T3-L1 preadipocytes.[31] Therefore, it cannot be excluded that PDGF-BB–driven adipocyte differentiation by orbital fibroblasts involves c-ABL activity.

The adipogenesis-enhancing effect of PDGF-BB on orbital fibroblasts was abrogated by a low concentration (0.04 μg/mL) of dasatinib, a clinically available TKI that exhibits high inhibitory potency (pIC50) for the PDGF-receptor,[10] as well as by the specific PDGF receptor TKI tyrphostin AG1296. Moreover, dasatinib also downregulated PPAR-γ mRNA expression in cultured GO orbital tissues. This effect of dasatinib in the orbital tissue culture experiments did not reach statistical significance (P = 0.08), which is most likely related to the small number of tissues tested (n = 3). Nevertheless, the data clearly illustrated the potential of dasatinib in targeting adipogenesis in GO, and inhibition of the PDGF-receptor might be involved in this. As dasatinib not only targets the PDGF-receptor tyrosine kinase, we cannot exclude that dasatinib targeted other tyrosine kinase molecules in the orbital tissue as well.[10]

Previously, we proposed that PDGF-receptor targeting TKI could be of potential interest for the treatment of GO to block PDGF-induced cytokine and hyaluronan production, proliferation, and TSHR expression by orbital fibroblasts.[4,28] These data and our current findings suggest that PDGF-receptor inhibition in GO can be expected to target several pathological processes in GO, including adipogenesis. However, it also has been described that imatinib mesylate, another TKI that inhibits the PDGF-receptor, at a concentration of 5 μg/mL enhances adipogenesis by orbital fibroblasts cultured under pathological pressure.[32] Also proadipogenic effects of imatinib mesylate and dasatinib on multipotent mesenchymal stromal cells have been described.[33,34] In contrast to this, imatinib mesylate at a concentration of 2.5 μg/mL

(the highest nontoxic concentration in our hands) blocked adipogenesis by orbital fibroblasts in our culture system (Supplementary Fig. S1), although not as efficient as dasatinib at that concentration. Although these differences may be related to different methodological approaches between studies, it stresses our incomplete understanding of GO pathogenesis and orbital fibroblast biology, and indicates that novel drugs to treat GO, such as TKI, should be implemented with caution. However, alternative approaches to interfere with PDGF signaling in GO can be thought of, for instance via PDGF-neutralization with specific neutralizing antibodies or soluble receptor molecules, or blockage of the PDGF-receptor with neutralizing antibodies or dominant negative ligands.[1]

In summary, we report that PDGF-BB enhances adipogenesis by orbital fibroblasts exposed to a proadipogenic culture environment. Therefore, PDGF-BB can be expected to contribute to the orbital adipose tissue expansion in GO. Collectively, our previous observations of elevated orbital PDGF-BB expression in GO, the stimulatory effects of PDGF-BB on proliferation, cytokine, and hyaluronan production, and TSHR expression by orbital fibroblasts, and our current finding that PDGF-BB enhances adipogenesis suggests that PDGF-BB represents an important contributor to the pathogenesis of GO. Consequently, PDGF-BB, the PDGF-receptors and their downstream signaling molecules may represent potential targets for therapy in GO.

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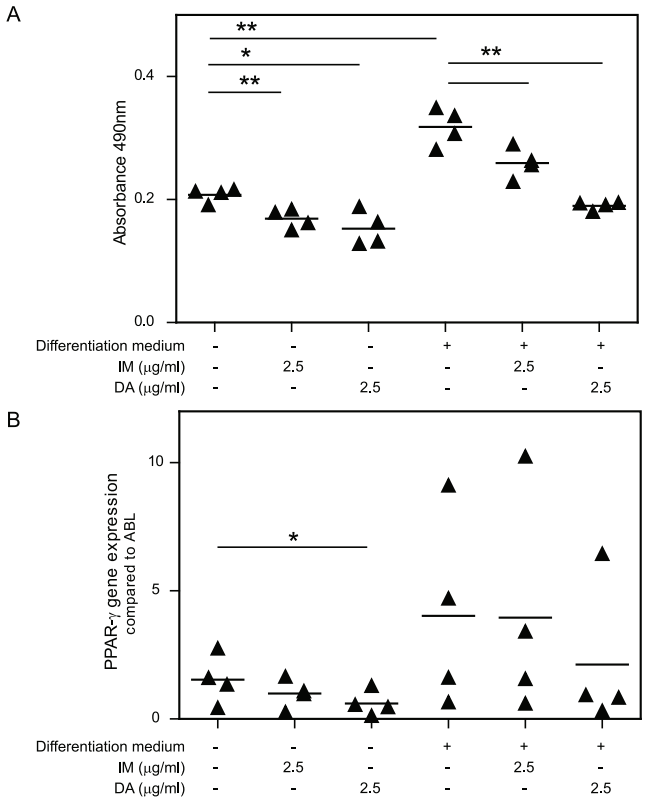
Supplemental data



Supplemental figure 1. Effect of tyrosine kinase inhibitors on adipogenesis in orbital fibroblasts. A: Oil-Red-O staining was quantified in eluate from orbital fibroblasts (controls n=2, GO patients n=2) cultured for 14 days in non-differentiation medium or adipocyte differentiation medium either in the presence or absence of imatinib mesylate or dasatinib (both 2.5μg/ml). B: PPAR-γ mRNA expression was determined by RQ-PCR in orbital fibroblasts from controls (n=2) and GO patients (n=2) cultured for 14 days in non-differentiation medium or adipocyte differentiation medium either in the presence or absence of imatinib mesylate or dasatinib. Each dot represents the orbital fibroblast strain from one individual and horizontal bars represent the mean values within a group. Data were analyzed using the paired Student's t-test. * and ** represent p value of < 0.05 and < 0.01, respectively.

Chapter 5

Basic FGF and PDGF-BB synergistically stimulate hyaluronan and IL-6 production by orbital fibroblasts: a rationale for multitarget therapy in Graves' ophthalmopathy?

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Manuscript submitted

Abstract

Orbital fibroblast activation is a central pathologic feature of Graves' ophthalmopathy (GO). Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been proposed to contribute to GO, but their effects on orbital fibroblasts are largely unknown.

We found that bFGF stimulated proliferation and hyaluronan production, but not IL-6 production by orbital fibroblasts, while VEGF hardly affected orbital fibroblast activity. Remarkably, co-stimulation of orbital fibroblasts with bFGF and PDGF-BB synergistically enhanced IL-6 and hyaluronan production and displayed an additive effect on proliferation compared to either bFGF or PDGF-BB stimulation. Nintedanib, a FGF- and PDGF-receptor targeting drug, more efficiently blocked bFGF+PDGF-BB-induced IL-6 and hyaluronan production than dasatinib that only targets PDGF-receptor.

In conclusion, bFGF may contribute to orbital inflammation and tissue remodeling in GO, especially through synergistic interaction with PDGF-BB. Multi-target therapy directed at the bFGF and PDGF pathways may potentially be of interest for the treatment of GO.

Introduction

Graves' Ophthalmopathy (GO) is the most prevalent extra thyroidal manifestation of Graves' disease (GD), which occurs in up to 50 % of GD patients. GO is clinically characterized by upper eyelid retraction, proptosis, edema and erythema of the conjunctivae and expansion of periorbital tissues resulting from adipose/connective tissue and extraocular muscle inflammation [1, 2]. Up to now medical treatment strategies for GO are limited and far from optimal. On a pathophysiological level, GO is an inflammatory fibro-proliferative disease in which orbital fibroblasts represent a central role [3, 4]. Activation of orbital fibroblasts by autoantibodies directed against thyrotropin-receptor (TSHR) and insulin-like growth factor-I receptor as well as factors such as cytokines, inflammatory lipids and growth factors leads to enhanced proliferation, secretion of cytokines and extracellular matrix (ECM) components (especially hyaluronan) and differentiation into adipocytes and profibrotic myofibroblasts [2, 4]. These insights have led to the general concept that orbital fibroblast activating factors, their specific receptors or downstream signalling molecules represent attractive targets for medical treatment of GO [5]. However, the net biological effect of orbital fibroblast activating factors in the pathophysiology of GO can be complex and is far from completely understood, as is for instance illustrated by the divergent effects of interleukin (IL)-4 and interferon (IFN)-γ on IL-1β-induced hyaluronan and prostaglandin E2 production [6]. Interplay between orbital fibroblast activating factors will thus clearly impact pathogenesis, but it also indicates that proper understanding of such interactions is required to implement safe and effective medical strategies to treat GO.

Receptor tyrosine kinases (RTK) represent a family of cell membrane expressed receptors that are generally activated via ligand-induced receptor-chain dimerization, which juxtaposes cytoplasmic tyrosine kinase domains. This juxtapositioning facilitates autophosphorylation of tyrosine residues which subsequently results in conformational changes, recruitment and activation of other downstream signalling molecules thereby initiating specific cellular responses [7]. The RTK family includes, amongst others, epidermal growth factor receptor, platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs) and vascular endothelial derived growth factor receptors (VEGFRs). Currently small molecule inhibitors of RTK, tyrosine kinases inhibitors (TKIs), are effectively applied for targeted cancer therapy [7, 8]. There is ample evidence that indicates involvement of different PDGF isoforms and PDGFR activation in orbital fibroblasts in the pathophysiology of GO. PDGF-A and PDGF-B chains are elevated in GO orbital tissue as are serum PDGF-AA levels [9-11]. In addition, especially PDGF-BB strongly induces proliferation, TSHR expression, adipogenesis, hyaluronan and cytokine production by orbital fibroblasts, indicative of its contribution to (autoimmune) inflammation and tissue expansion in GO [12-14]. *In vitro* studies demonstrated that the TKI imatinib mesylate, nilotinib and dasatinib, that all target the PDGFRs, inhibit PDGF-induced orbital fibroblast activity as well as hyaluronan and cytokine production by orbital tissue from GO patients [10-13, 15, 16]. These data

suggest that TKI with specificity for the PDGFRs may represent treatment options for GO.

Basic (b)FGF and VEGF have also been proposed to contribute to GO. Both bFGF and VEGF levels are increased in serum from GO patients compared to GD patients without GO and control subjects, with serum levels being highest in active GO [9, 17, 18]. Immunohistochemical analysis of orbital tissues from GO patients revealed bFGF expression by fibroblasts, adipocytes and endothelial cells [19, 20]. Orbital bFGF expression as well as bFGF serum levels were found to correlate positively with the clinical activity score (CAS) [19, 20]. Also a positive correlation between orbital VEGF levels and CAS has been reported [20]. This indicates that bFGF and VEGF levels may reflect the degree of orbital inflammatory activity in GO, but whether and how they contribute to this process is mostly unclear.

Although extensive studies on the effects of PDGF-BB on orbital fibroblasts have been performed, the influence of bFGF on orbital fibroblasts is only examined to a very limited extent so far [21, 22], while to our knowledge no such studies have been conducted for VEGF at all. Moreover, the combined effects of PDGF-BB, bFGF and VEGF on orbital fibroblasts has not been explored so far. Insight into this is however relevant considering that PDGFR targeting TKI have been proposed as potential treatment options for GO based on observations in *in vitro* studies [12]. Moreover, the TKI nintedanib that, besides PDGFRs, also targets FGFRs and VEGFRs was found effective in the treatment of idiopathic pulmonary fibrosis, an until now untreatable disease, while other TKI with RTK specificity limited to PDGFRs were less efficient [23, 24]. This emphasizes the need to investigate the interplay between PDGF-BB, bFGF and VEGF on orbital fibroblast activation and study the potential of nintedanib to interfere with this in GO.

Material and Methods

Orbital fibroblast culture

Orbital fibroblasts were cultured from four patients with GO at an inactive stage of disease who underwent orbital decompression surgery and from two controls without thyroid or inflammatory disease and undergoing orbital surgery for other reasons, as described previously [10]. GO patients were euthyroid and had not received steroid or other immunosuppressive treatment for at least three months prior to orbital decompression surgery. All orbital tissues were obtained at 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 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and antibiotics (penicillin and streptomycin; Cambrex BioWhittaker, Verviers, Belgium) [10]. Orbital fibroblasts were serially passaged with gentle treatment of trypsin/EDTA and used for experiments between the 4^{th} and 12^{th} passage.

PDGF-receptor, FGF-receptor and VEGF-receptor mRNA expression by orbital fibroblasts

Orbital fibroblasts were harvested from culture to determine growth factor receptor expression levels. Messenger RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturers' protocol and converted into cDNA as described previously [10]. Expression levels of PDGF-receptor α (*PDGFRA*), *PDGFRB*, FGF-receptor-1 (*FGFR1*), *FGFR2*, *FGFR3*, *FGFR4,* VEGF-receptor-1 (*VEGFR1*) and *VEGFR2* were determined by real-time quantitative (RQ)-PCR (7900 PCR system; Applied Biosystems, Foster City, CA) and normalized to the control gene *ABL* [10]. Primer-probe combinations used are listed in table 1.

Orbital fibroblast proliferation assay

Orbital fibroblasts were seeded at 6.0 x 10^3 cells/well in 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) in DMEM containing 1% FCS and allowed to adhere overnight. Subsequently orbital fibroblasts were stimulated with recombinant human PDGF-BB (220-BB; R&D Systems, Abingdon, UK), bFGF (233-FB; R&D Systems) or VEGF (293-VE; R&D Systems) at concentrations ranging from 0.1 to 50 ng/ml. Six replicates were performed per condition and proliferation was assessed after 24 and 48 hours by colorimetric assay based on uptake and subsequent release of methylene blue dye. Proliferation was calculated as percentage above control by comparing stimulated to unstimulated conditions, as described before [10]. To study the effects of combined growth factor stimulation on proliferation, orbital fibroblasts were simultaneously stimulated for 48 hours with combinations of PDGF-BB (50 ng/ml), bFGF (20 ng/ml) and VEGF (50 ng/ml).

IL-6 and Hyaluronan production by orbital fibroblasts

Orbital fibroblasts were seeded at 5.0 x 10^4 cells/well into 12-well plates (Thermo Fisher Scientific) in DMEM 10% FCS until fully confluent monolayers were established. Orbital fibroblast monolayers were then put overnight in DMEM 1% FCS and subsequently stimulated with PDGF-BB, bFGF or VEGF at concentrations ranging from 0.1 to 50 ng/ml. Supernatant was collected after 24 and 48 hours and IL-6 and hyaluronan concentrations were determined by ELISA according to the manufacturer's protocol (Invitrogen, Frederick, MD, USA and R&D Systems, respectively). To study the effects of combined growth factor stimulation on IL-6 and hyaluronan production orbital fibroblasts were simultaneously stimulated for 48 hours with combinations of PDGF-BB (50 ng/ml), bFGF (20 ng/ml) and VEGF (50 ng/ml).

IL-6, hyaluronan synthase and hyaluronidase mRNA expression in orbital fibroblasts

Orbital fibroblasts from three GO patients were seeded at 3.5 x 10^5 cells/well into 6-well plates (Thermo Fisher Scientific) in DMEM 1% FCS and allowed to adhere overnight and subsequently stimulated with PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) for 1, 2, 4, 6 and 24 hours. Messenger RNA was isolated and converted into cDNA[10]. Transcript levels of IL-6 (*IL6*), hyaluronan synthase-1 (*HAS1*), *HAS2, HAS3* and hyaluronidase-1 (*HYAL1*), *HYAL2*, *HYAL3* and *HYAL4* were determined by RQ-PCR and normalized to the control gene *ABL* [10]. Primer-probe combinations and specific Tagman gene expression assays (Life Technologies, Foster City, CA, USA) used are listed in table 1.

Effects of nintedanib and dasatinib on orbital fibroblast activation

The effects of the tyrosine kinase inhibitor (TKI) nintedanib that exhibits high inhibitory specificity for the tyrosine kinase of the PDGFRs and the FGFRs (and also VEGFRs), on PDGF-BB and/or FGF induced orbital fibroblast proliferation, IL-6 and hyaluronan production was examined. The effects of nintedanib were compared to that of dasatinib, a TKI that exhibits high inhibitory capacity for the PDGFRs but not the FGFRs and which was previously found to effectively inhibit PDGF-BB-induced orbital fibroblast activation [15, 25]. Hereto, orbital fibroblasts obtained from two control subjects and four GO patients were stimulated with PDGF-BB (50 ng/ml) or bFGF (20 ng/ml) or PDGF-BB (50 ng/ml) + bFGF (20 ng/ml) for 48 hours, either with or without the presence of nintedanib (BIBF 1120; S1010; molecular weight 539.62, Selleckchem, Houston, TX, USA) or dasatinib (S1021; molecular weight 488.01, Selleckchem). The inhibitory potential of nintedanib (concentration range from 0.008-0.5 μM) was first tested on the proliferative effect that PDGF-BB and bFGF induced individually in orbital fibroblasts. Nintedanib concentrations used were non-toxic to the cells as was determined by lactate dehydrogenase (LDH) release (Cytotoxicity Detection Kit (LDH); 11644793001, Roche, Basel, Switzerland) and microscopic appearance (data not shown). Proliferation, IL-6 and hyaluronan levels in culture supernatant were determined as described above.

Statistical analysis

Growth factor receptor expression by orbital fibroblasts was analyzed using paired Student's t-test. Data from orbital fibroblast stimulation were analyzed using ANOVA and subsequently analyzed with the Mann Whitney U test or Wilcoxon match-pairs signed rank test. A P-value <0.05 was considered statistically significant.

Results

PDGF-receptor, FGF-receptor and VEGF-receptor mRNA expression by orbital fibroblasts

Orbital fibroblasts expressed both PDGFRα and PDGFRβ mRNA, but the mRNA levels for PDGFRβ were significantly (P <0.001) higher (Figure 1A). FGFR1 mRNA was also abundantly expressed, while mRNA levels for FGFR2, FGFR3 and FGFR4 were present at far lower levels (Figure 1B). Messenger RNA levels for VEGFR1 and VEGFR2 mRNA were expressed at comparable but very low levels (Figure 1C). No difference in the mRNA expression pattern of these growth factor receptors was observed between orbital fibroblasts from GO patients and healthy controls (Figure1).

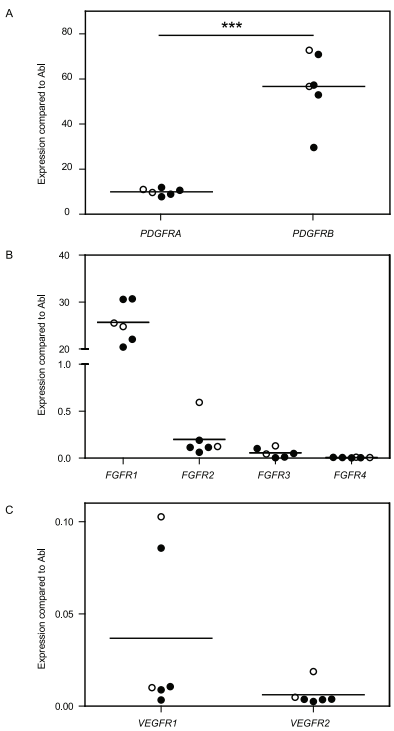

Figure 1. Orbital fibroblasts express PDGF-Receptor, FGF-Receptor and VEGF- Receptor.

Orbital fibroblasts from GO (n = 4; black circle) and controls (n = 2; open circle) were examined for PDGF-receptor α (*PDGFRA*), and *PDGFRB* (A), FGF-receptor-1 (*FGFR1*), *FGFR2*, *FGFR3*, *FGFR4* (B) and VEGF-receptor-1 (*VEGFR1*) and

VEGFR2 (C) mRNA expression by RQ-PCR and normalized to the control gene *ABL*. Each dot represents the orbital fibroblast strain from one individual and horizontal bars represent the mean values. Data were analyzed using the paired Student's t-test. *** indicates p-value of <0.001.

Effect of PDGF-BB, bFGF and VEGF on orbital fibroblast proliferation

PDGF-BB stimulated orbital fibroblast proliferation after 24 hours in a concentration-dependent manner, being already significant from a concentration of 0.1 ng/ml ($P < 0.05$; Figure 2A) which did not further increase upon 48 hours of stimulation (Figure 2B). bFGF stimulation for 24 hours only resulted in marginal orbital fibroblast proliferation, albeit in a concentration-dependent manner and reaching statistical significance from concentrations of 1.0 ng/ml and higher ($P < 0.05$; Figure 2A). bFGF stimulation for 48 hours further increased orbital fibroblast proliferation, and equaled the proliferation levels obtained after stimulation with PDGF-BB (Figure 2B). VEGF did not significantly affect orbital fibroblast proliferation after 24 nor after 48 hours of stimulation.

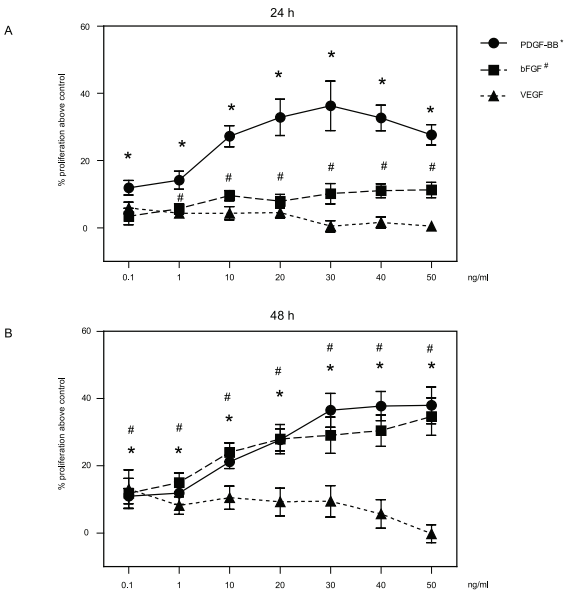

Figure 2. PDGF-BB and bFGF, but not VEGF, stimulate on orbital fibroblast proliferation.

Orbital fibroblasts from GO (n=2-4) and controls (n=2) were stimulated with recombinant human PDGF-BB (circle), bFGF (square) or VEGF (triangle) at indicated concentrations and proliferation was assessed after 24 (A) and 48 (B) hours by colorimetric assay based on uptake and subsequent release of methylene blue dye. Results are presented as mean value (percentage above

the unstimulated condition) with error bars representing the standard error of the mean (SEM). Data were analyzed using ANOVA followed by Wilcoxon match-pairs signed tank test. * and # indicate a p-value of <0.05 for PDGF-BB and bFGF stimulation compared to the unstimulated condition, respectively.

Effect of PDGF-BB, bFGF and VEGF on IL-6 production by orbital fibroblasts

PDGF-BB induced IL-6 production by orbital fibroblasts after 24 hours of stimulation in a concentration-dependent manner, reaching statistical significance from a concentration of 30 ng/ml and higher ($P < 0.05$; Figure 3A). The PDGF-BB induced IL-6 production further increased after 48 hours of stimulation, again in a concentration dependent manner, with significant induction of IL-6 production from PDGF-BB concentrations of 20 ng/ml and higher ($P < 0.05$; Figure 3B). bFGF and VEGF did not affect IL-6 production by orbital fibroblasts after 24 and 48 hours of stimulation (Figure 3A and B) nor after 72 hours of stimulation (data not shown).

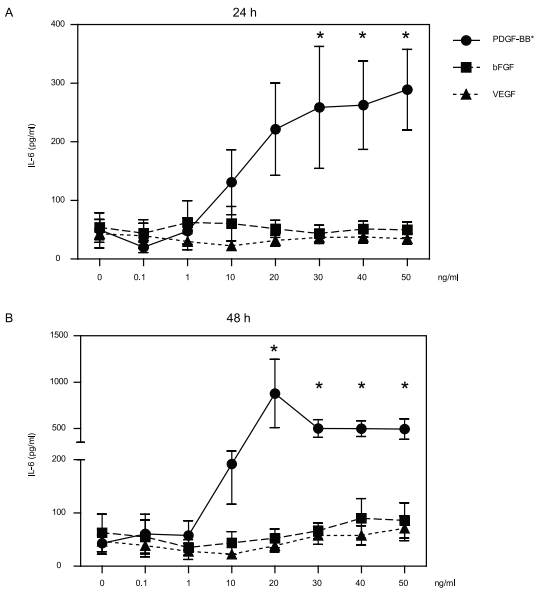

Figure 3. PDGF-BB, but not bFGF and VEGF, stimulates IL-6 production by orbital fibroblasts.

Orbital fibroblasts from GO (n=2-4) and controls (n=2) were stimulated with recombinant human PDGF-BB (circle), bFGF (square) or VEGF (triangle) at indicated concentrations. IL-6 levels were assessed after 24 (A) and 48 (B) hours

by ELISA. Results are presented as the mean value with error bars (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test. * indicates a p-value of <0.05 for PDGF-BB stimulation compared to the unstimulated condition.

Effect of PDGF-BB, bFGF and VEGF on hyaluronan production by orbital fibroblasts

PDGF-BB induced hyaluronan production by orbital fibroblasts after 24 hours of stimulation in a concentration dependent manner, reaching statistical significance from a concentration of 10 ng/ml and higher ($P < 0.05$; Figure 4A). PDGF-BB-induced hyaluronan production further increased after 48 hours of stimulation, again in a concentration dependent manner (Figure 4B). After 24 hours of stimulation bFGF induced hyaluronan production by orbital fibroblasts in a dose dependent manner and reaching significance from a concentration of 10 ng/ml and higher ($P < 0.01$), which increased slightly further after 48 hours of stimulation (Figure 4A and B). The hyaluronan production induced by bFGF was however less than that induced by PDGF-BB ($P < 0.01$). VEGF did not induce hyaluronan production by orbital fibroblasts after 24 hours of stimulation but did induce hyaluronan production after 48 hours of stimulation, reaching statistical significance from a concentrations of 20 ng/ml and higher ($P < 0.05$; Figure 4B), albeit at slightly lower level than bFGF stimulation and at significantly far lower levels than PDGF-BB stimulation ($P < 0.01$; Figure 4B).

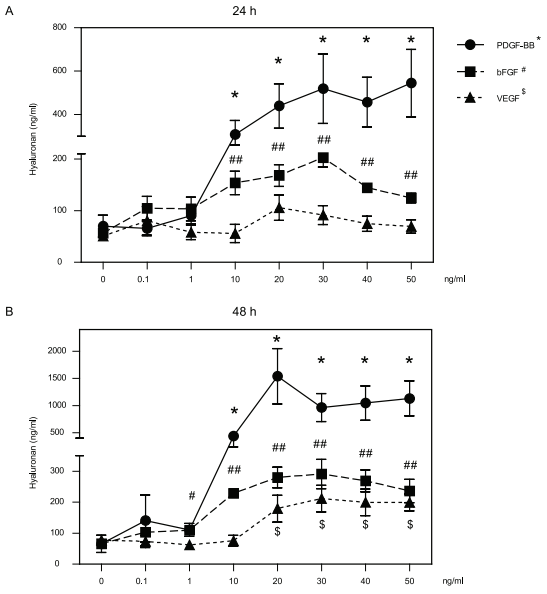

Figure 4. PDGF-BB, bFGF and VEGF stimulate hyaluronan production by orbital fibroblasts.

Orbital fibroblasts from GO (n=4) and controls (n=2) were stimulated with recombinant human PDGF-BB (circle), bFGF (square) or VEGF (triangle) at indicated concentrations. Hyaluronan levels were assessed after 24 (A) and 48 (B) hours by ELISA. Results are presented as the mean value with error bars (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test. * indicates a p-value of <0.05 for PDGF-BB stimulation compared to the unstimulated condition, $ indicates a p-value of <0.05 for VEGF stimulation compared to the unstimulated condition, and # and ## indicates p-value of <0.05 and <0.01 for bFGF stimulation compared to the unstimulated condition, respectively.

Co-stimulatory effect of PDGF-BB, bFGF and VEGF on orbital fibroblast proliferation, IL-6 and hyaluronan production

Co-stimulation experiments were performed for 48 hours using the following concentrations: PDGF-BB 50 ng/ml, bFGF 20 ng/ml and VEGF 50 ng/ml, based on the results presented above. Co-stimulation of orbital fibroblasts with PDGF-BB and bFGF resulted in significantly higher proliferation compared to that induced by PDGF-BB ($P < 0.01$) or bFGF ($P < 0.001$) alone, which was of additive nature (Figure 5A). While bFGF by itself hardly affected IL-6 production it appeared to synergistically enhance the effect of PDGF-BB on IL-6 production, although this was not significant ($P = 0.1$; Figure 5B). A strong synergistic effect was observed on hyaluronan production when orbital fibroblasts were stimulated with the combination of PDGF-BB and bFGF compared to PDGF-BB ($P < 0.01$) or bFGF stimulation alone ($P < 0.01$) (Figure 5C). VEGF in combination with PDGF-BB and/or bFGF had no additional effect on proliferation, IL-6 and hyaluronan production induced by either of the factors alone (Figure 5A-C).

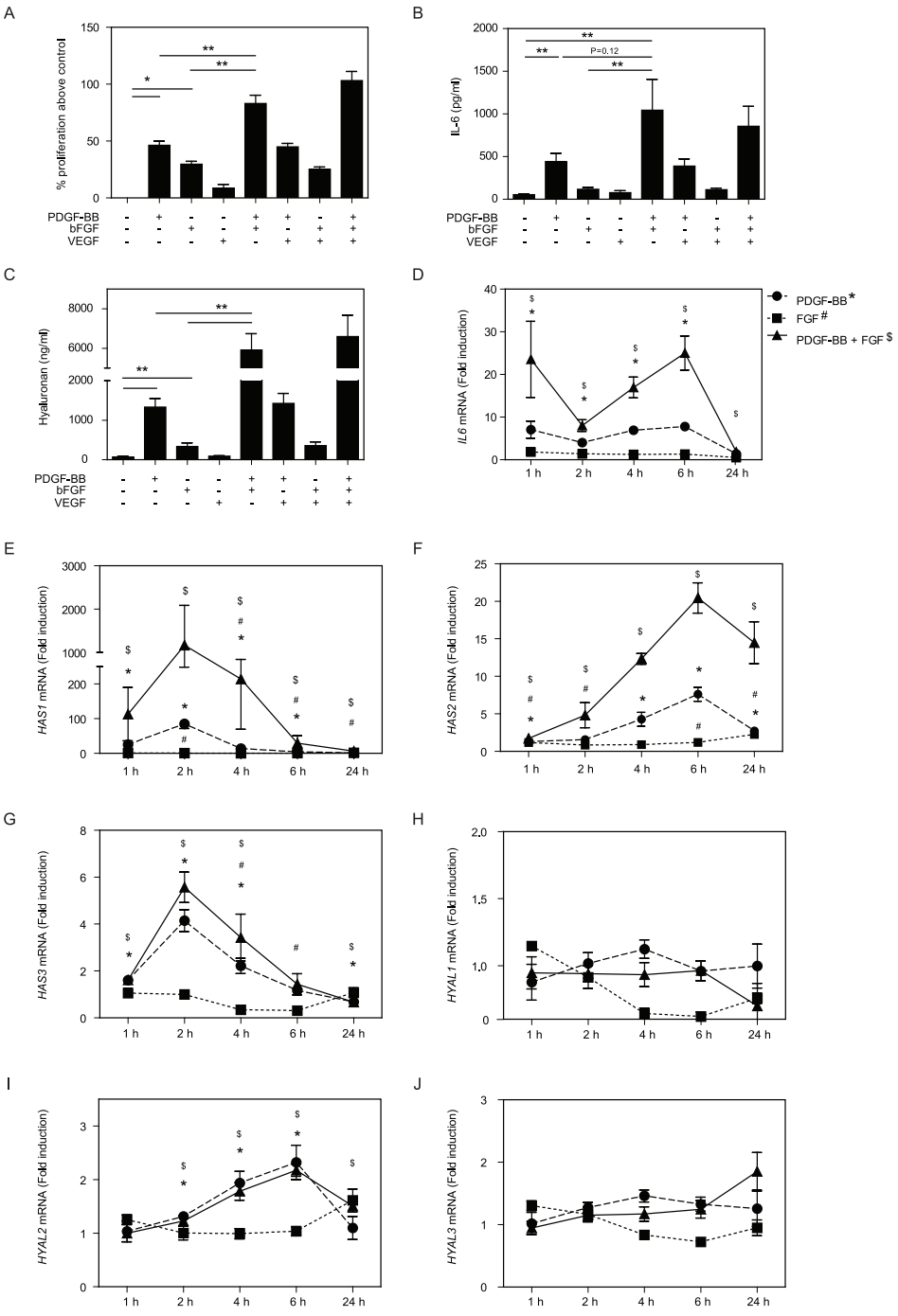
A
% proliferation above control
PDGF-BB
bFGF
VEGF
B
IL-6 (pg/ml)
P=0,12
C
Hyaluronan (ng/ml)
D
IL6 mRNA (Fold induction)
PDGF-BB*
FGF#
PDGF-BB + FGF$
E
HAS1 mRNA (Fold induction)
F
HAS2 mRNA (Fold induction)
G
HAS3 mRNA (Fold induction)
H
HYAL1 mRNA (Fold induction)
I
HYAL2 mRNA (Fold induction)
J
HYAL3 mRNA (Fold induction)
1 h
2 h
4 h
6 h
24 h

Figure 5. bFGF synergistically amplified the effect of PDGF-BB on IL-6 and hyaluronan production by orbital fibroblasts and additively stimulates PDGF-BB-induced proliferation while VEGF did not modify the effects induced by bFGF or PDGF-BB.

(A-C) Orbital fibroblasts from GO (n = 4) and controls (n = 2) were simultaneously stimulated with combinations of recombinant human PDGF-BB (50 ng/ml), bFGF (20 ng/ml) and VEGF (50 ng/ml), and proliferation (A), IL-6 (B) and hyaluronan production (C) were measured after 48 hours. Results are presented as mean value with error bars (SEM). (B-C) Data were analyzed using ANOVA and subsequently analyzed with the Wilcoxon matched- pairs signed rank test or Mann Whitney U test. * and ** indicate p-value of <0.05 and <0.01. (D-J) Orbital fibroblasts from GO (n = 3) were stimulated with recombinant human PDGF-BB (50 ng/ml; circle), bFGF (20 ng/ml; square) or PDGF-BB+bFGF (triangle) for 1, 2, 4, 6 and 24 hours. Transcript levels of *IL6* (D), hyaluronan synthase-1 (*HAS1*) (E), *HAS2* (F), *HAS3* (G) and hyaluronidase-1 (*HYAL1*) (H), *HYAL2* (I), *HYAL3* (J) and *HYAL4* mRNA expression were determined by RQ-PCR and normalized to the control gene *ABL*. Results are presented as the mean value with error bars (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test.*, # and $ indicate a p-value of <0.05 compared to the unstimulated condition.

IL-6 mRNA expression in orbital fibroblasts

PDGF-BB significantly induced *IL6* mRNA expression by orbital fibroblast reaching maximum level of induction at 6 hours after stimulation (Figure 5D; P <0.05), which is in line with our previous observation [11]. bFGF did not induce *IL6* mRNA expression by orbital fibroblast at the time points measured in this experiment (Figure 5D). Co-stimulation of orbital fibroblasts with PDGF-BB and bFGF resulted in synergistic enhancement of *IL6* mRNA expression compared to that induced by PDGF-BB alone, and reaching maximum expression level at 6 hours after stimulation (P < 0.05; Figure 5D).

Hyaluronan synthase and hyaluronidase mRNA expression in orbital fibroblasts

In order to further investigate the effect of PDGF-BB and/or bFGF stimulation on hyaluronan production *HAS1, HAS2* and *HAS3* mRNA expression by orbital fibroblasts was measured at various time points. PDGF-BB transiently induced *HAS1, HAS2* and *HAS3* mRNA expression by orbital fibroblasts, reaching maximum level of induction at 2, 6 and 2 hours after stimulation, respectively (P < 0.05; Figure 5E-G), which is in line with our previous observations [11]. bFGF slightly induced *HAS2*, reaching significant induction from 6 hours after stimulation (P < 0.05; Figure

5F) while it did not affect *HAS1* and *HAS3* mRNA levels. Co-stimulation of the orbital fibroblasts with PDGF-BB and bFGF synergistically enhanced the mRNA expression level of especially *HAS1* ($P < 0.05$; Figure 5E) and to a lesser extent *HAS2* ($P < 0.05$) when compared to PDGF-BB stimulation alone (Figure 5F). The synergistic enhancement of HAS expression observed under PDGF-BB/bFGF co-stimulation suggests that increased synthesis of hyaluronan could account for the strong rise in hyaluronan levels detected in the culture medium under these conditions (Figure 5C). In order to determine whether alterations in hyaluronan degradation could have contributed as well, the mRNA expression levels of four main hyaluronidases (*HYAL1*, *HYAL2*, *HYAL3* and *HYAL4*) were measured. Orbital fibroblasts all expressed *HYAL1*, *HYAL2* and *HYAL3* mRNA while *HYAL4* mRNA was undetectable. PDGF-BB significantly induced *HYAL2*, not *HYAL1* and *HYAL3*, mRNA expression, reaching a maximum level at 6 hours after stimulation ($P < 0.05$; Figure 5H-J). bFGF did not alter *HYAL1*, *HYAL2* and *HYAL3* mRNA expression levels in orbital fibroblasts. Co-stimulation of orbital fibroblasts with PDGF-BB and bFGF had the same effect on *HYAL* mRNA expression as PDGF-BB alone (Figure 5H-J).

Effect of nintedanib and dasatinib on orbital fibroblast activity upon PDGF-BB and bFGF stimulation

As VEGF hardly influenced proliferation, IL-6 and hyaluronan production by orbital fibroblasts the effect of nintedanib and dasatinib was studied on orbital fibroblasts stimulated with PDGF-BB and/or bFGF. Nintedanib was used at a concentration of 0.5 μM, the highest non-toxic concentration tested that significantly blocked PDGF-BB as well as bFGF-induced orbital fibroblast proliferation (Supplementary figure 1) and its efficacy was compared to that of dasatinib (0.5 μM).

Nintedanib and dasatinib both inhibited PDGF-BB-induced orbital fibroblast proliferation, IL-6 and hyaluronan production with the same efficacy ($P < 0.01$; Figure 6A-C). Both nintedanib and dasatinib inhibited bFGF-induced orbital fibroblast proliferation ($P < 0.01$; Figure 6A). Nintedanib also significantly blocked bFGF-induced hyaluronan ($P < 0.01$) production by orbital fibroblasts, while dasatinib did not significantly inhibit bFGF-induced hyaluronan production by orbital fibroblasts (Figure 6B). Both TKI suppressed the additive/synergistic effect of PDGF-BB/bFGF-induced orbital fibroblast proliferation, IL-6 and hyaluronan production ($P < 0.01$); however, nintedanib was more potent in inhibiting PDGF-BB+bFGF-induced IL-6 ($P < 0.01$) and hyaluronan ($P < 0.01$) production than dasatinib (Figure 6B, C).

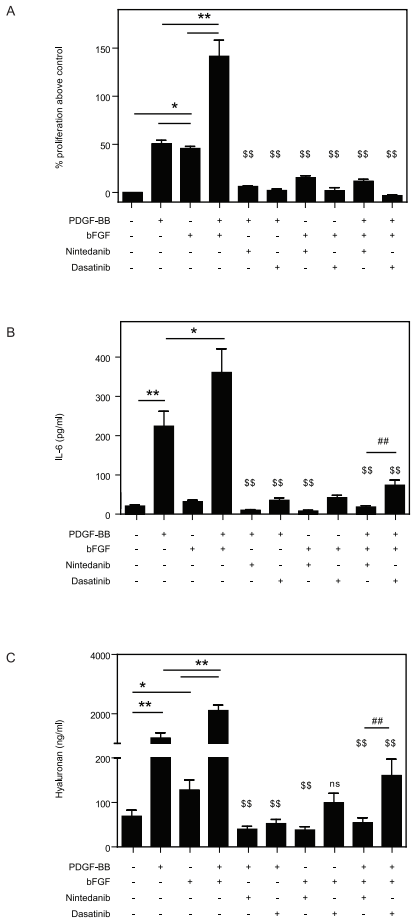

Figure 6. Nintedanib is more potent in inhibiting PDGF-BB+bFGF-induced IL-6 and hyaluronan than dasatinib.

Orbital fibroblasts from GO (n=4) and controls (n=2) were stimulated with recombinant human PDGF-BB (50 ng/ml) or bFGF (20 ng/ml) or PDGF-BB (50 ng/ml) + bFGF (20 ng/ml) for 48 hours, either with or without the presence of nintedanib (0.5 μM) or dasatinib (0.5 μM). Proliferation (A), IL-6 (B) and hyaluronan production (C) were measured after 48 hours. Results are presented as mean value with error bars (SEM). Data were analyzed using ANOVA and subsequently analyzed with the Wilcoxon matched-pairs signed rank test or Mann Whitney U test. * and ** indicate a p-value of <0.05 and <0.01, respectively. $$ indicates a p-value of <0.01 compared to the PDGF-BB and/or bFGF stimulation. ## indicates a p-value of <0.01.

Discussion

Activation of orbital fibroblasts by different types of mediators, including well known growth factors such as TGF-β and PDGF and subsequent excessive proliferation and production of cytokines and hyaluronan is considered to represent a major pathological pathway in GO [4]. Pathologic roles for bFGF and VEGF, growth factors that signal via receptor tyrosine kinases, have been suggested to contribute to GO but their effects on orbital fibroblast activity remained largely unclear [9, 17, 18]. In this study we demonstrated that VEGF marginally stimulated hyaluronan production by orbital fibroblasts, while it did not affect proliferation and IL-6 production. bFGF stimulated proliferation and hyaluronan production by orbital fibroblasts, but not IL-6 production. *In vivo* orbital fibroblasts in GO will however receive signals from a combination of different growth factors present, with the possibility that these growth factors interfere with each other individual biological effect (e.g. amplify or inhibit) [4, 6]. Here we demonstrated that VEGF did not modify the effects induced by bFGF and/or PDGF-BB. In contrast, bFGF synergistically amplified the effects of PDGF-BB, a growth factor well established to contribute to orbital fibroblast activation in GO, on IL-6 and hyaluronan production by orbital fibroblasts, while it showed an additive effect to PDGF-BB-induced proliferation. These data imply that effective therapies for GO might require multiple targets. This hypothesis is supported by our observation that nintedanib, a TKI that targets both PDGFRs and FGFRs was more effective in blocking PDGF-BB+bFGF-induced hyaluronan and IL-6 production than the TKI dasatinib that targets PDGFRs but not FGFRs.

VEGF belongs to the same growth factor family as PDGF and regulates physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions [26-28]. Dysfunctions in the VEGF signaling pathways contribute to the pathophysiology of many diseases, including tumor growth and macular degeneration [26, 29]. In this study VEGF hardly affected proliferation, IL-6 and hyaluronan production by orbital fibroblasts, which might well be related to the low expression of VEGFRs we found in orbital fibroblasts. Therefore, although increased systemic and orbital VEGF levels have been observed in GO [17, 18], our current data argue against a major role for VEGF in activating pathological processes in orbital fibroblasts. VEGF is however a strong activator of other cell types. This includes endothelial cells where it increases proliferation, migration and vascular permeability but also monocytes for which it can act as chemotactic factor [30, 31]. Therefore in GO the contribution of VEGF may be at the level of cell types other than orbital fibroblasts, for instance endothelial cells, monocytes and macrophages that abundantly express VEGFRs [26].

Basic FGF is expressed in nearly all tissues and is considered important in a wide range of physiological functions, such as the earliest stages of embryonic development, organogenesis, homeostasis and response to injury, where it controls cell proliferation, differentiation, migration, metabolism and survival [32]. So far several studies have suggested involvement of bFGF in the pathogenesis of GO [17, 19, 20].

Immunohistochemical studies on the lateral rectus muscle and orbital tissues from GO patients revealed bFGF expression by fibroblasts and adipocytes [19, 20], suggesting the possibility that bFGF is involved amongst others in regulating orbital fibroblast activity. Moreover the level of bFGF expressed in GO orbital tissue correlated positively to clinical disease activity and severity, indicating that it may reflect the degree of orbital inflammatory activity, tissue remodeling and fibrosis [19, 20]. In support of this we show that bFGF stimulates orbital fibroblast proliferation, an important determinant of orbital tissue expansion in GO [4]. This is in contrast with a previous study that found no mitogenic effect of bFGF on orbital fibroblasts, which may be related to methodological differences [21]. However in independent sets of experiments we consistently found that bFGF stimulated orbital fibroblast proliferation (Figure 2, 5A and 6A). Therefore we consider bFGF as true mitogen for orbital fibroblasts.

Our current and a previous study demonstrate that bFGF stimulates hyaluronan production by orbital fibroblasts [21]. Basic FGF itself hardly affected IL-6 production by orbital fibroblasts but we found it to synergistically enhance the effect of PDGF-BB on IL-6 production. A remarkably strong synergistic effect on hyaluronan production was observed when orbital fibroblasts were stimulated with bFGF and PDGF-BB simultaneously. This was accompanied by a synergistically enhanced and prolonged expression of especially HAS1 and HAS2, while expression of the hyaluronidase enzymes was not affected by bFGF. This data indicate that the strong synergistic enhancement that bFGF in combination with PDGF-BB exerts on hyaluronan production by orbital fibroblasts is due to increased synthesis rather than decreased degradation. How the synergistic enhancement is regulated at the level of intracellular signaling and transcriptional regulation is currently unclear, although enhanced and prolonged ERK activation might be involved as autocrine bFGF signaling enhanced and prolonged ERK activation in PDGF-BB-induced human smooth muscle cell proliferation [33]. A synergistic effect of PDGF-BB and bFGF on neovascularization and metastasis was found to depend on the induction of PDGFRα and PDGFRβ expression in capillary endothelial cells by bFGF and from the induction of FGFR1 expression in vascular smooth muscle cells by PDGF-BB [34]. We did not observe an effect of bFGF on PDGFRα and PDGFRβ mRNA expression nor an effect of PDGF-BB on FGFR1 mRNA expression in orbital fibroblasts (Supplementary figure 2). Nevertheless, together with the observed increased expression of bFGF in GO and the positive correlation with disease activity and severity our data so far clearly show that bFGF may represent a regulator of orbital inflammation and tissue remodeling in GO, especially in combination with PDGF-BB. Our data emphasize the importance to study the effect of combinations of growth factors/cytokines on orbital fibroblasts as we found that the contribution of bFGF to IL-6 production but especially hyaluronan production may greatly depend on the presence of PDGF-BB. A study that solely examined the effect of bFGF on hyaluronan and IL-6 production would thus have underestimated its importance in the pathogenesis of GO and consequently as therapeutic target for this disease.

The inhibitory effects of nintedanib on the additive/synergistic effects that PDGF-BB and bFGF exerted on orbital fibroblasts were tested in this study and compared to dasatinib. Nintedanib was more effective in inhibiting the combined effect of PDGF-BB and bFGF-induced orbital fibroblast activation than dasatinib. These data indicate that multiple RTK directed therapy might be promising to reduce excessive orbital fibroblast activity present in GO, for which a drug as nintedanib might be of potential interest. Remarkably, nintedanib treatment is also associated with less side effects than TKI as dasatinib and imatinib and more beneficial in the treatment of the aggressive pulmonary fibro-proliferative disease idiopathic pulmonary fibrosis [23, 24].

In conclusion, our current data suggests that in GO VEGF is unlikely to contribute to orbital inflammation and tissue remodeling via direct activation of orbital fibroblasts. In contrast bFGF may contribute to orbital inflammation and tissue remodeling in GO by stimulating proliferation, cytokine and hyaluronan production by orbital fibroblasts, especially through synergistic enhancement of PDGF-BB effects. These data indicate that therapy directed at the inhibition of both bFGF and PDGF induced orbital fibroblast activity may be of interest for the treatment of GO, for which for instance a multiple RTK targeting drug as nintedanib could be considered.

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Supplementary table 1. Real-time quantitative PCR primer-probe combination

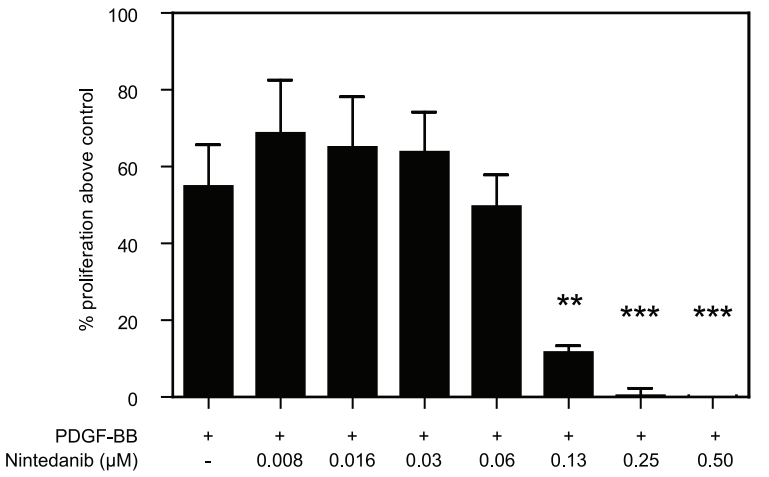
Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′ FAM - 3′ TAMRA)
ABL	TGGAGATAACATCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT
FGFR1	TaqMan Gene Expression Assays (Hs00915142_m1), Life technologies, Foster City, CA.		
FGFR2	TaqMan Gene Expression Assays (Hs01552918_m1), Life technologies.		
FGFR3	TaqMan Gene Expression Assays (Hs00179829_m1), Life technologies.		
FGFR4	TaqMan Gene Expression Assays (Hs01106908_m1), Life technologies.		
HAS1	GCAAGCGCGAGGTCATGT	CGGGGGTCCTCGTCCA	ACTACGTGCAGGTCTGTGACTCGGACAC
HAS2	AATGGGGTGGAAAAAGAGAAGTC	CAACCATGGGATCTTCTTCTAAAAC	TCCACACTTCGTCCCAGTGCTCTGA
HAS3	AAGGCCCTCGGCGATTC	CCCCCGACTCCCCCTACT	ACATCCAGGTGTGCGACTCTGACACTGTG
Hyal1	TaqMan Gene Expression Assays (Hs00201046_m1), Life technologies.		
Hyal2	TaqMan Gene Expression Assays (Hs01117343_g1), Life technologies.		
Hyal3	TaqMan Gene Expression Assays (Hs00185910_m1), Life technologies.		
Hyal4	TaqMan Gene Expression Assays (Hs00202177_m1), Life technologies.		
IL6	TAGCCGCCCCACACAGA	GTGCCTCTTTGCTGCTTTCAC	AGCCACTCACCTCTTCAGAACGAATTGACA
PDGFRA	TGAAGGCAGGCACATTTACATCTA	TACAGGAGTCTCGGGATCAGTTG	TGCCAGACCCAGATGTAGCCTTTGTACCTC
PDGFRB	GGGGACAGGGAGGTGGATT	ATTCCCGATCACAATGCACA	TCTACAGACTCCAGGTGTCATCCATCAACGTC
VEGFR1 (FLT1)	TaqMan Gene Expression Assays (Hs01052961_m1), Life technologies.		
VEGFR2 (KDR)	TaqMan Gene Expression Assays (Hs00911700_m1), Life technologies.		

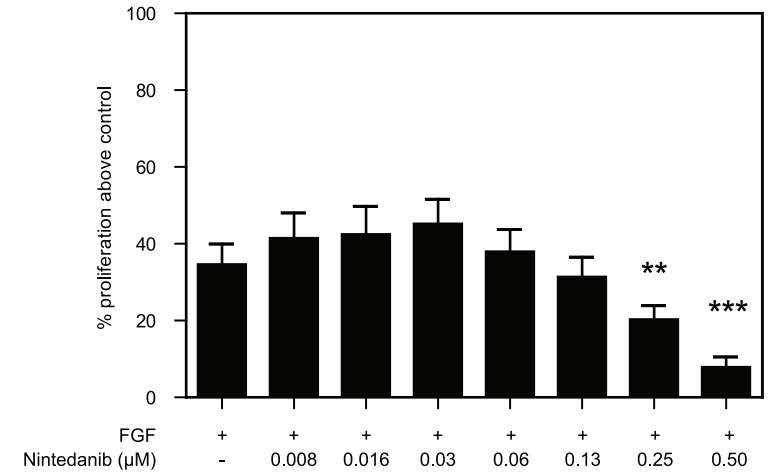
Supplementary table 2. Summary on the effect of growth factor(s) on orbital fibroblast activation

Growth factor(s) stimulation	Proliferation	IL-6 production	Hyaluronan production
PDGF-BB	+	+	+
bFGF	+	=	+
VEGF	=	=	+
PDGF-BB + bFGF	++	+++	+++
PDGF-BB + VEGF	+	+	+
bFGF + VEGF	+	+	+
PDGF-BB + bFGF + VEGF	++	+++	+++

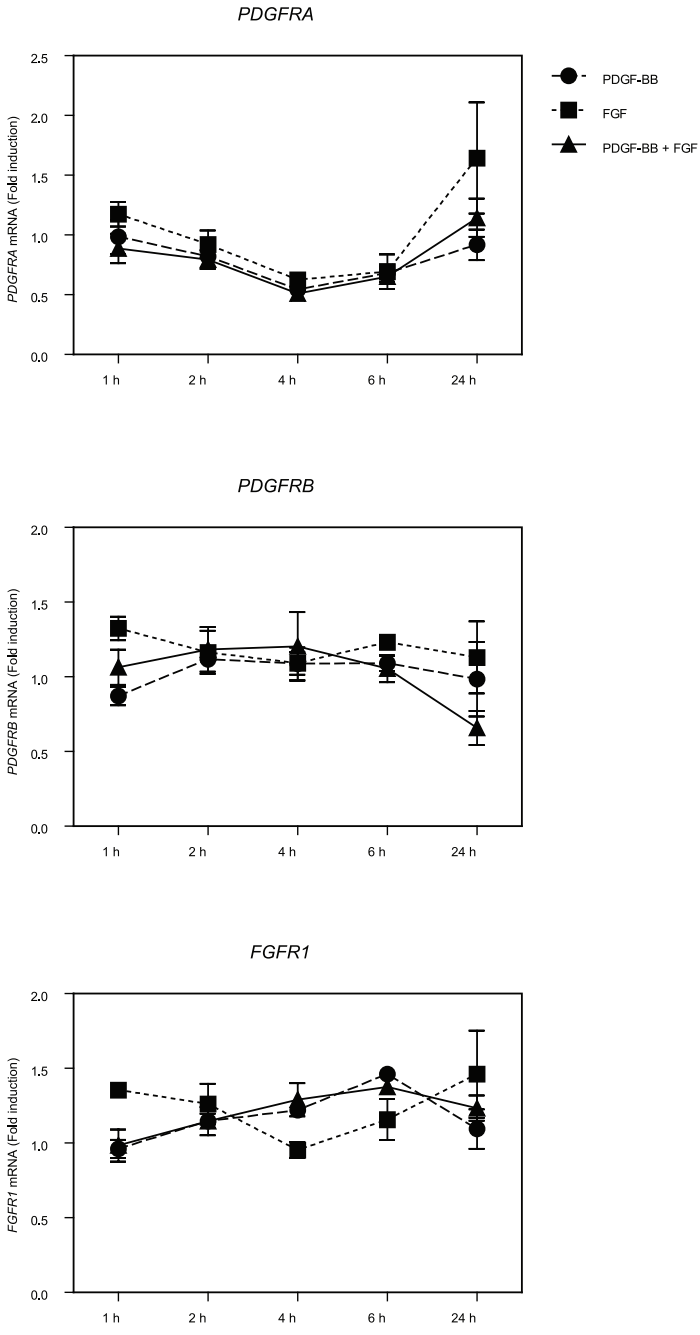
+ represents “inducing effect”, ++ represents “additive effect”, +++ represents “synergistic effect”, = represents “no effect”.

Supplementary figure 1.





Supplementary Figure 2.



Chapter 6

Autocrine PDGF-BB signaling is involved in IL-6 and hyaluronan production by orbital fibroblasts co-stimulated with basic FGF and PDGF-BB

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Manuscript submitted

Abstract

Orbital fibroblast activation by cytokines, growth factors and stimulatory autoantibodies is the central pathologic event in Graves' ophthalmopathy (GO). Basic fibroblast growth factor (bFGF) was previously found to stimulate orbital fibroblast proliferation and hyaluronan production, but not IL-6 production while platelet-derived growth factor (PDGF)-BB stimulated proliferation, hyaluronan and IL-6 production. Remarkably co-stimulation of orbital fibroblasts with bFGF and PDGF-BB synergistically enhanced IL-6 and hyaluronan production by orbital fibroblasts, while the effect on proliferation was of additive nature. In this study we explored the mechanism involved and demonstrate that co-stimulation of orbital fibroblasts with bFGF and PDGF-BB results in prolonged elevation of PDGF-B mRNA levels. A neutralizing antibody directed against PDGF-BB did not significantly alter the effect of bFGF on hyaluronan production but did significantly reduce the effect of combined bFGF and PDGF-BB stimulation on IL-6 and hyaluronan production, up to the level induced by bFGF alone. Our data suggest that the synergistic effect that bFGF in combination with PDGF-BB exerts on hyaluronan and IL-6 production depends on autocrine PDGF-BB release.

Introduction

Activation of orbital fibroblasts by cytokines, growth factors and thyrotropin receptor (TSHR) and insulin like growth factor-1 receptor (IGF-1R) autoantibodies followed by excessive proliferation, production of inflammatory mediators and hyaluronan is considered an important pathogenic event in Graves' ophthalmopathy (GO) [1]. For decades studies by many investigators put effort in elucidating the effect that individual mediators, that were proposed to contribute to GO, have on orbital fibroblast activation [1]. Although this yielded important insight into GO pathogenesis, a major drawback of this approach is its oversimplification of the *in vivo* situation where orbital fibroblasts will integrate signals from different mediators at once. However, studies that explored the effect of combinations of different mediators on orbital fibroblast activity have hardly been conducted so far [1-3].

In GO increased levels of PDGF-BB and bFGF are reported in the orbital tissue and serum, suggesting a role of these growth factors in the pathophysiology of GO [4-6]. Of these, the contribution of PDGF-BB to GO has been extensively studied and *in vitro* studies demonstrated that PDGF-BB is strong activator of proliferation, TSHR receptor expression, cytokine and hyaluronan production, and adipogenesis by orbital fibroblasts [4, 7-9]. The contribution of bFGF to orbital fibroblast activation is only poorly studied to date. Recently we found that bFGF stimulated proliferation and hyaluronan production but not IL-6 production by orbital fibroblasts (manuscript submitted, chapter 5). The most striking observation was however the synergistic effect that bFGF in combination with PDGF-BB exerted on the production of hyaluronan and IL-6 by orbital fibroblasts.

Currently the mechanism underlying this synergy of bFGF and PDGF-BB is unclear, but studies in various other cell types suggested a close relationship between PDGF-BB and bFGF. In human foreskin fibroblasts PDGF-BB was found to induce FGF-receptor-1 (*FGFR1*) mRNA expression [10] and a recent study demonstrated that bFGF can upregulate PDGF-receptor β expression in human pericytes [11]. In addition, PDGF-BB induced smooth muscle cell proliferation via autocrine bFGF release [12] and its effect on neovascularization and metastasis in mice was synergistically enhanced by bFGF [13]. Also, the activated FGF-receptor can transactivate the PDGF-receptor [14]. These data suggest existence of an intricate interplay between PDGF-BB signaling and bFGF signaling.

Previously we found no effect of PDGF-BB on FGF-receptor expression nor of bFGF on PDGF-receptor expression by orbital fibroblasts (manuscript submitted, chapter 5). Therefore, in this study we examined whether autocrine release of PDGF-BB or bFGF was involved in the synergistic effect that bFGF and PDGF-BB have on hyaluronan and IL-6 production by orbital fibroblasts.

Material and Methods

Orbital fibroblast culture

Orbital fibroblasts were cultured from four patients with GO at an inactive stage of disease who underwent orbital decompression surgery and from two controls without thyroid or inflammatory disease and undergoing orbital surgery for other reasons, as described previously [2]. GO patients were euthyroid and had not received steroid or other immunosuppressive treatment for at least three months prior to orbital decompression surgery. All orbital tissues were obtained at 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 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and antibiotics (penicillin and streptomycin; Cambrex BioWhittaker, Verviers, Belgium) [2]. Orbital fibroblasts were serially passaged with gentle treatment of trypsin/EDTA and used for experiments between the 4^{th} and 12^{th} passage.

PDGF-A, *PDGF-B* and *bFGF* mRNA expression by orbital fibroblasts

Orbital fibroblasts from three GO patients were seeded at 3.5 x 10^5 cells/well into 6-well plates (Thermo Fisher Scientific, Roskilde, Denmark) in DMEM 1% FCS and allowed to adhere overnight and subsequently stimulated with PDGF-BB (50 ng/ml; 220-BB; R&D Systems, Abingdon, UK) and/or bFGF (20 ng/ml; 233-FB; R&D Systems) for 1, 2, 4, 6 and 24 hours. PDGF-BB and bFGF concentrations were based on our previous studies (manuscript submitted, chapter 5). Messenger RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and converted into cDNA as described previously [2]. Expression levels of PDGF-A, PDGF-B and bFGF were determined by real-time quantitative (RQ)-PCR (7900 PCR system; Applied Biosystems, Foster City, CA) and normalized to the control gene *ABL* [2]. Primer-probe combinations used are listed in table 1.

IL-6 and hyaluronan production by orbital fibroblasts

Orbital fibroblasts from two controls and three GO patients were seeded at 5.0 x 10^4 cells/well into 12-well plates (Thermo Fisher Scientific) in DMEM 10% FCS until fully confluent monolayers were established. Orbital fibroblast monolayers were then put overnight in DMEM 1% FCS and subsequently stimulated with recombinant human PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) in the presence or absence of an anti-human PDGF-BB neutralizing antibody (AB-220- NA; R&D Systems) or goat IgG control (AB-108-C; R&D Systems) at concentrations of 0.5 and 5.0 µg/ml. Supernatant

was collected after 48 hours and IL-6 and hyaluronan concentrations were determined by ELISA according to the manufacturer's protocol (Invitrogen, Frederick, MD, USA and R&D Systems, respectively).

IL-6 and hyaluronan synthase-2 mRNA expression in orbital fibroblasts

Orbital fibroblasts from three GO patients were seeded at 3.5 x 10^5 cells/well into 6-well plates (Thermo Fisher Scientific) in DMEM 1% FCS and allowed to adhere overnight and subsequently stimulated with PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) for 6 hours in the presence or absence of an anti-human PDGF-BB neutralizing antibody or goat IgG control at concentrations of 0.5 and 5.0 µg/ml. Messenger RNA was isolated and converted into cDNA as described previously (6). Transcript levels of IL-6 (*IL6*) and hyaluronan synthase-2 (*HAS2*) were determined by RQ-PCR and normalized to the control gene *ABL* [2]. Primer-probe combinations used are listed in table 1.

Statistical analysis

Data from orbital fibroblast stimulation were analyzed using ANOVA and subsequently analyzed with the Mann Whitney U test. A P-value <0.05 was considered statistically significant.

Results

The effect of PDGF-BB and bFGF on PDGF-A, PDGF-B and bFGF mRNA expression by orbital fibroblasts

PDGF-A mRNA expression was transiently induced by PDGF-BB ($P < 0.05$) reaching the peak at 2 hours while bFGF appeared to significantly downregulate *PDGF-A* mRNA expression by orbital fibroblasts after 4 hours (Figure 1A). Co-stimulation of orbital fibroblasts with PDGF-BB and bFGF revealed *PDGF-A* mRNA kinetics comparable to that observed with PDGF-BB stimulation alone (Figure 1A). *PDGF-B* mRNA expression was transiently induced by PDGF-BB ($P < 0.05$) and bFGF ($P < 0.05$), but the level of induction was significantly higher for PDGF-BB than bFGF stimulation ($P < 0.05$), reaching peak levels at 2 and 4 hours, respectively (Figure 1B). Co-stimulation of orbital fibroblasts with PDGF-BB and bFGF did not result in significant higher induction of *PDGF-B* mRNA compared to stimulation with PDGF-BB alone, but was associated with a less rapid decrease of the elevated PDGF-B mRNA levels over the period between 2 and 24 hours after stimulation (Figure 1B, grey area). PDGF-BB stimulation did not impact *bFGF* mRNA expression levels in orbital fibroblasts while bFGF appeared to significantly downregulate *bFGF* mRNA expression by orbital fibroblasts after 4 hours (Figure 1C). However, co-stimulation of orbital fibroblasts with PDGF-BB and bFGF transiently induced *bFGF* mRNA expression ($P < 0.05$) reaching the peak at 4 hours (Figure 1C). No increase in PDGF-BB and bFGF protein levels was observed in culture supernatants from orbital fibroblasts co-stimulated with PDGF-BB and FGF after 12 and 24 hours, as determined by ELISA (data not shown).

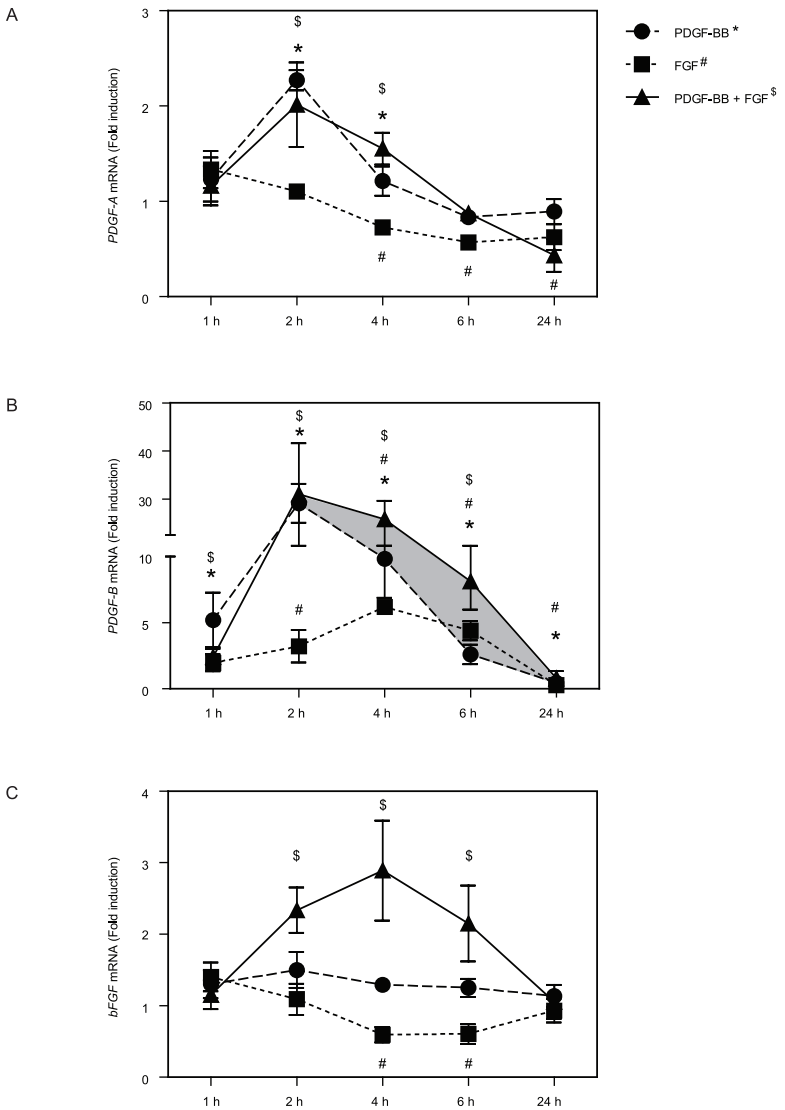


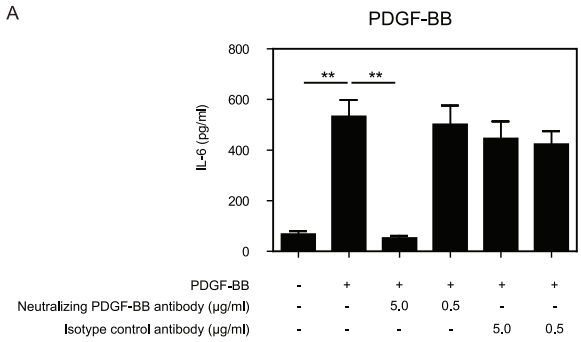
Figure 1. *PDGF-A*, *PDGF-B* and *bFGF* mRNA expression upon PDGF-BB and/or bFGF stimulation.

Orbital fibroblasts from three GO patients were stimulated with recombinant human PDGF-BB (50 ng/ml; circle), bFGF (20 ng/ml; square) or PDGF-BB+bFGF (triangle) for 1, 2, 4, 6 and 24 hours. Transcript levels of *PDGF-A* (A), *PDGF-B* (B) and *bFGF* (C) mRNA were determined by RQ-PCR and normalized to the control gene *ABL*. Results are presented as the mean value with error bars indicating the standard error of the

mean (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test. *, # and $ indicate a p-value of <0.05 compared to the unstimulated condition.

Effect of PDGF-BB neutralization on IL-6 production by orbital fibroblasts upon PDGF-BB and/or bFGF stimulation

Because the mRNA data suggest that orbital fibroblast stimulation with the combination of PDGF-BB and bFGF may result in prolonged autocrine PDGF-BB exposure we examined the effect of a PDGF-BB neutralizing antibody on IL-6 production. PDGF-BB strongly induced IL-6 production by orbital fibroblasts (P <0.01) and the stimulatory effect of PDGF-BB was abrogated (P <0.01) by the highest concentration (5ug/ml) of the neutralizing antibody (Figure 2A). bFGF slightly enhanced IL-6 production by orbital fibroblasts, which was not affected by the PDGF-BB neutralizing antibody (Figure 2B). In line with our previous finding, co-stimulation of orbital fibroblasts with PDGF-BB and bFGF resulted in higher production of IL-6 compared to that induced by PDGF-BB alone, and this was blocked by the highest concentration of the PDGF-BB neutralizing antibody, (P <0.01; Figure 2C) up to levels found with bFGF stimulation alone. In all cases no effect was observed with the low concentration of PDGF-BB neutralizing antibody nor with the isotype control at both concentrations analyzed (Figure 2 and Supplemental figure 1). Also at the mRNA level co-stimulation with PDGF-BB and bFGF resulted in greater induction of *IL6* mRNA than that induced by PDGF-BB alone, although this did not reach statistical significance (P = 0.2; Figure 2D). The PDGF-BB neutralizing antibody inhibited the effect of PDGF-BB/bFGF co-stimulation up to the level induced by bFGF alone (Figure 2D).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IL-6 (pg/ml)
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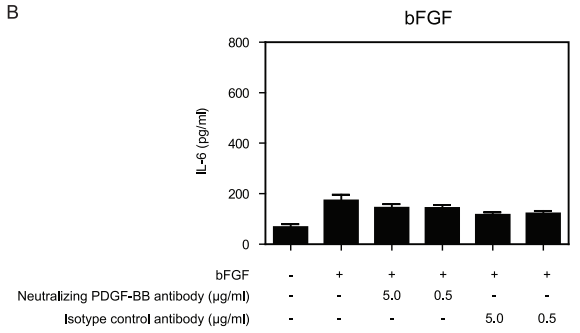
Isotype control antibody (µg/ml) - - - - 5.0 0.5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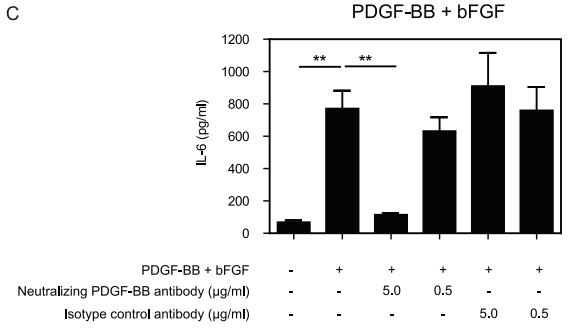
C
PDGF-BB + bFGF
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Neutralizing PDGF-BB antibody (µg/ml) - - 5.0 0.5 - -
Isotype control antibody (µg/ml) - - - - 5.0 0.5

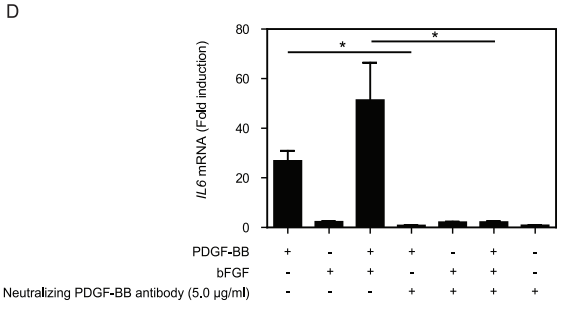
D
IL6 mRNA (Fold induction)
80
60
40
20
0
*
*
PDGF-BB + - + + - + -
bFGF - + + - + + -
Neutralizing PDGF-BB antibody (5.0 µg/ml) - - - + + + +

Figure 2. The effect of neutralizing PDGF-BB antibody on PDGF-BB and/or bFGF-induced IL-6 production by orbital fibroblast.

(A-C) Orbital fibroblasts from GO (n=3) and controls (n=2) were stimulated with recombinant human PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) in the presence or absence of PDGF-BB neutralizing antibody or goat IgG control at concentrations of 0.5 or 5.0 µg/ml. IL-6 levels were assessed after 48 hours by ELISA. (D) Orbital fibroblasts from three GO patients were stimulated with recombinant human PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) in the presence or absence of PDGF-BB neutralizing antibody at concentrations of 0.5 or 5.0 µg/ml for 6 hours. Transcript levels of *IL6* mRNA expression were determined by RQ-PCR and normalized to the control gene *ABL*. Results are presented as the mean value with error bars (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test. * and ** indicate a p-value of <0.05 and <0.01, respectively.

Effect of PDGF-BB neutralization on hyaluronan production by orbital fibroblasts upon PDGF-BB and/or bFGF stimulation

PDGF-BB strongly induced hyaluronan production by orbital fibroblasts (P <0.01) and this was blocked to the level of basal production by the highest concentration of PDGF-BB neutralizing antibody (P <0.01; Figure 3A). bFGF significantly (P <0.01) induced hyaluronan production by the orbital fibroblasts, although at far lower level than PDGF-BB, and this was not abrogated by the PDGF-BB neutralizing antibody (Figure 3B). Co-stimulation of orbital fibroblasts with PDGF-BB and bFGF resulted in synergistic enhancement of hyaluronan production when compared to that induced by PDGF-BB or bFGF alone (Figure 3C), which is in line with our previous study (manuscript submitted, chapter 5). The highest concentration of the PDGF-BB neutralizing antibody significantly (P <0.01) inhibited the effect of PDGF-BB/bFGF co-stimulation on hyaluronan production, although this inhibition was not complete but was up to the level of hyaluronan production induced by bFGF alone (Figure 3C vs Figure 3B). Because HAS2 is considered the main HAS involved in hyaluronan synthesis by orbital fibroblasts [15, 16], we also examined *HAS2* mRNA expression. PDGF-BB enhanced *HAS2* mRNA expression approximately 10 fold, which was abrogated by the PDGF-BB neutralizing antibody (P <0.05; Figure 3D). bFGF enhanced *HAS2* mRNA expression ~1.5 fold, which was not reduced by the PDGF-BB neutralizing antibody (Figure 3D). Co-stimulation of the orbital fibroblasts with PDGF-BB and bFGF induced *HAS2* mRNA levels ~17 fold, which was significantly (p <0.05) reduced by the PDGF-BB neutralizing antibody up to the level of induction achieved by bFGF alone (Figure 3D). In all cases no effect was observed with the low concentration of PDGF-BB neutralizing antibody and not with the isotype control at both concentrations analyzed (Figure 3 and Supplemental figure 1).

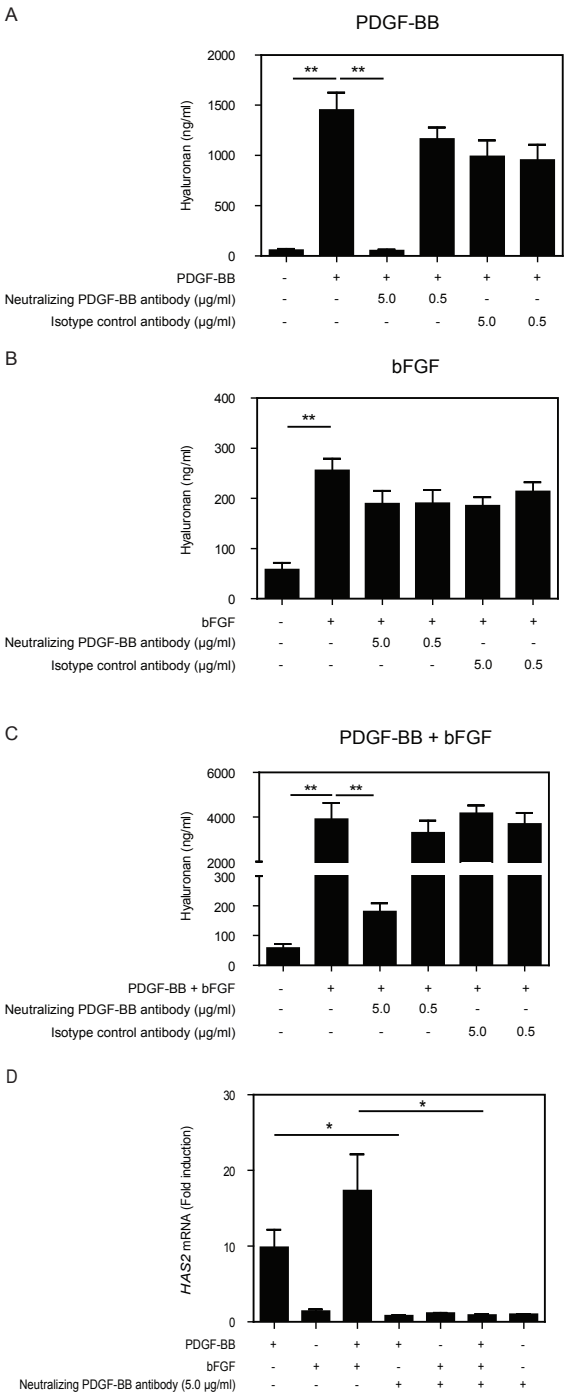
A
PDGF-BB
Hyaluronan (ng/ml)
2000
1500
1000
500
0
**
**
PDGF-BB - + + + + +
Neutralizing PDGF-BB antibody (µg/ml) - - 5.0 0.5 - -
Isotype control antibody (µg/ml) - - - - 5.0 0.5
B
bFGF
Hyaluronan (ng/ml)
400
300
200
100
0
**
bFGF - + + + + +
Neutralizing PDGF-BB antibody (µg/ml) - - 5.0 0.5 - -
Isotype control antibody (µg/ml) - - - - 5.0 0.5
C
PDGF-BB + bFGF
Hyaluronan (ng/ml)
6000
4000
2000
300
200
100
0
**
**
PDGF-BB + bFGF - + + + + +
Neutralizing PDGF-BB antibody (µg/ml) - - 5.0 0.5 - -
Isotype control antibody (µg/ml) - - - - 5.0 0.5
D
HAS2 mRNA (Fold induction)
30
20
10
0
*
*
PDGF-BB + - + + - + -
bFGF - + + - + + -
Neutralizing PDGF-BB antibody (5.0 µg/ml) - - - + + + +

Figure 3. The effect of neutralizing PDGF-BB antibody on PDGF-BB and/or bFGF-induced hyaluronan production by orbital fibroblast.

(A-C) Orbital fibroblasts from GO (n=3) and controls (n=2) were stimulated with recombinant human PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) in the presence or absence of PDGF-BB neutralizing antibody or goat IgG control at concentrations of 0.5 or 5.0 µg/ml. Hyaluronan levels were assessed after 48 hours by ELISA. (D) Orbital fibroblasts from three GO patients were stimulated with recombinant human PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) in the presence or absence of PDGF-BB neutralizing antibody at concentrations of 0.5 or 5.0 µg/ml for 6 hours. Transcript levels of *HAS2* mRNA expression were determined by RQ-PCR and normalized to the control gene *ABL*. Results are presented as the mean value with error bars (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test. * and ** indicate a p-value of <0.05 and <0.01, respectively.

Discussion

In GO orbital fibroblasts will encounter activation signals by different mediators at once, and once these activation signals are integrated by the orbital fibroblast they may result in a biological response different from that induced by a single mediator [1]. PDGF-BB and bFGF have been proposed to contribute to GO [4, 7]. Previously we reported that PDGF-BB and bFGF synergistically enhanced IL-6 but especially hyaluronan production by orbital fibroblasts. In this study we showed that co-stimulation of orbital fibroblasts with PDGF-BB and bFGF prolonged the duration of elevated PDGF-B mRNA levels in the orbital fibroblasts compared to stimulation with PDGF-BB or bFGF. Studies with a PDGF-BB neutralizing antibody inhibited the synergistic effect of PDGF-BB/bFGF co-stimulation on IL-6 and hyaluronan production up to the level of that induced by bFGF alone. Our data therefore support involvement of autocrine PDGF-BB signaling in the synergistic enhancement of IL-6 and hyaluronan when orbital fibroblasts are co-activated with PDGF-BB and bFGF.

In this study we observed prolonged *PDGF-B* mRNA expression in orbital fibroblasts co-stimulated with PDGF-BB and bFGF compared to PDGF-BB or bFGF stimulation alone. Unexpectedly we could not detect elevated PDGF-BB levels in the culture supernatants after orbital fibroblasts were stimulated for 12 or 24 hours with PDGF-BB and bFGF (Supplemental figure 2). Similarly, although mRNA expression data revealed an induction of *PDGF-B* mRNA expression upon bFGF stimulation, PDGF-BB was also not detected by ELISA from the supernatant when culturing orbital fibroblasts with bFGF. Although this lack of PDGF-BB detection might be related to technical limitations of the detection assay used or the experimental set-up, the data generated with the neutralizing PDGF-BB antibody support involvement of PDGF-BB produced and secreted by the orbital fibroblasts when co-stimulated with PDGF-BB and bFGF.

In our previous study (manuscript submitted, chapter 5) we could not find an effect of PDGF-BB on *FGFR1* mRNA expression nor of bFGF on *PDGFRA/B* mRNA expression at all time points observed in this study, which is in contrast to other studies, [10, 11]. It is however well recognized that orbital fibroblasts have unique features and can respond differently to specific stimuli than fibroblasts from other anatomical regions [17]. Previous studies from our group revealed a positive correlation between PDGFR mRNA levels and protein expression in human retinal pigment epithelial cells [18] which is in line with a study in human dermal fibroblasts and neuroblastoma cell lines [19, 20]. Although PDGFR protein expression was not examined in our current study and a *PDGFB* knockout orbital fibroblast model is not available to date it is so far tempting to hypothesize that the combined effect of bFGF and PDGF-BB on hyaluronan and IL-6 production involves prolonged autocrine

PDGF-BB signaling that is not regulated by changes in PDGF-receptor expression.

The effect of bFGF alone on hyaluronan and to a lesser extend IL-6 production by the orbital fibroblasts was not inhibited by the PDGF-BB neutralizing antibody, despite the small enhancement in PDGF-B mRNA expression induced by bFGF (figure 1B). Moreover, the PDGF-BB neutralizing antibody inhibited the synergistic effect of PDGF-BB/bFGF up to the level of that induced by bFGF alone. This clearly indicates that bFGF induces effects that are independent of induction of PDGF-BB production by the orbital fibroblasts. Comparable, PDGF-BB independent effects of bFGF on skeletal muscle growth and differentiation have been described [21]. From our data it cannot be ruled out that a process like PDGFR transactivation is involved in bFGF-induced hyaluronan production [14]. This is however unlikely as we previously observed that the tyrosine kinase inhibitor (TKI) dasatinib that inhibits the tyrosine kinase activity of the PDGFR but not FGFR did not inhibit bFGF-induced hyaluronan and IL-6 production by orbital fibroblasts (manuscript submitted, Chapter 5).

Several studies suggested tyrosine kinase inhibitors (TKI) that inhibit the PDGFR, including imatinib mesylate and dasatinib, as potential drugs for the treatment of GO [2, 7, 22]. Also PDGF-BB neutralizing antibodies, that are currently however not yet available for clinical application, can be considered as potential treatment option for GO [4, 23]. However these approaches cannot be expected to completely interfere with the effect of bFGF on hyaluronan and IL-6 production by orbital fibroblasts which fits our previous notion that therapy directed at the inhibition of both bFGF- and PDGF-induced orbital fibroblast activity may be of interest for the treatment of GO, for instance with a TKI that targets both the FGF-receptors and PDGF-receptors.

In conclusion, we demonstrate that the synergistic effect of bFGF and PDGF-BB on hyaluronan and IL-6 production by orbital fibroblasts involves prolonged PDGF-BB production and thus autocrine PDGF-BB signaling by orbital fibroblasts.

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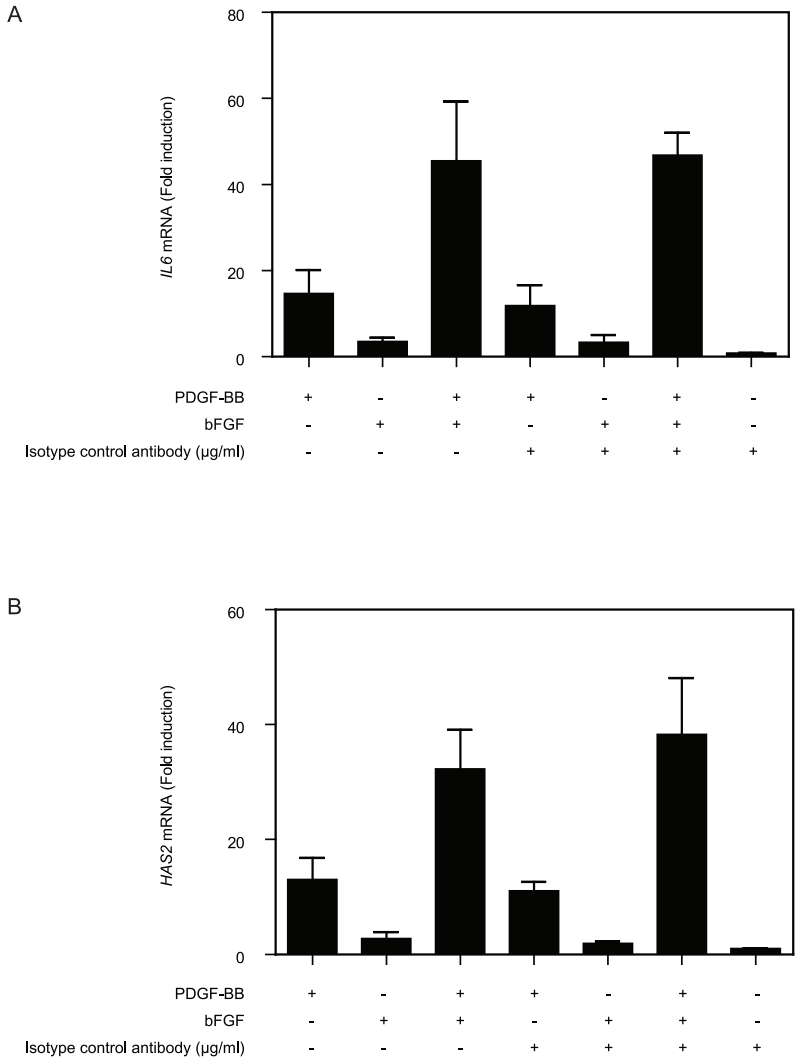
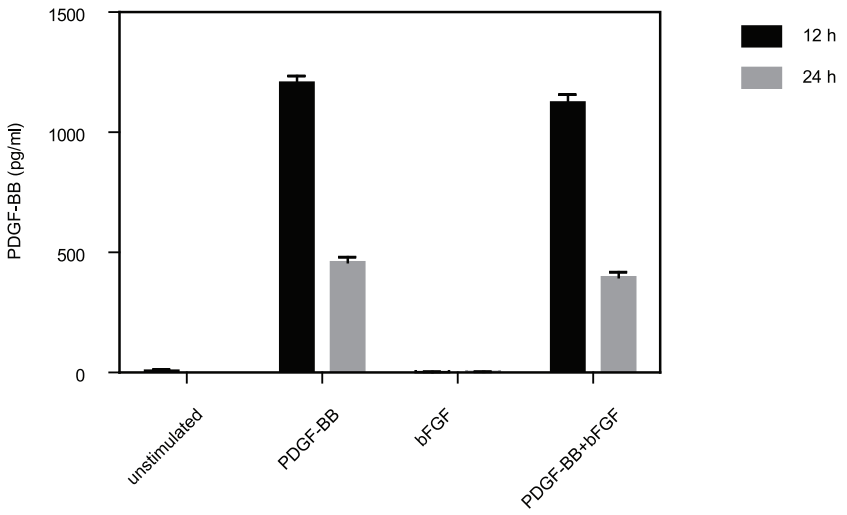


Supplemental figure 1. The effect of isotype control antibody on PDGF-BB and/or bFGF-induced *IL6* and *HAS2* mRNA expression by orbital fibroblast.

Orbital fibroblasts from three GO were stimulated with recombinant human PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml) in the presence or absence of goat IgG control at concentrations of 0.5 or 5.0 µg/ml for 6 hours. Transcript levels of *IL6* (A) and *HAS2* (B) mRNA expression were determined by RQ-PCR and normalized to the control gene *ABL*. Results are presented as the mean value with error bars (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test.



Supplemental figure 2. PDGF-BB production upon PDGF-BB and/or bFGF stimulation.

Orbital fibroblasts from GO (n=4) and controls (n=2) were stimulated with recombinant human PDGF-BB (50 ng/ml) and/or bFGF (20 ng/ml). PDGF-BB levels were assessed after 12 and 24 hours by ELISA. Results are presented as the mean value with error bars (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test.

Supplementary table 1. Real-time quantitative PCR primer-probe combination

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′ FAM - 3′ TAMRA)
ABL	TGGAGATAACATCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT
bFGF	CGGGGGCTTCTTCCTGC	GCCAGGTAACGGTTAGCACAC	TTGTAGCTTGATGTGAGGGTCGCTCTTCTC
FGFR1	TaqMan Gene Expression Assays (Hs00915142_m1), Life technologies, Foster City, CA.		
HAS2	AATGGGGTGGAAAAAGAGAAGTC	CAACCATGGGATCTTCTTCTAAAAC	TCCACACTTCGTCCCAGTGCTCTGA
IL6	TAGCCGCCCCACACAGA	GTGCCTCTTTGCTGCTTTCAC	AGCCACTCACCTCTTCAGAACGAATTGACA
PDGF-A	CGGGGTCCATGCCACTAA	GGGGCCAGATCAGGAAGTTG	AGCTTCCTCGATGCTTCTCTTCCTCCG
PDGF-B	TCCCGAGGAGCTTTATGAGATG	CGGGTCATGTTCAGGTCCAAC	AGTGACCACTGATCGCTCCTTTG
PDGFRA	TGAAGGCAGGCACATTTACATCTA	TACAGGAGTCTCGGGATCAGTTG	TGCCAGACCCAGATGTAGCCTTTGTACCTC
PDGFRB	GGGGACAGGGAGGTGGATT	ATTCCCGATCACAATGCACA	TCTACAGACTCCAGGTGTCATCCATCAACGTC

Chapter 7

Histamine induces NF-KB controlled cytokine secretion by orbital fibroblasts via histamine receptor type-1

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Manuscript submitted

Abstract

Mast cells and their products are likely to be involved in regulating orbital fibroblast activity in Graves' ophthalmopathy (GO). Histamine is abundantly present in granules of mast cells and is released upon mast cell activation. However, the effect of histamine on orbital fibroblasts has not been examined so far. Orbital tissues from GO patients and controls were analyzed for the presence of mast cells using toluidine blue staining and immunohistochemical detection of CD117 (stem cell factor receptor). Orbital fibroblasts were cultured from GO patients and healthy controls, stimulated with histamine and cytokines (IL-6, IL-8, CCL2, CCL5, CCL7, CXCL10 and CXCL11) were measured in culture supernatants. Also hyaluronan levels were measured in culture supernatants and hyaluronan synthase (*HAS*) and hyaluronidase (*HYAL*) gene expression levels were determined. In addition histamine receptor subtype gene expression levels were examined as well as the effect of the histamine receptor-1 (HRH1) antagonist loratadine and NF-κB inhibitor (SC-514) on histamine-induced cytokine production. Mast cell numbers were increased in GO orbital tissue. Histamine stimulated the production of IL-6, IL-8 and CCL2 by orbital fibroblasts, while it had no effect on the production of CCL5, CCL7, CXCL10, CXCL11 and hyaluronan. Orbital fibroblasts expressed HRH1 and loratadine and SC-514 both blocked histamine-induced IL-6, IL-8 and CCL2 production by orbital fibroblasts. In conclusion, this study demonstrates that histamine can induce the production of NF-κB controlled-cytokines by orbital fibroblasts, and supports a role for mast cells in GO.

Introduction

Graves' ophthalmopathy (GO) is an extra-thyroidal manifestation of Graves' disease (GD) and is characterized by an orbital infiltrate, consisting of T cells, macrophages, some B cells and plasma cells [1-3]. These immune cells produce cytokines, growth factors as well as stimulatory autoantibodies against thyrotropin-receptor (TSHR) and insulin-like growth factor-I receptor (IGF1R) that stimulate orbital fibroblasts to proliferate, produce excess glycosaminoglycans (mainly hyaluronan) and inflammatory mediators and to differentiate into adipocytes and myofibroblasts [1, 4]. All this contributes to orbital inflammation, tissue expansion/remodeling and fibrosis typical of GO [1, 2, 4].

Mast cells have also been observed in GO orbital tissue, especially in the late phase of disease that is associated with tissue remodeling and fibrosis [3, 5-7]. Moreover, recruitment of mast cells into orbital tissue has been observed in a murine GO model [8]. The cause of mast cell accumulation 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 this [9]. Serum levels of IgE can be increased in GD patients and positive correlations between elevated serum IgE levels and the presence of GO have been described [10, 11]. In addition to this, immunohistochemical studies demonstrated the presence of IgE in orbital muscle fibers from GO patients [6]. IgE binds and cross-links FcRε on mast cells, resulting in mast cell degranulation and the release of a plethora of mediators [12]. IgE molecules with specificity to the TSHR have been described in GO patients, which may possibly be involved in regulating orbital mast cell recruitment and degranulation [13]. Although the above data suggest involvement of mast cells in the pathogenesis of GO their contributing role is so far poorly studied.

Orbital tissue mast cells are often localized in close proximity to orbital fibroblasts or adipocytes and show features of degranulation [14]. Within the secretory granules mast cells store an extensive variety of preformed mediators, including many different cytokines and growth factors [15]. Co-cultures of orbital fibroblasts with the mast cell line HMC-1 revealed that mast cell-derived prostaglandin D2 (PGD_2) stimulated hyaluronan and prostaglandin E2 (PGE_2) production by orbital fibroblasts [16, 17]. Previous studies from our group showed that mast cells in orbital tissue from GO patients are a rich source of platelet-derived growth factor (PDGF)-BB [3], a potent mitogen and stimulus of production of hyaluronan, cytokines, adipogenesis and TSHR expression by orbital fibroblasts [3, 4, 18-21]. Although these data support a role for mast cell derived mediators in the activation of orbital fibroblasts in GO further investigation is warranted, both from a pathophysiological as well as therapeutic point of view.

Histamine is a biogenic amine that is highly expressed in granules of mast cells and released upon their activation and which causes vasodilation, bronchoconstriction, increased capillary permeability, and smooth muscle contraction, all phenomena commonly associated with allergic and inflammatory reactions [15]. Moreover, histamine has been found to induce chemotaxis, proliferation, extracellular matrix molecule and

inflammatory mediator synthesis by fibroblasts, thereby contributing to wound healing and tissue remodeling but also fibrosis [22-37]. Histamine effects can be mediated through four types of G-protein coupled histamine receptors; HRH1 to HRH4 [38] but differences in specific histamine receptor involvement may exist between fibroblasts from different anatomical regions [36, 39]. Although orbital fibroblast activation is at the heart of GO pathogenesis and there is data to implicate that mast cell-derived factors contribute to this [40], the contribution of histamine to GO, especially with regard to orbital fibroblast activation has not been examined so far.

The purpose of the present study was to evaluate the effect of histamine on the production of cytokines (IL-6, IL-8, CCL2, CCL5, CCL7, CXCL10 and CXCL11) previously implicated in GO as well as hyaluronan by orbital fibroblasts. Histamine receptor subtype involvement and NF-κB signaling was further investigated using pharmacological inhibitors.

Materials and Methods

Patients and control subjects

Orbital tissues were obtained at the Rotterdam Eye Hospital (Rotterdam, The Netherlands) from five euthyroid GO patients who underwent orbital decompression surgery at an inactive stage of disease and three control subjects without known thyroid or inflammatory disease who underwent orbital surgery for other reasons. The patients had not received steroid or other immunosuppressive treatment for at least three months prior to surgery. Informed consent was obtained in accordance with the principles of the Declaration of Helsinki and the protocol was approved by the local medical ethics committee. Orbital tissue was partly snap-frozen for (immuno)histological studies, the remaining orbital tissue was used for orbital fibroblast isolation as described previously [19]. Orbital fibroblasts were retained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and antibiotics (penicillin and streptomycin; Cambrex BioWhittaker, Verviers, Belgium) and used for experiments between the 4^{th} and 12^{th} passage.

Mast cell detection in orbital tissues

Snap-frozen orbital tissue from GO patients and controls was sectioned (7 μm) and put onto glass slides and kept overnight at room temperature in humidified atmosphere. The slides were air-dried for 1 hour prior to staining. For toluidine blue staining, the sections were fixed in ethanol for 4 minutes and allowed to dry for 10 minutes. Hereafter, tissue sections were stained with toluidine blue solution (0.5 % toluidine blue in 0.5M HCl) for 3 minutes, subsequently rinsed with tap-water and embedded with glycerol-gelatin. For CD117 (stem cell factor receptor) staining, sections were fixed in acetone containing 0.02% H_2O_2 and air-dried for 10 minutes. Slides were then washed with phosphate-buffered saline (PBS) and incubated overnight in humidified atmosphere with a mouse-anti-human CD117 antibody (YB5.B8, BD Biosciences, Erembodegem, Belgium) or a mouse IgG_1 isotype control (Santa Cruz Biotechnologies, Heidelberg, Germany). Subsequently secondary biotin-labeled horse-anti-mouse antibody (Vector laboratories Ltd, Peterborough, UK) and tertiary horse-radish-peroxidase (HRP)-labeled avidin–biotin complex (ABC/HRP; Dako, Heverlee, Belgium) were added for 1 hour at room temperature. HRP activity was developed by incubating slides with 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at room temperature. After adequate washing in PBS the slides were counterstained with haematoxylin, embedded in glycerol-gelatin and visualized using an Axiovert (Zeiss, Oberkochen, Germany) and photographed at 200x or 400x magnification using an AxioCam MR5 (Zeiss).

IL-6 and hyaluronan production by orbital fibroblast cultures

Orbital fibroblasts from four GO patients and three controls were used. Orbital fibroblasts were seeded at a density of 2.5 x 10^5 cell/well in 12-well plates in DMEM 10 % FCS and allowed to reach confluence. Once a fully confluent monolayer was obtained the medium was substituted with DMEM 1 % FCS overnight. Hereafter, the orbital fibroblasts were incubated in DMEM 1 % FCS in the presence or absence of histamine (59964; Sigma-Aldrich) at concentrations of 1.25, 2.5 and 5.0 mM for 24 and 48 hours. Culture supernatant was collected and analyzed by ELISA for IL-6 (Invitrogen, Frederick, MD, USA) and hyaluronan (R&D Systems, Abingdon, UK).

Hyaluronan synthase and hyaluronidase mRNA expression by orbital fibroblast cultures

Orbital fibroblasts from three GO patients and three controls were used. Orbital fibroblasts were seeded at 4.0×10^5 cells/well into 6-well plates in DMEM 1 % FCS and allowed to adhere overnight. Hereafter, the orbital fibroblasts were incubated in DMEM 1 % FCS in the presence or absence of histamine (5.0 mM) for 2, 4, 6, 8 and 24 hours. Messenger RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and converted into cDNA as described previously [19]. Hyaluronan synthase (*HAS1*, *HAS2* and *HAS3*) and hyaluronidase (*HYAL1*, *HYAL2*, *HYAL3* and *HYAL4*) transcript levels were 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 [19]. Primer-probe combinations used are listed in table 1.

Histamine receptor expression by orbital fibroblasts

Messenger RNA was isolated from four GO and three control orbital fibroblast stains and reverse transcribed into cDNA. Expression levels of the histamine receptors (*HRH1*, *HRH2, HRH3 and HRH4*) was determined by RQ-PCR and normalized to the control gene Abelson. Primer-probe combinations used are listed in table 1.

Histamine receptor inhibition

Orbital fibroblasts from five GO patients and two controls were used. Orbital fibroblasts were seeded at a density of 2.5 x 10^5 cell/well in 12-well plates in DMEM 10 % FCS and allowed to reach confluence. Once a fully confluent monolayer was obtained the medium was substituted with DMEM 1 % FCS overnight. Hereafter, the orbital fibroblasts were incubated in DMEM 1 % FCS in the presence or absence of histamine (5.0 μM) and either in the presence or absence of the HRH1 blocker Loratadine (C 14648000; Dr. Ehrenstorfer GmbH, Augsburg, Germany) at concentrations of 5, 50 and 100 μM for 48 hours. Hereafter culture supernatant was collected and analyzed by ELISA for IL-6, IL-8 (Invitrogen), CCL2, CCL5, CCL7, CXCL10 and CXCL11 (R&D Systems).

NF-κB inhibition

Orbital fibroblasts from four GO patients and three controls were seeded at a density of 2.5 x 10^5 cell/well in 12-well plates in DMEM 10 % FCS and allowed to reach confluence. Once a fully confluent monolayer was obtained the medium was substituted with DMEM 1 % FCS overnight. Hereafter, the orbital fibroblasts were incubated in DMEM 1 % FCS with histamine (5.0 mM; Sigma-Aldrich) in the presence or absence of IKK-2 Inhibitor (SC-514; Calbiochem, La Jolla, CA, USA) at concentrations ranging from 1 to 10 μM for 48 hours. Culture supernatant was collected and analyzed by ELISA for IL-6, IL-8 and CCL2.

Statistical analysis

Data from orbital fibroblast stimulation were analyzed using ANOVA and subsequently analyzed with the Mann Whitney U test. A P-value <0.05 was considered statistically significant.

Results

Mast cell detection in orbital tissues

Toluidine blue staining clearly revealed that mast cells are more abundantly present in GO than control orbital tissue (Figure 1A), which is in line with previous observations [14] and was not further quantified. Mast cells localized next to clusters of fibroblast-like cells as well as adipocytes (Figure 1A). Staining for the mast cell surface marker CD117 (c-Kit/stem cell factor receptor) showed a similar pattern of mast cell numbers and distribution (arrows; Figure 1B).

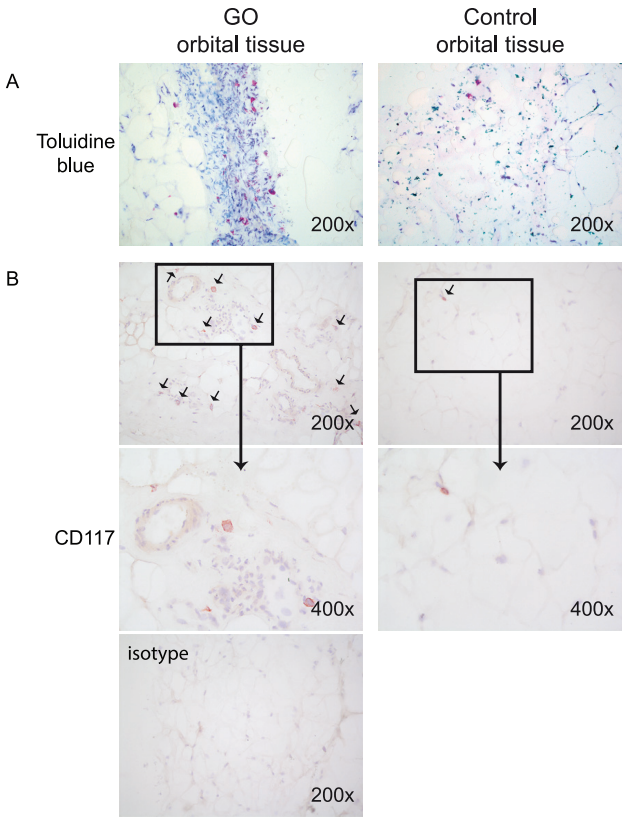

Fig. 1. Mast cells in orbital tissue.
Mast cells were detected in orbital tissue from GO patients (left panels) and control individuals (right panels) by (A) toluidine blue staining (mast cells stain purple) and (B) immunohistochemical detection of the mast cell marker CD117 (red staining and indicated by arrows). Representative pictures from a GO patient and a healthy control are shown.

The effect of histamine on IL-6 production by orbital fibroblasts

Histamine significantly induced IL-6 production by orbital fibroblasts after 24 hours in a concentration-dependent manner (P <0.01), with the strongest induction of IL-6 with the highest histamine concentration (5 mM) tested (Figure 2A). Histamine-induced IL-6 production by orbital fibroblasts did not further increase after 48 hours of stimulation. No difference in response was observed between fibroblasts from GO and controls.

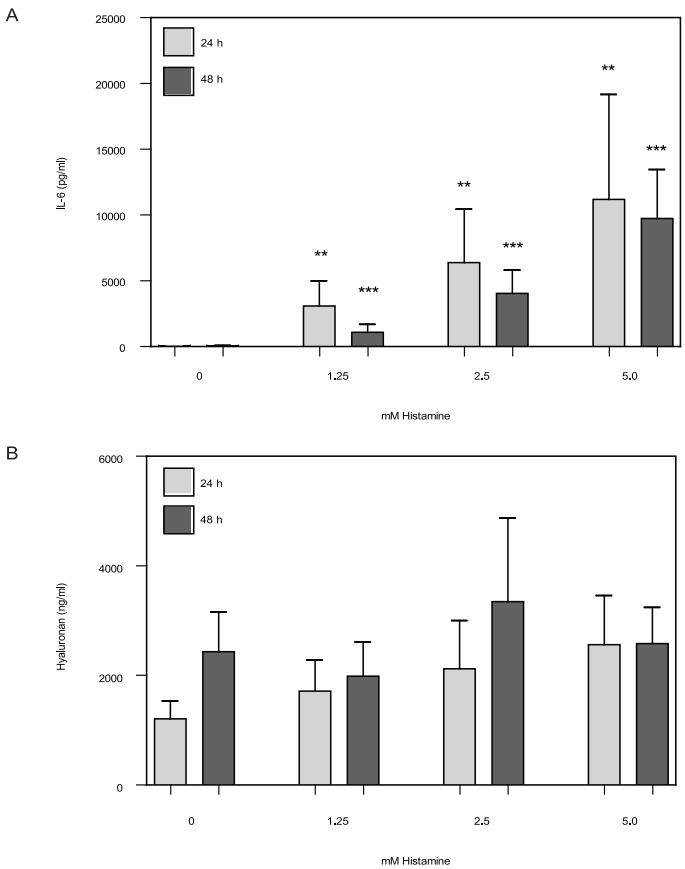

Fig. 2. The effect of histamine on IL-6 and hyaluronan production by orbital fibroblasts.

Orbital fibroblasts (GO n = 4, controls n = 3) were stimulated with histamine (concentration range: 1.25-5.0 mM) for 24 and 48 hours. Culture supernatants were analyzed for IL-6 (A) and hyaluronan (B). Each bar represents the mean value and standard error of the mean (SEM). Data were analyzed using ANOVA followed by Mann Whitney U test. ** and *** indicate p-value of <0.01 and <0.001, respectively.

The effect of histamine on hyaluronan production by orbital fibroblasts

Histamine stimulation for 24 hours slightly, yet non-significantly, induced hyaluronan production by the orbital fibroblasts, reaching the highest level of production at the concentration of 5mM. Prolonged histamine stimulation for 48 hours did not further affect hyaluronan production (Figure 2B). No difference in response was observed between fibroblasts from GO and controls.

To further investigate the effect of histamine on hyaluronan production, three isoforms of hyaluronan synthase (*HAS*) were measured at the mRNA expression level. Histamine transiently enhanced the *HAS1* and *HAS3* mRNA expression level by orbital fibroblasts reaching significance for *HAS1* at 2 hours ($P < 0.01$; Figure 3A) and 4 hours ($P < 0.001$; Figure 3A) and for *HAS3* at 8 hours ($P < 0.05$; Figure 3C). Histamine did not significantly affect *HAS2* mRNA expression level by orbital fibroblasts at all time points detected in this experiment (Figure 3B). In order to examine whether histamine stimulation might alter hyaluronan degradation the mRNA expression level of the four hyaluronidases (*HYAL1*, *HYAL2*, *HYAL3* and *HYAL4*) was determined. Orbital fibroblasts expressed *HYAL1, HYAL2* and *HYAL3* (Figure 3D-E) while *HYAL4* mRNA was undetectable and the expression levels were not affected by histamine (data not shown).

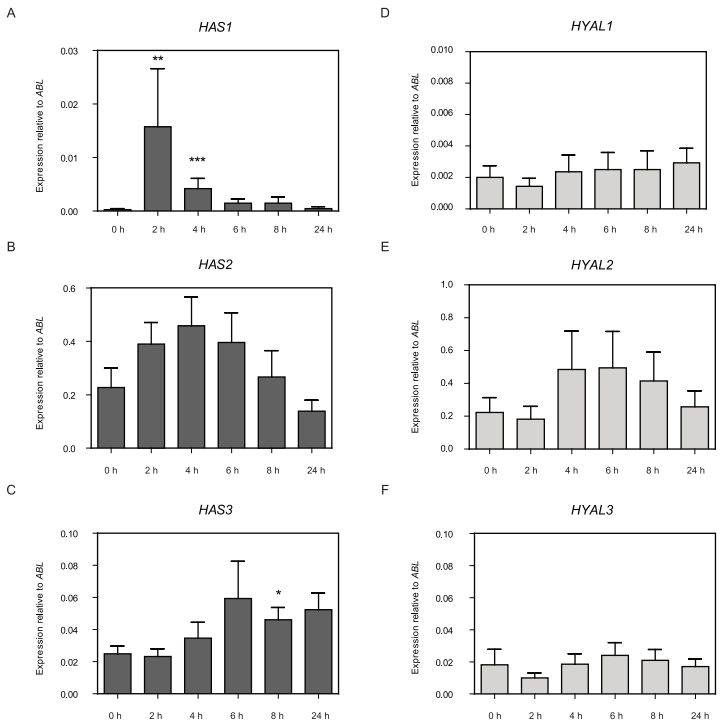

Fig. 3. The effect of histamine on hyaluronan synthase and hyaluronidase mRNA expression by orbital fibroblasts.

Orbital fibroblasts (GO n = 3, controls n = 3) were stimulated with histamine (5 mM) for 2, 4, 6, 8 and 24 hours. Transcript levels of hyaluronan synthase (*HAS)1* (A), *HAS2* (B), *HAS3* (C) and hyaluronidase-1 (*HYAL1*) (D), *HYAL2* (E) and *HYAL3* (F) were determined by RQ-PCR and normalized to the control gene *ABL*. Each bar represents the mean value and the SEM. Data were analyzed using ANOVA followed by Mann Whitney U test. *, ** and *** indicate p-value of <0.05, <0.01 and <0.001, respectively.

The effect of HRH1 inhibition on histamine-induced cytokine production by orbital fibroblasts

Orbital fibroblasts from both GO and controls clearly expressed HRH1 mRNA, while HRH2 mRNA was expressed at very low level (Figure 4A) and HRH3 and HRH4 mRNA were not expressed (data not shown). Therefore we further examined whether histamine induced cytokine production by orbital fibroblasts involved activation of HRH1. Hereto the effect of histamine (5mM) in the presence or absence of the HRH1 blocking agent loratadine (concentration range: 5-100 μM) on the production of IL-6, IL-8, CCL2, CCL5, CCL7, CXCL10 and CXCL11 was examined. Histamine significantly stimulated

IL-6 (P <0.001), IL-8 (P <0.05) and CCL2 (P <0.05) production by orbital fibroblasts (Figure 4B-D) while it did not affect the production of CCL5, CCL7, CXCL10 and CXCL11 (data not shown). The effect of histamine-induced IL-6, IL-8 and CCL2 production by orbital fibroblast was significantly abrogated in a concentration dependent manner by the HRH1 antagonist loratadine (Figure 4B-D). The HRH2 antagonist ranitidine at the same concentrations did not inhibit the histamine-induced IL-6, IL-8 and CCL2 production by the orbital fibroblasts (data not shown).

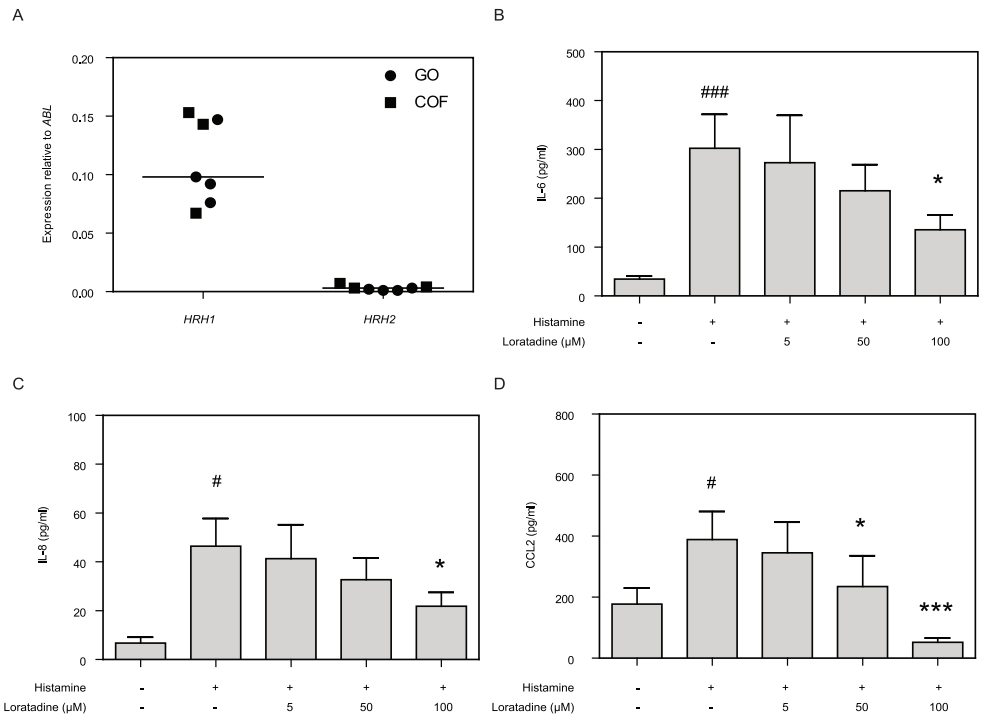

Fig. 4. The contribution of histamine receptor type-1 to histamine-induced cytokine production by orbital fibroblasts.
(A) Transcript levels of histamine receptor (*HRH*)-1, *HRH2*, *HRH3* and *HRH4* were determined in orbital fibroblasts from GO patients (n = 4; circle) and controls (n = 2; square) by RQ-PCR and normalized to the control gene *ABL*. Results are presented as the mean value with error bars (SEM). Orbital fibroblasts (GO n = 5, controls n = 2) were stimulated with histamine (5 mM) in the presence or absence of loratadine (concentration range 5-100 μM) for 48 hours. Culture supernatants were analyzed for IL-6 (B), IL-8 (C) and CCL2 (D). Each bar represents the mean value and the SEM. Data were analyzed using ANOVA followed by Mann Whitney U test. # and ### indicate p-value of <0.05 and <0.001, respectively,

compared to the unstimulated condition. * and *** indicate p-value of <0.05 and <0.001, respectively, compared to histamine stimulation.

The effect of NF-κB inhibition on histamine-induced cytokine production by orbital fibroblasts

To investigate whether histamine-induced NF-κB activity was involved in cytokine production orbital fibroblasts were stimulated with histamine after pre-incubation with the IKK2 inhibitor SC-514 (that blocks NF-κB activation). SC-514 significantly reduced histamine-induced IL-6 and IL-8 production by orbital fibroblast in concentration-dependent manner (P <0.01) (Figure 5A-B). SC-514 also reduced histamine-induced CCL2 production by orbital fibroblasts, although this did not reach statistical significance (Figure 5C). SC-514 at the concentration of 1 μM did not decrease basal production of these cytokines while at a concentration of 10 μM it significantly diminished basal IL-6 and IL-8 production by the orbital fibroblasts (Figure 5).

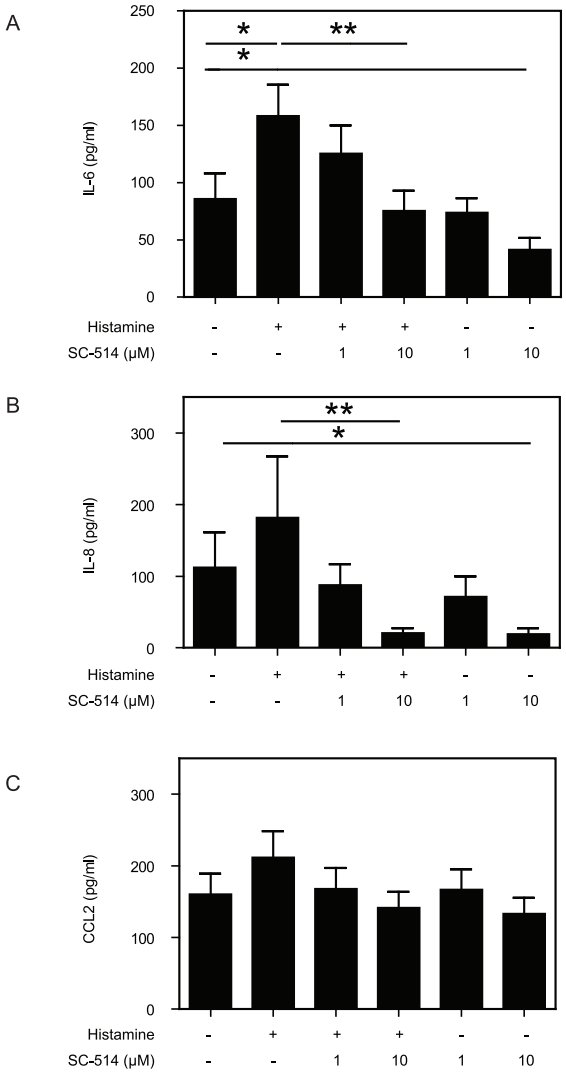

Fig. 5. The effect of NF-κB inhibition on histamine-induced cytokine production by orbital fibroblasts.

Orbital fibroblasts (GO n = 4, controls n = 3) were stimulated with histamine (5 mM) in the presence or absence of the IKK2 inhibitor SC-514 (concentration range 1-10 μM) for 48 hours. Culture supernatants were analyzed for IL-6 (A), IL-8 (B) and CCL2 (C). Each bar represents the mean value and the SEM. Data were analyzed using ANOVA followed by Mann Whitney U test. * and ** indicate p-value of <0.05 and <0.01, respectively.

Discussion

Increased mast cell numbers in orbital tissues from patients with GO have already been noticed for a long time [5], but their role in GO pathogenesis remains highly enigmatic. In line with previous reports we here demonstrate that in orbital tissue from GO patients the infiltrated mast cells are located closely to orbital fibroblasts and adipocytes [14]. Moreover, mast cells in orbital tissue from GO patients were found to display signs of degranulation, indicative of their state of activation [13, 14]. Collectively these data suggest that mediators released by mast cells might be involved in regulating orbital fibroblast activity in GO. In support of this we here demonstrate for the first time that histamine stimulates the production of IL-6, IL-8 and CCL2, cytokines involved in the pathophysiology of GO, by orbital fibroblasts.

IL-6 is a multifunctional cytokine that regulates immune responses, it attracts and activates B-lymphocytes and stimulates plasma cell differentiation and immunoglobulin production [41]. IL-6 levels are elevated in orbital tissue from GO patients and stimulates TSHR expression on orbital fibroblasts and drives adipocyte differentiation by orbital fibroblasts, which is a major component of GO pathology [18, 42, 43]. IL-8 is a major mediator of the inflammatory response and is a powerful chemoattractant, mostly for neutrophils [44]. IL-8 is elevated in orbital tissue from GO patients and elevated serum levels have been associated with hyperthyroidism and GO [45-47]. CCL2 is a potent chemoattractant for monocytes and macrophages, which are abundantly present in orbital tissue from GO patients [3, 48, 49]. Remarkably of the T-cell chemoattractants (CCL5, CXCL10 and CXLL11) implicated in GO [20, 50-52] and analyzed in our study none was induced by histamine. This data might suggest that histamine predominantly regulates monocyte/macrophage infiltration in GO by inducing the production of monocyte/macrophage chemoattractants by orbital fibroblasts, including CCL2. Positive correlations between the amount of monocyte/macrophage infiltration and CCL2 expression in orbital tissue from GO patients have indeed been observed [48].

The stimulatory effect of histamine that we observed on the production of IL-6, IL-8 and CCL2 by orbital fibroblasts is in line with observations in fibroblasts from other anatomical regions [53-55]. Previous studies in a.o. nasal and gingival fibroblasts found clear relationships between HRH1, NF-κB signaling and cytokine production, including IL-6 [53, 55, 56]. The HRH1 blocker loratadine prevented histamine-induced production of IL-6, IL-8 and CCL2 by orbital fibroblasts and inhibition of NF-κB completely abrogated histamine-induced IL-6 and IL-8 production, while CCL2 production was only partially reduced. This latter finding suggests that histamine induces also NF-κB independent activities that control CCL2 production by orbital fibroblasts, which is in line with previous notions that NF- κB requires additional transcriptional regulators to optimally induce CCL2 production by orbital fibroblasts [20]. Indeed next to NF- κB additional signaling molecules and pathways downstream of HRH1 have been described,

including ATP release, Ca^{2+} mobilization, cGMP, p38, ERK and JNK [36, 38, 53, 57]. Also the inability of histamine to stimulate the production of CCL5, CCL7, CXCL10 and CXCL11 indicates that histamine-induced HRH1 activation in orbital fibroblasts is not sufficiently activating signal pathways and transcriptional machinery required for the production of these chemokines, which requires for instance the transcription factor interferon regulatory factor (IRF)-3 [58].

Hyaluronan production by orbital fibroblasts plays an important role GO [4, 40]. Histamine did enhance *HAS1* and *HAS3* mRNA expression by orbital fibroblasts, yet did not result in increased hyaluronan production. This latter is most likely not related to increased hyaluronan degradation as histamine did not enhance mRNA levels for four main hyaluronidases in orbital fibroblasts. Histamine did however not enhance the expression level of *HAS2*, which is considered to represent the main HAS isoform involved in hyaluronan synthesis by orbital fibroblasts in GO [40, 59].

In conclusion our data indicate that the mast cell mediator histamine can induce the production of NF-κB controlled-cytokines by orbital fibroblasts and as such can contribute to the pathologic orbital environment in GO. Our findings are relevant in relation to the improvement of tearing, itching and dryness of the eyes observed in a small cohort of GO patients treated with the HRH1 antagonist cetirizine in combination with the leukotriene receptor antagonist montelukast [60]. Our data are therefore in support of the hypothesis that orbital fibroblast activation by mast cell-derived products is an important mechanism in the pathogenesis of GO and that mast cells, their mediators and downstream receptors might represent therapeutic targets in GO.

Acknowledgments

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Table 1. Real-time quantitative PCR primer-probe combinations

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′ FAM - 3′ TAMRA)
ABL	TGGAGATAACATCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT
HRH1	TaqMan Gene Expression Assays (Hs00911670_s1), Life technologies, Foster City, CA.		
HRH2	TaqMan Gene Expression Assays (Hs00254569_s1), Life technologies.		
HRH3	TaqMan Gene Expression Assays (Hs00200610_m1), Life technologies.		
HRH4	TaqMan Gene Expression Assays (Hs00222094_m1), Life technologies.		
HAS1	GCAAGCGCGAGGTCATGT	CGGGGGTCCTCGTCCA	ACTACGTGCAGGTCTGTGACTCGGACAC
HAS2	AATGGGGTGGAAAAAGAGAAGTC	CAACCATGGGATCTTCTTCTAAAAC	TCCACACTTCGTCCCAGTGCTCTGA
HAS3	AAGGCCCTCGGCGATTC	CCCCCGACTCCCCCTACT	ACATCCAGGTGTGCGACTCTGACACTGTG
HYAL1	TaqMan Gene Expression Assays (Hs00201046_m1), Life technologies.		
HYAL2	TaqMan Gene Expression Assays (Hs01117343_g1), Life technologies.		
HYAL3	TaqMan Gene Expression Assays (Hs00185910_m1), Life technologies.		
HYAL4	TaqMan Gene Expression Assays (Hs00202177_m1), Life technologies.		

Chapter 8

Limited, but potentially predictable effect of imatinib mesylate in systemic sclerosis using Interferon type I activation and type III procollagen N-terminal propeptide

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Manuscript submitted

Abstract

Introduction
Systemic sclerosis (SSc) is an often therapy refractory autoimmune disease characterized by excessive collagen deposition. In this study we examined the effect of imatinib mesylate (IM) in the treatment of SSc and determined whether the effect could be predicted using markers of collagen synthesis and Interferon type I induced gene expression, the so-called IFN type I signature.

Methods
10 previously therapy refractory SSc patients were treated with IM 400 mg orally. Response was assessed by using overall SSc activity score and Modified Rodnan Skin Score (MRSS). Serum N-terminal propeptide of type III collagen (PIIINP) was used as a biomarker for collagen synthesis. Interferon (IFN) type I signature expression was used as a biomarker for disease activity.

Results
Only one patient showed a decrease of 2 points in overall SSc activity score. Two patients had a clinically relevant decrease in MRSS. The two patients with a skin response had significantly higher PIIINP levels at baseline and were the only ones to show an IFN-type I signature.

Discussion
IM 400 mg daily has limited effect in SSc. The patients that did respond with softening of the skin had significantly higher markers of collagen synthesis and showed a IFN-type I signature. The latter results might help in selecting patients that are responsive to therapy.

Introduction

Systemic sclerosis (SSc) is a difficult to treat, severely debilitating, autoimmune disease characterized by vasculopathy, immune activation and fibrosis of the skin and internal organs. Imatinib mesylate (IM), is used as a treatment for therapy-refractory SSc with high variability in therapeutic outcomes ranging from ineffective/toxic responses to extremely encouraging clinical improvement.[1] This high variability in treatment outcomes stresses the need for a biomarker identifying SSc patients likely to respond to IM treatment.

We conducted a study to determine the effect of IM in a group of patients with SSc who had failed on previous immunosuppressive drug treatment. Furthermore we examined whether the effect of treatment could be predicted using biomarkers of collagen synthesis and disease activity. Serum N-terminal propeptide of type III collagen (PIIINP) that has been reported increased in the skin and serum of SSc patients was used as a biomarker for collagen synthesis.[2] Interferon type I induced gene expression in monocytes, the IFN type I signature, has been shown to correlate with disease activity in various autoimmune diseases including SSc and was used as a biomarker for disease activity.[3]

Material and methods

We performed an open label study in which we intended to treat 10 SSc patients with IM 400mg/day for one year. All patients included in this open-label trial met the American College of Rheumatology criteria for SSc.[4] Patients (over 18 years of age) had to be refractory to therapy with either cyclophosphamide, Methotrexate or Mycophenolic acid. Adequate end organ function, defined as: total bilirubin <1.5 x ULN, SGOT and SGPT < 2.5 x ULN (or <5 x ULN if hepatic disease involvement is present), creatinine < 1.5 x ULN, ANC >1.5 x 10^9/L, platelets > 100 x 10^9/L, was required. All patients gave written informed consent. The Medical Ethics committee of Erasmus MC approved the study.

The initial primary endpoint for response to IM was defined as a significant decrease in SSc mean Modified Rodnan Skin Score (MRSS).[5] A 10-point reduction or normalisation in Rodnan skin score at 12 months was regarded a major response. Assuming a standard deviation in the difference between the two measurements of 10, this study has a power of 0.8 to detect a 10-point difference with a significance level of 0.05. Furthermore absence of progression in pulmonary, skin and renal disease after one year (all required) was considered necessary for a major response.

However, as three patients included in the study had a Rodnan skin score at baseline below 10 the primary endpoint was redefined as a reduction of at least 2 points in overall SSc severity score using the Medsger Disease Severity Scale.[6] Secondary efficacy parameters were total lung capacity (TLC) and diffusing capacity for carbon monoxide (DLCO) and Modified Rodnan skin score.

In addition to these parameters, blood and serum were collected prior to IM treatment and at 3, 6, 9 en 12 months of treatment. Interferon type I signature expression and B-cell activating factor (BAFF) mRNA expression were determined in monocytes as described previously[7]. PIIINP was measured using the UniQ ® PIIINP RIA (Orion Diagnostica, Espoo, Finland). Assay was performed following the manufacturer's instructions.

Results

Patient characteristics are summarized in Table 1. Disease activity as measured by overall SSc severity score varied between 4 and 11. Two patients stopped treatment within 3 months as they did not notice any improvement and where no longer motivated to continue. In terms of the redefined primary endpoint only one patient showed a response. Two other patients had a one-point reduction in overall SSc severity. None of the patients had an increase in overall severity score. One of the patients with a one-point decrease in SSc severity score showed a reduction of more than 10 points on MRSS. The other patient with a one-point reduction had substantial improvement in gastrointestinal function. One additional patient had a decrease of 8 points on MRSS after one year. This patient also showed a remarkable improvement in pulmonary function (TLC baseline: 77% of predicted, end of study: 92%; Diffusion capacity baseline: 74%, end of study: 88%).

We isolated monocytes from 8 of the SSc patients included who completed the study and analysed the IFN type I signature expression. Only the monocytes from the 2 patients who showed a decrease in MRSS displayed a positive IFN type I signature at baseline (Figure 1A). The IFN type I signature remained the same during IM treatment (Figure 1A-B).

Previously, we found B cell activating factor (BAFF) mRNA to strongly correlate with the presence of the IFN type I signature in Sjögren's syndrome patients[7] and BAFF serum levels were found to correlate with the extent of SSc skin fibrosis.[8] We therefore assessed BAFF mRNA expression in monocytes from the SSc patients in our study. The two skin responders showed the highest levels of BAFF mRNA at baseline, which decreased significantly upon IM treatment (Figure 1C-D).

Baseline and follow-up PIIINP levels were assessed in serum of 7 of the SSc patients. The two skin responders showed much higher baseline PIIINP levels than the other SSc patients, decreasing drastically upon 6 months of IM treatment (Figure 1E).

Discussion

In the present study we treated 10 patients with therapy refractory SSc with IM 400 mg. Although none of the patients deteriorated only one had a reduction of more than 2 points on overall SSc severity score. Additionally, two patients showed a reduction in MRSS. Previous studies have focussed mainly on skin effects. The reported response to treatment with tyrosine kinase inhibitors in these studies varies considerably. A double-blinded study in patients with extensive cutaneous involvement did not show any benefit of IM treatment [9] whereas an open label study showed a statistically significant decrease in mean MRSS from 21 to 16 in three years.[10] Interestingly, the two patients in our study that showed a clinically relevant decrease in MRSS where those with a high PIIINP level. In line with this, a beneficial clinical response to IM has previously been reported when the SSc skin displays excessive deposition of collagen type III.[11] These two patients also showed a clear IFN type I signature and associated high BAFF mRNA levels. The latter also decreased under therapy. Unfortunately neither the patient that responded with a two point decrease in overall activity score nor the patient with an isolated decrease in gastrointestinal complaints could be distinguished based on either PIIINP level or IFN-signature.

This study has several drawbacks. First of all, it is not placebo controlled which increases the risk for observer bias. In order to limit this we decided to use a relatively high cut-off level for total and skin response. Furthermore, the study is small. Nevertheless the association of response with biomarkers is striking and needs further exploration.

The results of our study suggest that IM therapy is effective in a limited number of patients. The patients most likely to respond with skin improvement are those with biochemical evidence for excessive collagen synthesis and active disease as determined by high BAFF mRNA levels. Using targeted therapy, responding patients may potentially be selected based on BAFF mRNA and PIIINP levels thereby avoiding expensive and potentially toxic treatment in others. Our results need to be corroborated in larger controlled studies.

Acknowledgements

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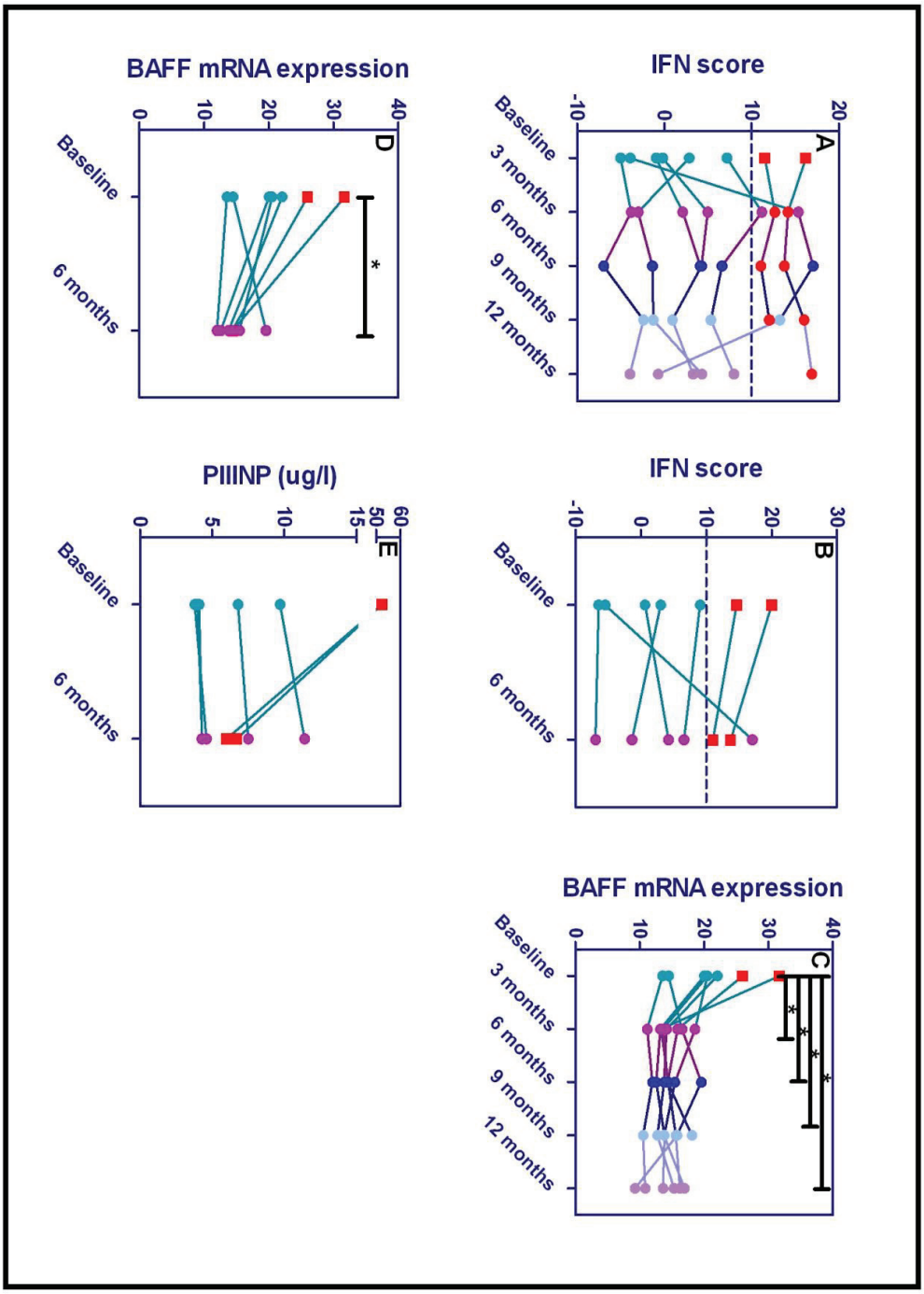
Table 1. Baseline characteristics

patient	Gender (M/F)	Age (years)	SSc score (start)	MRSS (start)	Completed study (yes/no)
1	F	57	7	2	yes
2	F	49	4	23	yes
3	F	72	5	23	yes
4	M	45	6	2	yes
5	F	33	10	34	no
6	F	35	4	10	yes
7	F	49	8	24	yes
8	M	62	8	17	yes
9	F	55	8	1	yes
10	M	39	11	14	no

M = male, F= female, SSc score = systemic sclerosis activity score, MRSS = modified Rodnan skin score

Figure 1. (A) IFN scores in monocytes from IM treated SSc patients (n=8) at baseline, 3 months, 6 months, 9 months and 12 months of treatment. (B) IFN scores in IM treated SSc patients (n=8) at baseline and 6 months of treatment. (C) BAFF mRNA expression levels in monocytes of IM treated SSc patients (n=8) at baseline, 3 months, 6 months, 9 months and 12 months of treatment. (D) BAFF mRNA expression levels in monocytes of IM treated SSc patients (n=8) at baseline and 6 months of treatment. (E) PIIINP levels in serum from IM treated SSc patients (n=7) at baseline and 6 months of treatment.

Data from the two patients who responded to IM is displayed in red. In A and C the Kruskal-Wallis test was performed followed by Mann Whitney U test in A-E. Only significant differences are shown where (*) represents *P*-values <0.05.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Chapter 9

General discussion

Graves' disease (GD) may be complicated by Graves' ophthalmopathy (GO), a condition that remains difficult to treat in a significant number of patients, as many of these patients do not respond to currently available therapeutic options. Treatment options for GO available so far are limited, which is most likely related to the complex pathophysiology of the disease. Currently, the most common and effective treatment for active moderate-to-severe and sight-threatening GO is (high dose) intravenous glucocorticoids [1]. However, glucocorticoid treatment shows high success rates only if it is introduced in an active inflammatory phase of the disease [2, 3]. It has been recognized that up to 40% of patients do not respond to the currently used immunosuppressive treatment with glucocorticoids [4], and orbital decompression surgery is applied when patients fail to respond to glucocorticoids or for rehabilitating purposes [2, 3]. This clearly indicates the need for improved insight into the pathophysiological processes involved in GO as this will help to facilitate development of novel therapeutic interventions. Currently, orbital fibroblast activation followed by excessive proliferation, hyaluronan and inflammatory mediator production and differentiation of orbital fibroblasts into adipocytes and pro-fibrotic myofibroblasts are considered central processes in the (immune) pathogenesis of GO [5]. Consequently, it has been hypothesized that therapies targeting orbital fibroblast activity could be promising treatment options in GO [6]. PDGF-BB was previously found to be elevated in orbital tissues from GO patients and it plays an important role in orbital fibroblast activation by inducing inflammatory and tissue remodeling processes. Along this line the tyrosine kinase inhibitors (TKIs) imatinib mesylate and nilotinib that target the platelet derived growth factor (PDGF)-receptor (PDGF-R) have been proposed as treatment options for GO [7]. However, for optimal clinical application of such TKIs in GO a better understanding of the orbital fibroblast activating properties of PDGF along with that of other growth factors is required. Alternatively, other ways of interfering with receptor tyrosine kinase activation can be thought of, for example with small molecules that target downstream signaling pathways such as PI3K and MAPK [8, 9]. Moreover, additional factors such as mediators produced by mast cells that might be able to activate orbital fibroblasts need to be identified as they may represent therapeutic targets as well, either alone or in combination with other mediators. The studies performed in this thesis generated new insights into the complex biological processes involved in GO. This could be of relevance for the development of novel therapeutic interventions and will be further discussed hereunder. In addition, important directions for future research are indicated.

NOVEL INSIGHTS IN THE PATHOGENESIS OF GRAVES' OPHTHALMOLOGY

In GO the clinical symptoms are driven by three main pathologic features: 1) inflammation, 2) increased accumulation of extracellular matrix components (ECM) (mostly hyaluronan), and 3) enhanced adipogenesis [5]. These dynamic processes all

occur within the context of the space limited orbital cavity, resulting in typical clinical features, including upper eye lid retraction, edema, erythema of the periorbital tissues and conjunctivae, and proptosis [10, 11]. PDGF-BB was previously found to be elevated in orbital tissues from GO patients, at all stages of disease, suggestive for a role in inflammation as well as tissue remodeling [7]. In support of this it was demonstrated that PDGF-BB:

1) stimulates orbital fibroblasts to produce chemokines and cytokines involved in recruitment, activation and differentiation of immune cells, including monocytes, macrophages, T lymphocytes and B lymphocytes [12]

2) stimulates orbital fibroblast proliferation and hyaluronan production, processes that will largely contribute to tissue volume expansion [7, 13]

3) enhances the expression of thyroid stimulating hormone (TSH) receptors (TSHR) on orbital fibroblasts, which increases susceptibility of orbital fibroblasts to activation by TSHR stimulatory autoantibodies resulting in increased production of cytokines and hyaluronan [14].

These data clearly link PDGF-BB and the autoimmune inflammatory process involved in GO. However, another major process involved in orbital tissue volume expansion in GO is the differentiation of orbital fibroblasts into adipocytes [10, 11]. Several mediators have been proposed to enhance adipogenesis by orbital fibroblasts in GO, including interleukin (IL)-1β, IL-6, prostaglandin D2 (PGD_2) and TSHR stimulatory autoantibodies [15-19]. Studies in this thesis demonstrated that PDGF-BB enhanced adipogenesis by orbital fibroblasts that were cultured in a pro-adipogenic environment (Chapter 4). This effect was not mediated via induction of autocrine IL-6 signaling. It is also unlikely that IL-1β production and signaling was involved as PDGF-BB does not stimulate IL-1β production by orbital fibroblasts [12]. However, so far it cannot be excluded that other factors induced by PDGF-BB are involved in this. Remarkably, for other cell types, including adipose-derived stem cells and mesenchymal stromal cells, PDGF-BB has been identified as anti-adipogenic factor, which involved activation of signaling molecules such as extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC)α/δ [20-23]. On the other hand, PDGF also activates the signaling molecule c-Abl, which is a tyrosine kinase that was recently identified to be indispensable for adipocyte differentiation by murine 3T3-L1 preadipocytes [24-26]. Although c-Abl activation by PDGF-BB in orbital fibroblasts has not been examined so far, it cannot be excluded that PDGF-BB-driven adipocyte differentiation by orbital fibroblasts involves c-Abl activity.

What exactly creates the pro-adipogenic environment in orbital tissue from GO patients is still far from clear, but there are indications that mostly Thy1- orbital fibroblasts differentiate into adipocytes. Studies exploring the effects of PDGF-BB on adipogenesis by Thy1- and Thy1+ orbital fibroblasts would therefore

be of interest. A recent study by Li *et al*. demonstrated that Thy1- orbital fibroblasts cultured under pathological pressure in a three-dimensional collagen matrix differentiate into adipocytes [27]. This *in vitro* model mimics the increased mechanical pressure that orbital fibroblasts from GO patients encounter within the context of the noncompliant and space limited orbital cavity, which thus provides pro-adipogenic signals. It would be of interest to examine the contribution of PDGF-BB in this model.

Fibrocytes are bone marrow derived cells that can be considered as connective tissue progenitors that circulate in the blood and infiltrate damaged tissue where they contribute to inflammation, tissue remodeling and fibrosis [28-30]. In GO, fibrocytes were found to infiltrate the orbital tissue where they further contribute to the heterogeneity of the orbital fibroblast pool [31]. Fibrocytes were demonstrated to express TSHR and to spontaneously differentiate into adipocytes which was associated with a further increase in TSHR expression [31]. Fibrocytes express PDGF-R and the PDGF-Rβ-PDGF-BB axis has been proposed to contribute to the recruitment of fibrocytes into tissue [32]. Whether PDGF-BB, or other PDGF isoforms, also drive adipogenesis by fibrocytes has not been explored so far but such studies would be of relevance to further delineate GO pathogenesis.

Nevertheless, the data presented in this thesis demonstrate that PDGF-BB has the ability to stimulate adipogenesis by a heterogeneous pool of orbital fibroblasts. This, along with the already observed effects of PDGF-BB on proliferation, hyaluronan, cytokine/chemokine production and TSHR expression by orbital fibroblasts indicates that PDGF-BB may represent a key factor in the pathogenesis of GO. This notion is further strengthened by the observations from chapter 5 where the actions of PDGF-BB were explored in combination with other growth factors implicated in GO.

Basic fibroblast growth factor (bFGF) is a growth factor that is implicated in organ fibrosis, especially via the stimulation of proliferation and ECM production by fibroblasts [33-37]. In orbital tissues from GO patients, elevated bFGF expression was observed and immunohistochemical studies identified orbital fibroblasts, adipocytes and endothelial cells as producing sources [38, 39]. Also elevated serum bFGF levels have been observed in GO patients [40], but the contribution of bFGF to orbital fibroblast activation has been hardly studied. The study in chapter 5 demonstrated that bFGF synergistically enhanced the capacity of PDGF-BB to stimulate hyaluronan and IL-6 production by orbital fibroblasts. Importantly, bFGF alone did not stimulate IL-6 production by orbital fibroblasts, which underlines the importance of studying combined effects of growth factors in GO. In other cell types synergistic effects of PDGF and bFGF were observed as well and depended on prolonged activation of signaling molecules, enhanced growth factor receptor expression and autocrine release of additional growth factors. In human smooth muscle cells autocrine bFGF signaling enhanced and prolonged ERK activation in PDGF-BB-induced cell proliferation [41]. In addition, induction of PDGF-R expression upon bFGF stimulation and the induction of FGF-R1 expression upon PDGF-BB stimulation have been observed in various cell types such as vascular smooth muscle cells, endothelial cells, pericytes and foreskin fibroblasts [42-44]. We did not observe an

effect of bFGF on PDGF-Rα and PDGF-Rβ mRNA expression nor an effect of PDGF-BB on FGF-R1 mRNA expression in orbital fibroblasts. Activated FGF-R can also transactivate the PDGF-R [45]. It is however unlikely that transactivation of PDGF-R contributed to the synergistic enhancement observed in our studies as the TKI dasatinib, that inhibits the tyrosine kinase activity of the PDGF-R but not FGF-R, had no effect on bFGF-induced hyaluronan and IL-6 production by orbital fibroblasts (Chapter 5). Although usage of a *PDGFB* knockout orbital fibroblast model would have strengthened the study presented in chapter 6 it is still feasible to hypothesize that the synergistic effect of bFGF and PDGF-BB on hyaluronan and IL-6 production involves prolonged autocrine PDGF-BB signaling (Chapter 6).

The data from chapter 5 also demonstrated that bFGF did not synergistically enhance orbital fibroblast proliferation induced by PDGF-BB but only had an additive effect on this. This stresses the importance of studying combined effects of growth factors on several aspects of orbital fibroblast activation in GO, as different outcomes can be expected. Further studies that explore the effects of growth factors alone or combinations thereof are therefore warranted.

Transforming growth factor (TGF)-β_1 was previously found to be elevated in GO orbital tissue and stimulated hyaluronan production by orbital fibroblasts. Moreover, it was demonstrated that co-stimulation of orbital fibroblasts with TGF-β_1 and PDGF-BB showed an additive effect of TGF-β_1 to PDGF-BB on hyaluronan production [7]. However, the effect of TGF-β_1 on adipogenesis in orbital fibroblasts remains controversial [46, 47]. A preliminary study exploring the effect of TGF-β_1 alone and in combination with PDGF-BB on adipogenesis was therefore performed. This revealed that TGF-β_1 did not affect adipogenesis in orbital fibroblast cultures when compared to differentiation medium alone (Figure 1 and 2A), nor did it induce expression of the adipocyte predominant transcription factor peroxisome proliferator-activated receptor gamma (*PPARγ*) by orbital fibroblasts (Figure 2B). Remarkably, TGF-β_1 significantly inhibited PDGF-BB-induced adipogenesis (Figure 2). In line with this, TGF-β_1 is known to downregulate TSHR expression by orbital fibroblasts [47], while PDGF-BB enhances TSHR expression, which is associated with adipogenesis of orbital fibroblasts [14, 48]. These data are thus clearly in support of opposing effects of TGF-β_1 and PDGF-BB on adipogenesis by orbital fibroblasts. It is currently unknown how TGF-β_1 inhibits the pro-adipogenic effect of PDGF-BB, but activation of SMAD family member 3 (SMAD3) might be involved as previous studies demonstrated inhibition of adipogenesis by TGF-β_1 stimulated SMAD3 activity [49].

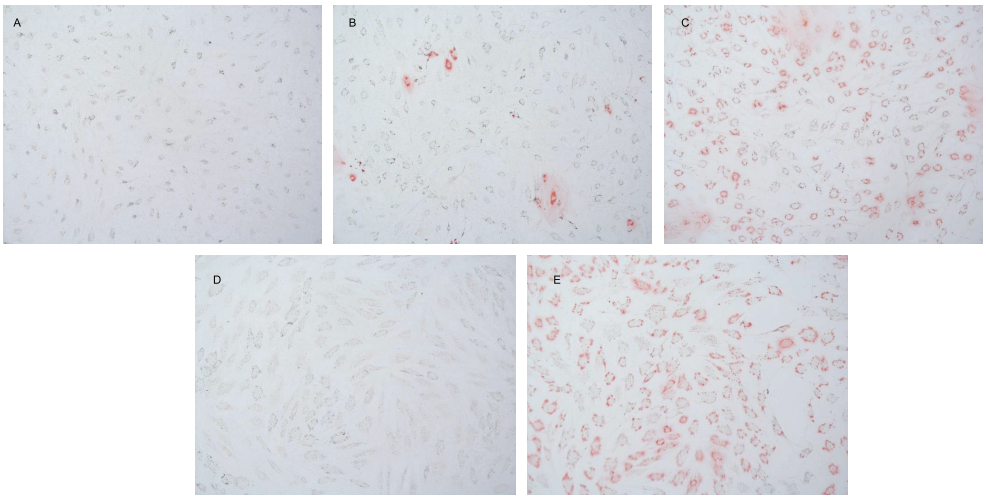

Figure 1. Oil-Red-O staining on orbital fibroblasts.
Orbital fibroblasts were cultured in nondifferentiation medium (A), adipocyte differentiation medium (B), adipocyte differentiation medium in the presence of PDGF-BB (50 ng/ml) (C), adipocyte differentiation medium in the presence of TGF-β_1 (10 ng/ml) (D) and adipocyte differentiation medium in the presence of PDGF-BB and TGF-β_1 (E). Oil-Red-O staining was performed after 14 days of differentiation. Results from a representative orbital fibroblast culture are displayed.

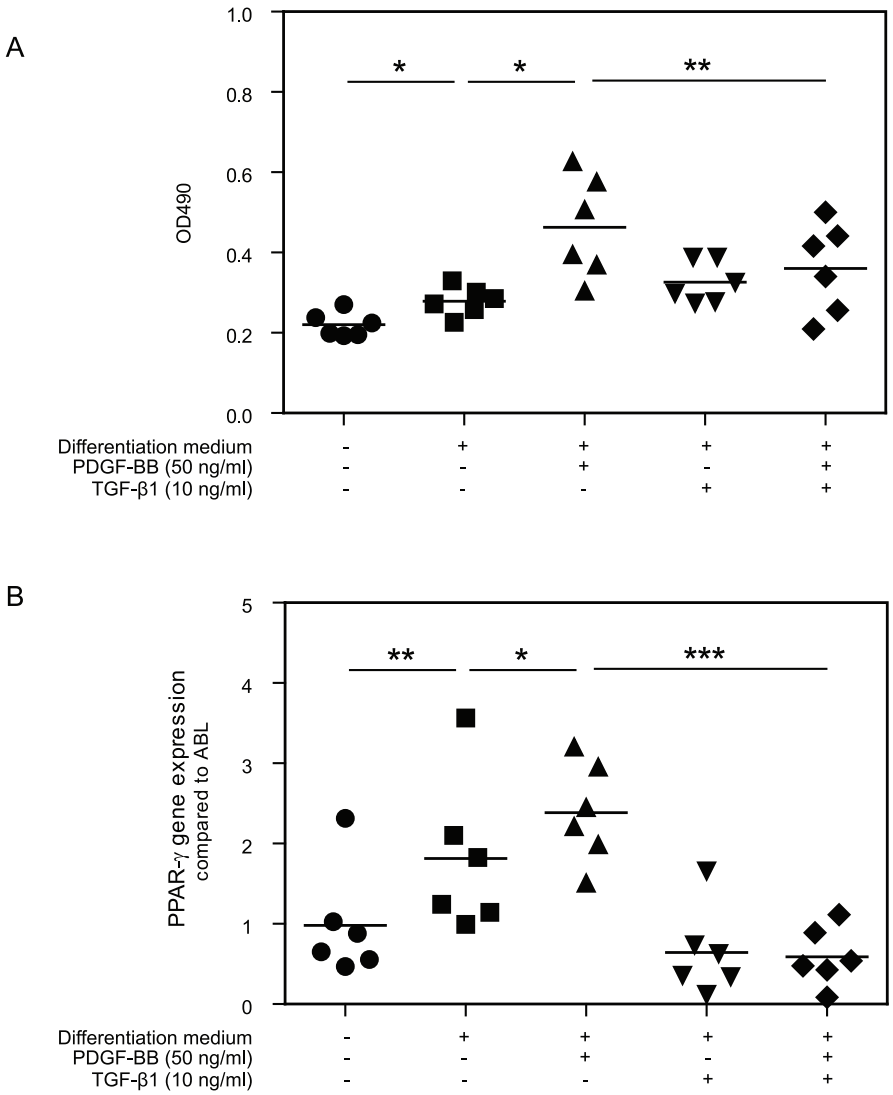

Figure 2. Quantification of adipogenesis in orbital fibroblasts.
(A) Oil-Red-O staining was quantified in eluate after 14 days of differentiation of orbital fibroblasts from controls (n=3) and GO patients (n=3) using a spectrophotometer at 490 nm. (B) Expression of PPAR-γ mRNA was determined by RQ-PCR and normalized to the control gene ABL in orbital fibroblasts from controls (n=3) and GO patients (n=3) after 14 days of adipocyte differentiation. Each dot represents the orbital fibroblast strain from one individual and horizontal bars represent the mean values within a group. Data were analyzed using ANOVA followed by Mann Whitney U test.

Although increased systemic and orbital vascular endothelial growth factor (VEGF) levels have been observed in GO [40, 50], the data presented in this thesis do not support a strong orbital fibroblast activating effect of VEGF, which may be related to low VEGF-R expression by orbital fibroblasts. It can however not be excluded that in GO VEGF activates other cell types, for instance endothelial cells, monocytes and macrophages that abundantly express VEGF-Rs [51]. VEGF is a strong activator of endothelial cells where it increases proliferation, migration and vascular permeability and VEGF is also a chemotactic factor for monocytes [52, 53]. Vascular changes around the ocular muscles have been found in GO [54] and monocytes and macrophages are abundantly present in GO orbital tissue [13, 55, 56]. In order to identify the precise role of VEGF in the pathophysiology of GO additional studies are required.

Mast cells are abundantly present in GO, especially during the later stages of disease [13, 57-59], but their role in GO pathogenesis remains poorly studied. In chapter 7 it was demonstrated that mast cells in orbital tissue from GO patients are localized closely to orbital fibroblasts and adipocytes, which is in line with previous reports by other groups that in addition described signs of mast cell degranulation [60, 61]. Therefore, the effects of histamine, which is highly present in mast cell granules, on orbital fibroblasts were examined in more detail (Chapter 7). Histamine stimulated the production of IL-6, IL-8 and chemokine (C-C motif) ligand (CCL) 2, cytokines previously indicated in the pathophysiology of GO, but did not stimulate hyaluronan production by orbital fibroblasts. These data suggest that in GO histamine might be predominantly involved in the inflammatory response and less likely to be involved in the excessive production of hyaluronan. Interestingly, histamine did not stimulate the production of the T lymphocyte chemoattractants CCL5, C-X-C motif ligand (CXCL) 10 and CXCL11 that have previously been implicated in GO [62-64]. From these data it can be hypothesized that histamine is involved in orbital monocyte/macrophage recruitment in GO by inducing the production of specific chemoattractants, including CCL2, by orbital fibroblasts. Positive correlations between macrophage infiltration and CCL2 expression in GO orbital tissue have indeed been described [13, 55, 56], but the exact contribution of histamine to this requires further studies.

Besides histamine, the granules of mast cells contain many other factors, including tryptase and chymase that have previously been linked to tissue remodeling and fibrotic conditions [65-67]. Tryptase for instance stimulates proliferation, collagen and cytokine production by cardiac, bronchial, conjunctival and synovial fibroblasts, mostly via activation of protease-activated receptor 2 (PAR2) [65, 67-72]. Mast cell derived chymase activates latent TGF-β_1, which subsequently results in SMAD signaling, proliferation and ECM production by fibroblasts [73, 74]. Previously it was found that TGF-β_1-induced hyaluronan production by orbital fibroblasts involves SMAD signaling [7]. Studies into the effects of tryptase and chymase on orbital fibroblast activation are therefore of interest and these might further support the notion that mast cells are important contributors to GO.

The regulation of mast cell accumulation in GO is unclear, but stem cell factor (SCF) and IL-33 might be involved as they represent important growth and survival factors for mast cells [75-77]. Increased levels of SCF and IL-33 have indeed been observed in serum from GO patients [78, 79]. Studies have demonstrated that SCF expressed by dermal fibroblasts provides a survival signal for mast cells via activation of the SCF-receptor (C-kit/CD117) expressed at the mast cell surface [80]. This pro-survival effect was further enhanced when dermal fibroblasts were treated with PDGF-BB, which increased the SCF expression. In line with this, preliminary studies conducted in our laboratory demonstrated that PDGF-BB significantly enhanced *SCF* mRNA expression by orbital fibroblasts (Figure 3), suggesting that such a mechanism can be involved in mast cell accumulation in GO orbital tissue. Importantly, mast cells were previously identified as an important source of PDGF-BB in GO orbital tissue [13]. These data along with the observed effects of histamine suggest interaction between mast cells and orbital fibroblasts in GO where they control each other's biological behaviour. This complex interaction between mast cells and orbital fibroblasts requires further study, for instance using co-culture trans-well approaches to delineate involvement of soluble and membrane expressed molecules as well as a phenomenon like trans-granulation [81].

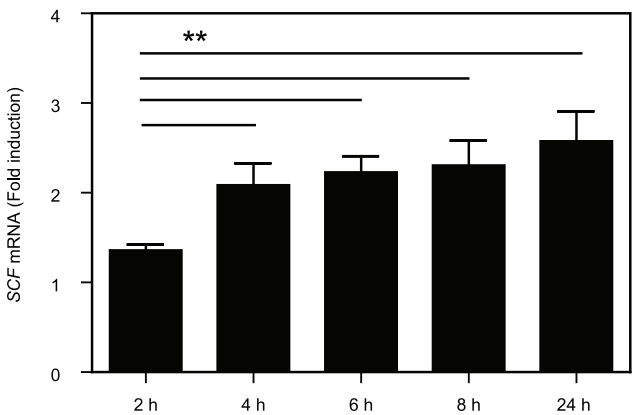

Figure 3. Effect of PDGF-BB on *SCF* mRNA expression by orbital fibroblasts.
SCF mRNA expression by orbital fibroblasts (n=5) was determined by RQ-PCR after stimulation with PDGF-BB (50ng/ml) for the indicated time periods, normalized to the control gene *ABL* and expressed relative to the unstimulated situation. Data were analyzed using ANOVA followed by Mann Whitney U test. A P-value of <0.05 was considered as statistically significant. ** indicates a p-value of <0.01.

Altogether the data in this thesis provide important additions to the pathogenic model of GO, as is summarized in figure 4. Based on these novel data on the pathogenesis, potential new treatment strategies could be suggested for patients with severe GO as will be discussed in the next paragraph.

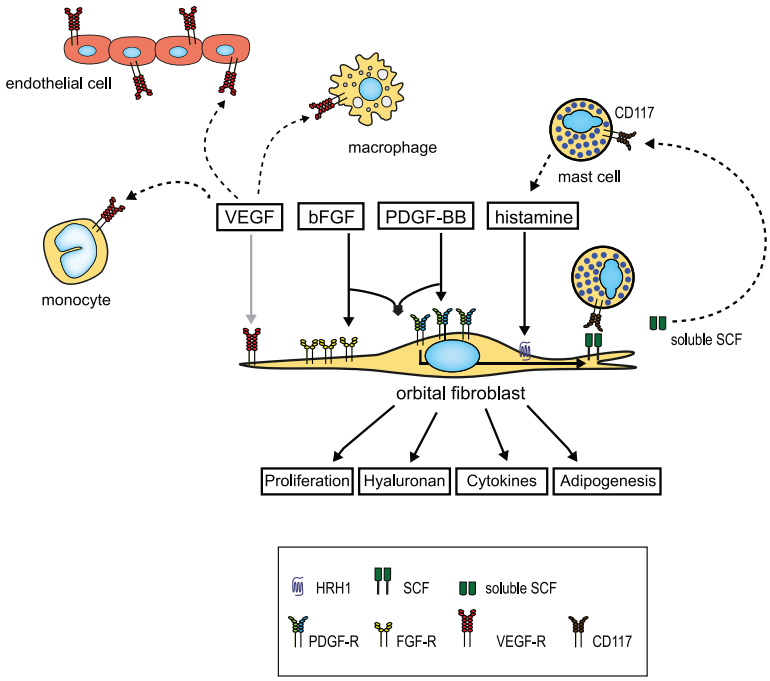

Figure 4. PDGF-BB, bFGF, VEGF and histamine in the pathogenesis of Graves' ophthalmopathy.

Proliferation, cytokine production, hyaluronan production and adipogenesis by orbital fibroblasts are central features of GO pathogenesis. PDGF-BB stimulates proliferation, the production of hyaluronan, cytokines and adipogenesis by orbital fibroblasts. In addition PDGF-BB enhances SCF expression by orbital fibroblasts. SCF is a cytokine that acts as survival factor for mast cells. SCF is mostly expressed at the cell membrane and it can be hypothesized that it provides survival signals to mast cells upon cellular interaction between orbital fibroblasts and mast cells via activation of SCF receptor (CD117/c-Kit). Alternatively this survival signal might also be generated by the soluble form of SCF (indicated by dashed line). bFGF induces orbital fibroblast proliferation and hyaluronan production, but not IL-6 production while VEGF hardly affects orbital fibroblast activation (indicated by grey line), presumably due to low VEGF-receptor (VEGF-R) expression in orbital fibroblasts. Monocytes, macrophages and endothelial cells express VEGF-R abundantly and therefore it is hypothesized that VEGF affects these cell types in GO (indicated by dashed lines). PDGF-BB and bFGF act synergistically on IL-6 and especially hyaluronan production by orbital fibroblasts, as indicated by the combined arrow. Orbital fibroblasts express histamine receptor subtype-1 (HRH1) and via this receptor histamine stimulates the production of NF-κB controlled cytokines, while it does not affect hyaluronan production.

POTENTIAL TREATMENT STRATEGIES FOR GRAVES' OPHTHALMOPATHY

❖ Imatinib mesylate versus Dasatinib

Based on our previous findings on the important role of PDGF in the pathogenesis of GO, TKIs imatinib mesylate and nilotinib that target the PDGF-R have been proposed as potential treatment options for GO [7]. However, the use of imatinib mesylate, and especially nilotinib, is associated with various side effects such as peri-orbital edema, peripheral arterial occlusive disease and cerebrovascular events when used in the treatment of chronic myeloid leukemia (CML) [82, 83]. As a result, imatinib mesylate and nilotinib may not represent preferable treatment options for GO, at least not when given in dosages comparable to those used to treat CML patients. Nevertheless, targeting the important pathogenic PDGF pathway in GO with other clinically available TKIs could still be considered for therapy-refractory patients or patients with sight-threatening disease. Dasatinib, a second-generation TKI, is a less specific TKI than imatinib mesylate and nilotinib, but it displays a considerably higher inhibitory potency for both PDGF-Rα and PDGF-Rβ chains when compared to imatinib mesylate and nilotinib [84]. In addition, dasatinib is associated with fewer side effects than imatinib mesylate in the treatment of CML [85, 86]. Moreover, in pre-clinical models of fibrotic diseases like systemic sclerosis, pulmonary fibrosis and cardiac fibrosis, dasatinib significantly reduced disease activity as demonstrated by the reduction in dermal thickness and lung fibrosis and the improvement in ventricular function [87-89]. These studies underline the potential clinical implications of dasatinib in fibrotic diseases. To provide a basis for the potential future introduction of dasatinib in the treatment of GO, the *in vitro*, *ex vivo* and clinical effects of dasatinib were investigated in this thesis and will be further discussed hereunder.

In chapters 3 and 4 *in vitro* and *ex vivo* studies are described, showing that dasatinib effectively inhibits PDGF-BB-induced proliferation, cytokine (CCL2, IL-6, IL-8) and hyaluronan production by orbital fibroblasts. Moreover, dasatinib also effectively inhibited adipogenesis and cytokine and hyaluronan production by whole orbital tissues derived from active GO patients [90, 91]. Interestingly, when compared to imatinib mesylate, significantly lower dosages of dasatinib were needed to inhibit PDGF-BB induced proliferation, cytokine and hyaluronan production by orbital fibroblasts [91]. This difference in effects could potentially be explained by the higher inhibitory potency of dasatinib for PDGF-Rα and PDGF-Rβ [84]. When these effects of dasatinib could be extrapolated to the *in vivo* situation, dasatinib could be a promising drug in the treatment of GO, as fewer side effects could be expected with the use of lower dosages, when compared to imatinib mesylate. Although dasatinib could be an interesting therapeutic option in GO, it should be taken into account that in other studies it was described that dasatinib stimulated adipogenesis in human bone marrow-derived mesenchymal stromal cells [92]. In GO, orbital fibroblasts with overlapping features of mesenchymal stem cells may be

present [93, 94] and adipogenesis could theoretically be stimulated by dasatinib treatment in GO as well. Although in our studies we did not demonstrate that dasatinib induced adipogenesis (Chapter 4) [90], further pre-clinical studies should be performed to determine the exact and cell-specific effects of dasatinib in GO tissues. Nevertheless, dasatinib might be a promising future treatment modality in patients with therapy-refractory or sight-threatening GO. It should however also be taken into account that GO may show a self-limiting course over time and this clinical course should be considered also with respect to the potential adverse effects of dasatinib that have been described such as pleural effusion, skin rash, vomiting, diarrhea, fatigue, headache, anemia, thrombocytopenia and neutropenia [91]. Introduction of dasatinib in clinical practice for patients with GO should therefore be performed with greatest caution and should be limited to patients with very active and/or severe disease. Based on our promising *in vitro* and *ex vivo* data, we studied in more detail on the clinical effects of dasatinib in a patient with a very severe and sight-threatening GO, that did not respond to conventional treatment modalities and required additional systemic therapy.

❖ **From *in vitro* to *in vivo***

Dasatinib for the treatment of very active and severe Graves' ophthalmopathy

Our patient was a 44-year old female, which we saw at the Endocrinology and Clinical Immunology outpatient clinics of the Rotterdam Thyroid Center for the first time in 2013. She was diagnosed with GD in 2003 and was initially successfully treated with so-called block and replace therapy, which consisted of thiamazole and levothyroxine. In 2006 a relapse of GD occurred and treatment with radioactive iodine was initiated. Consequent treatment with levothyroxine was terminated in 2012. Upon visit to our outpatient clinics, there were clinical signs of GO, accompanied by pretibial myxedema. Myxedema describes a specific form of cutaneous and dermal edema, secondary to increased deposition of connective tissue components. Although not fully understood, it is hypothesized that the increased deposition of connective tissue components is caused by fibroblast activation [95]. There were clear signs of proptosis, with an initial Clinical Activity Score (CAS, a tool to determine severity of eye involvement in GD) of 3 out of 7 (mild to moderate activity). Anti-TSHR antibody levels were 195.0 IU/l (upper limit of normal 0.9 IU/l), free thyroxine (fT4) was 18.6 pmol/ml (normal range 11-25 pmol/ml) and TSH was 0.033 mU/l (normal range 0.4-4.3 mU/l). Biochemical findings were compatible with subclinical hyperthyroidism. According to the international recommendations for treatment of GO [3], this patient was treated with intravenous glucocorticoid pulse therapy, receiving 6 infusions of 500 mg methylprednisolone followed by 6 infusions of 250 methylprednisolone. However, after this treatment, the patient experienced progressive complaints of GO, with proptosis and a decrease in visual activity. CAS went from 3/7 to 6/7 (severe activity). Because of sight-threatening GO, orbital decompression surgery became necessary. During surgery, orbital tissue was collected and taken into tissue

culture in order to evaluate whether other immunosuppressive therapies could be beneficial in this patient for future treatment of GO.

Orbital tissue obtained was divided into two parts and put in culture overnight in DMEM 1% FCS in the presence or absence of dasatinib (2.5 µg/mL) as described previously [91]. Messenger RNA was extracted, reversed transcribed into cDNA, and gene expression levels were determined by RQ-PCR. These preliminary data showed that dasatinib effectively decreased the mRNA expression levels of cytokine (*IL6*, *IL8*, *CCL2*, tumor necrosis factor alpha (*TNF*α) and *IL10*), hyaluronan synthase (*HAS*) 1-3, adhesion molecule (intercellular adhesion molecule 1 (*ICAM-1*)), and growth factor (*TGF-*β*1* and *PDGF-B*) genes in whole orbital tissue from the patient (Figure 5).

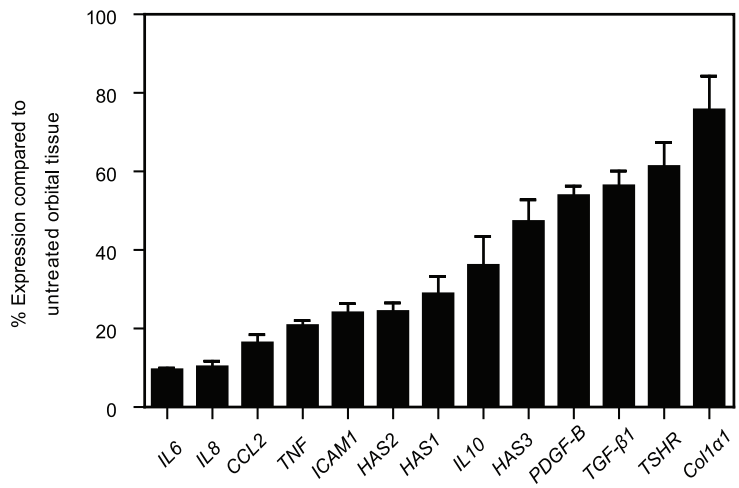

Figure 5. Effects of dasatinib on gene expression in whole orbital tissue from a patient with severe, sight-threatening GO.
Orbital tissues were put in culture in the presence or absence of dasatinib. mRNA expression levels from the orbital tissues were determined by RQ-PCR after 24 hours, normalized to the control gene ABL and expressed relative to the untreated tissue. Horizontal bars represent the mean values, error bars indicate the standard error of the mean. 100% represents a comparable expression compared to untreated orbital tissue and other values represent percentages of expression compared to the untreated tissue.

Based on these ex vivo data obtained from whole tissue cultures from the GO patient presented above and the fact that the patient still suffered from very severe, therapy-refractory GO which required systemic treatment after debulking surgery in order to prevent further expansion of orbital tissue, we decided, after obtaining written informed consent by the patient and approval by our institutional review board, to start treatment with dasatinib. Initially, a dose of 50 mg once daily was prescribed in order to

evaluate tolerability and side effects. After 4 weeks of treatment, the dosage was increased up to 100 mg once daily. Clinical evaluation after 6 weeks revealed a significant reduction of the CAS from 6/7 to 4/7 (Figure 6). Interestingly, her myxedema totally resolved over this period. TSHR autoantibody levels remained high with 98.4 IU/l, while thyroid function restored (fT4 13.9 pmol/ml and TSH 3.6 mU/l) (Figure 6). Because of significant clinical improvement and absence of side effects, treatment with dasatinib once daily 100 mg was continued. However, after 4 months of medical treatment the patient presented to the ophthalmologist with acute, sight-threatening progressive GO, for which acute decompression surgery was warranted. Treatment with dasatinib was terminated at that moment. Remarkably, we did not see any recurrence of her pretibial myxedema. After debulking surgery, oral glucocorticoid treatment was started in a tapering regimen. Her clinical condition remained stable and glucocorticoid treatment was terminated after 3 months. No need for additional systemic treatment was needed till date.

In conclusion, our *ex vivo* and clinical data on dasatinib support the potential clinical implication of the TKI dasatinib in patients with very severe and/or sight-threatening GO, in which conventional therapies are ineffective. There are however some limitations to our study. After initial improvement of GO, 4 months after start of treatment, there was significant progressive disease requiring acute decompression surgery. Several reasons for these findings could be hypothesized. First of all, it has been previously described that dasatinib stimulates adipogenesis in a cell-type specific manner [92]. Although we did not find such an effect of dasatinib on orbital fibroblasts *in vitro*, it cannot be ruled out that activation of mesenchymal stem cells-like orbital fibroblasts in the orbital tissue [93, 94] resulted in increased adipogenesis in our patient after several months of treatment, resulting in progressive GO. The initial response could then assign to the interference of dasatinib with the PDGF-signaling pathway. On the other hand, dasatinib is a rather selective TKI which does not target the FGF-R and as described in chapter 5 bFGF may significantly contribute to the pathogenesis of GO and may therefore be a target for therapy as well. Therefore, studies on novel TKIs, with a broader range of action should reveal whether these compounds could be of more interest as potential therapeutic options in GO. We also found a striking difference in the response to dasatinib in the patient between the orbital region and the skin (pretibial myxedema). It could be speculated that concentration differences between the orbital tissue and skin from the lower leg might be responsible for these site specific differences in effects of dasatinib when systemically administered. Current studies are evaluating potential differences in concentrations of dasatinib between the orbital tissue and skin lesions obtained from the patient described.

Although our initial findings on the effects of dasatinib in GO were promising, there is still need for broader therapeutic approaches in GO, as in our patient after 4 months of therapy progressive disease activity was experienced.

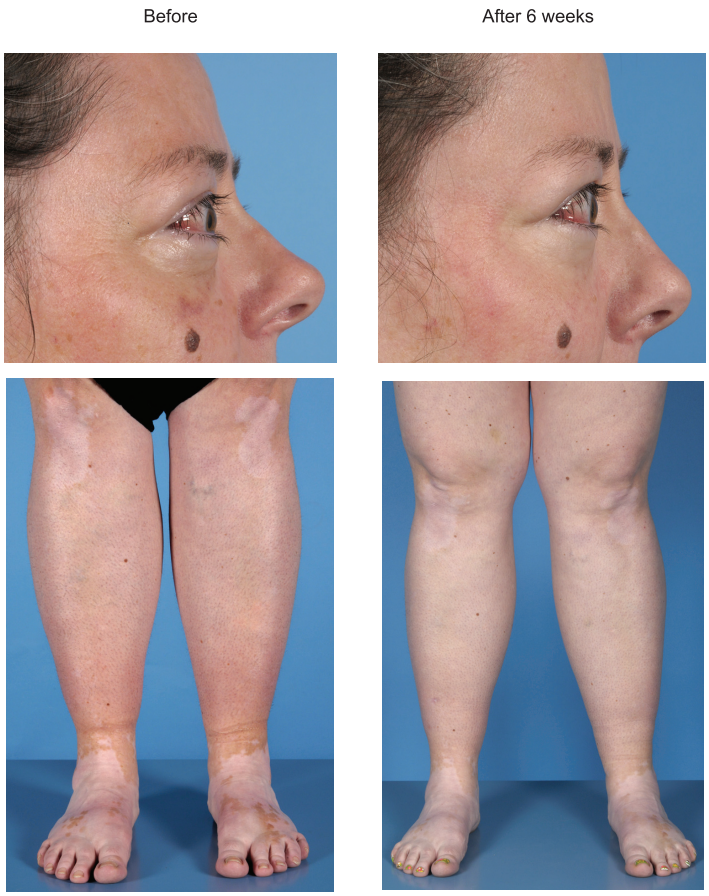

Figure 6. Clinical features of a patient with severe GO before and after 6 weeks of dasatinib treatment.
The patient presented with GO with CAS 6/7 (upper left picture) and pre-tibial myxedema (lower left picture). After 6 weeks of dasatinib treatment CAS had reduced from 6/7 to 4/7 (upper right picture) and pre-tibial myxedema completely resolved (lower right picture).

❖ Nintedanib

In studies described in chapters 5 and 6 we demonstrated that also other signaling pathways than the PDGF-cascade targeted by dasatinib could be of importance in the pathogenesis of GO, necessitating the exploration of other TKIs in the treatment of GO. bFGF signaling was found to induce orbital fibroblast proliferation and hyaluronan

production by orbital fibroblasts. Moreover, co-stimulation with PDGF-BB and bFGF resulted in the additive/synergistic induction of orbital fibroblast activation which highlights the importance of evaluating TKI that might block both PDGF and bFGF signaling pathways. Nintedanib, a TKI that targets PDGFRs, FGFRs and VEGFRs [96, 97], effectively blocked the additive/synergistic effects of PDGF-BB and bFGF on orbital fibroblasts while dasatinib, which targets only PDGFRs, only blocked the PDGF-BB effect (Chapter 5). These data indicate that multiple receptor tyrosine kinase (RTK) directed therapy could be promising in reducing excessive orbital fibroblast activity present in GO. In previous studies in fibrotic diseases it was demonstrated that nintedanib was very effective. Treatment with nintedanib significantly improved outcome in the aggressive pulmonary fibro-proliferative disease idiopathic pulmonary fibrosis (IPF) [97]. Remarkably, these data showed that nintedanib was able to reverse established fibrosis. In pre-clinical models for systemic sclerosis (SSc), nintedanib was found to inhibit fibroblast activation and to exert potent anti-fibrotic effects as well [98]. These last results have led to the recent start of a phase 3 clinical trial with nintedanib in patients with SSc related lung fibrosis (ClinicalTrials.gov NCT02597933). Moreover, the use of nintedanib was associated with minor side effects such as diarrhea and nausea when compared to other TKIs such as imatinib mesylate, nilotinib and dasatinib [83, 96, 97]. As a result, it would be of interest to investigate the effects of nintedanib in established GO. In our *in vitro* and *ex vivo models* nintedanib was found to effectively inhibit fibroblast proliferation and activation in GO. There could be however some drawbacks on the use of nintedanib in GO. The systemic blocking of FGF-R could interfere with various other pathways as FGF-Rs are expressed by fibroblasts, epithelial, mesenchymal and inflammatory cells involved in tissue repair. Targeting FGF-Rs could therefore interfere with processes essential after tissue injury [99]. In addition, FGF21 and FGFR1 have an important role in glucose intake [100-104] while FGFR4 is expressed by hepatocytes and mainly controls bile acid synthesis [105]. Collectively, it cannot be excluded that long-term nintedanib treatment might cause undesirable effects in response to tissue injury and might cause hypercholesterolemia, hyperbilirubinemia and type-2 diabetes [102-105]. Future safety and efficacy studies are needed to address these topics in more detail.

❖ **Other therapeutic interventions**

Chapter 8 describes the therapeutic effects of the TKI imatinib mesylate in a number of patients with SSc. SSc is a debilitating autoimmune disease with significant morbidity and mortality. It is characterized by a fibroproliferative vasculopathy, excessive production of ECM and an aberrant autoimmune activation resulting in skin and visceral organ fibrosis [106]. The underlying pathophysiologic mechanisms remain elusive and effective therapeutic options are limited. Progressive fibrosis is a hallmark of this disease and SSc could be used as a disease model for other diseases in which fibrosis is involved. Introduction of TKI treatment in SSc was based on several pre-clinical studies that showed beneficial effects from imatinib mesylate in targeting dermal fibroblast activity [107, 108]. In a recent report by Bournia *et al.* it was also demonstrated

that imatinib mesylate could be considered an individualized treatment approach in severe SSc [109].

In chapter 8 we describe a number of SSc patients who had failed on previous immunosuppressive drugs that were subsequently treated with imatinib mesylate. None of the patients deteriorated, and two patients showed a reduction in modified Rodnan skin score (mRSS). Biomarkers predicting which patients could respond to imatinib mesylate treatment were identified. Data in chapter 8 showed that patients who were positive for interferon (IFN) type I signature with high B-cell activating factor (BAFF) and high procollagen III N-terminal pro-peptide (PIIINP) levels responded to imatinib mesylate treatment. Even though the data suggested that IFN type I signature, BAFF and PIIINP level could predict the patients who are susceptible for imatinib mesylate treatment, and selected patients could thus benefit from such treatment, other alternative treatment regimens for SSc are needed. In previous, preliminary, studies we have demonstrated that fibroblast proliferation could be influenced by somatostatin (SS) analogues (unpublished results).

SS is a 14-amino acid neuropeptide with a variety of actions throughout the human body. Most of these actions are mainly inhibitory like, for instance on hormone secretion by the pituitary gland, thyroid gland and pancreas [110, 111]. Previous studies have shown that SS analogues also exert effects on various immune cells, where it influences chemotaxis, apoptosis and cytokine secretion [112, 113]. In addition, SS inhibits tumour growth, especially in neuroendocrine tumours [114, 115].

In order to evaluate the effects of SS on fibroblast activation and proliferation in SSc, as a model for fibrotic disease, 3 skin biopsies from affected skin were obtained from 3 patients with active and therapy-refractory SSc associated skin disease. Written informed consent and approval by our institutional review board were obtained. One whole tissue sample was cultured overnight in DMEM 1% FCS in the presence of imatinib mesylate (2.5 µg/ml), the second tissue sample was cultured in the presence of SS (10^{-8} M), and the third sample was put in culture without any treatment, serving as internal control. mRNA was extracted, reversed transcribed into cDNA, and gene expression levels were determined by RQ-PCR. In two out of three SSc patient tissues imatinib mesylate reduced *IL6*, *CCL2*, *TNF* and *IL10* mRNA expression levels while the expression levels of *TGF-β1* and collagen type 1 α 1 (*Col1α1*) were not affected by imatinib mesylate in all tissues (Figure 7A). Despite the fact that due to the small sized skin biopsies taken in this study, no further studies could be performed. It would be interesting to investigate the IFN type I signature in these biopsies and compare the results to the findings obtained in chapter 8. In one out of three SSc patient skin biopsies it was found that SS reduced *IL6*, *IL8*, *TNF* and *IL10* mRNA expression levels while the expression levels of *TGF-β1* and *Col1α1* were not affected by SS in all tissues (Figure 7B). As TGF-β_1 which can stimulate collagen production by skin fibroblasts and both *TGF-β_1* and *Col1α1* mRNA expression levels were not affected by both imatinib mesylate and SS [116, 117], these data suggest

that imatinib mesylate and SS might primarily target the inflammatory response by reducing pro-inflammatory cytokine production rather directly target on the process of excessive production of ECM. Further studies are needed to elucidate the effects of SS and its analogues in fibrotic diseases, like SSc. Moreover, combined treatment regimens, consisting of TKIs and SS analogues, could show additive or synergistic effects in inhibition of fibroblast activation and/or proliferation.

In one study in GO it was demonstrated that SS receptor 1 (SSTR1) expression was upregulated in orbital adipose tissue when compared to control tissue [118]. However, treatment of GO patients with SS or one of its analogues, octreotide and lanreotide which targets SSTR2 [119], did not show clinical improvement in GO [120-123]. These data suggested that SS and its analogues might be able to target SSTR2 expressed by primary orbital fibroblasts [124] and SSTR2 expressed by macrophages and dendritic cells [125], but SS and its analogues might not be able to reduce the overall orbital activation in GO. SS and its analogues also induce phosphatase activity which is associated with MAP kinase, AP-1 and NF-κB dephosphorylation [126, 127] which are also downstream signaling molecules of PDGF-Rs and FGF-Rs [8, 128]. However, to our knowledge, it is unknown whether this phosphatase activity induced by SS had any inhibitory effect on RTK such as PDGF-Rs and FGF-Rs. Combining SS with TKI in the treatment of GO might be beneficial in inhibiting RTK activity involved with orbital fibroblast activation in GO.

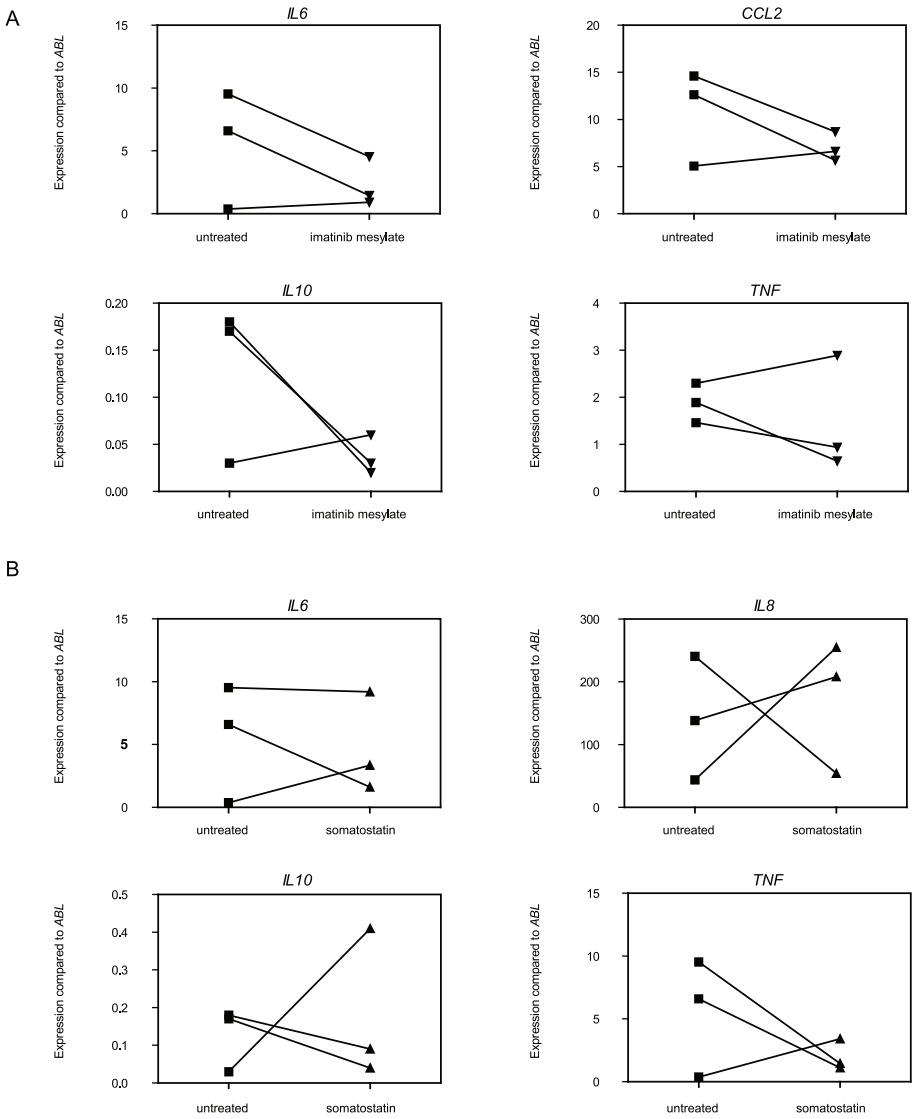

Figure 7 . Effects of imatinib mesylate and somatostatin on gene expression profiles in skin biopsies from 3 SSc patients.

Skin biopsies from 3 patients with active and therapy-refractory SSc associated skin disease were obtained and put in culture in the presence or absence of (A) imatinib mesylate (2.5 µg/ml) or (B) SS (10^{-8} M) for 24 hours. Gene expression levels were determined by RQ-PCR, normalized to the control gene ABL. Each line represents the response of tissue from an individual patient.

FUTURE RESEARCH DIRECTIONS

❖ Mast cells in Graves' ophthalmopathy

Increased mast cell numbers were observed in orbital tissues from patients with GO in this thesis. Increased levels of SCF and IL-33, important growth and survival factors for mast cells, have been observed in serum from GO patients [78, 79] which might be responsible for mast cell accumulation. Nevertheless, it is unknown whether PDGF-BB-induced SCF mRNA expression resulted in membrane-bound and/or soluble form. As a result, the pathogenic roles of both SCF (and IL-33) in GO require further investigation, for instance by using co-culture and trans-well approaches. Besides histamine, mast cell granules contain tryptase and chymase which have previously been linked to pathologic tissue remodeling [65-67]. Future studies on orbital fibroblast activating activity of both these substances is therefore of great interest.

❖ Animal model in Graves' ophthalmopathy

In this thesis the effects of PDGF-BB, bFGF, VEGF and histamine on orbital fibroblast activity were explored in a simplified *in vitro* single cell model or *ex vivo* orbital culture model. The contribution of PDGF-BB, bFGF and VEGF to GO as well as their inhibition by specific medication such as TKI needs further exploration in a recently developed pre-clinical mouse model for GO [129]. Also the contribution of mast cells and histamine to GO can be studied in this model as mast cells were found present in the orbital tissue from mice developing GO-like symptoms [129, 130]. In addition, such a model could be used to study the effect of (combined) therapies and would allow validation of the optimal route of drug administration, either systemic or locally.

❖ Epigenetics in Graves' ophthalmopathy

Epigenetics play an important role in the regulation of heritable gene expression via mechanisms including DNA methylation and histone modifications. For decades, epigenetics has shown to be important in cancers where DNA hypermethylation for instance can result in silencing of tumor suppressor genes and consequently rapid disease progression [131-133]. Recently, epigenetics has also been shown to play a role in autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and SSc as well as fibrotic disorders [134-139].

In idiopathic pulmonary fibrosis (IPF) DNA methylation has shown to be involved in the pathogenesis of the disease [140]. Interestingly, IPF fibroblasts exhibit differences in DNA methylation compared to healthy controls [141], including genes involved in proliferation and ECM production. Unexpectedly, genes involved with organ

morphogenesis and potassium ion channels also displayed altered DNA methylation resulting in overexpression, and they might represent novel candidate genes contributing to IPF. Synovial fibroblast activation and their contribution to inflammation is also regulated by DNA methylation. Transcription factor T-box transcription factor 5 (TBX5) was found to be hypomethylated in synovial fibroblasts from rheumatoid arthritis patients and overexpression of TBX5 resulted in production of chemokines, including IL-8, CXCL1 and CCL20 [142]. With Regard to disease severity it is of interest that dermal fibroblasts from diffuse SSc (dSSc) patients and limited SSc (lSSc) patients displayed different DNA methylation profiles, which involved genes related to ECM receptor interaction and focal adhesion [143].

In GD DNA methylation has been studied in $CD4^+$ and $CD8^+$ T lymphocytes, and genes involved in T lymphocyte signaling were found hypermethylated [144]. Because orbital fibroblasts are considered to be the central cell type in the pathophysiology of GO [5] and the comparison of epigenetic profiles from normal and diseased tissue might represent a novel predictive model for disease outcomes [145], studies into DNA methylation in orbital fibroblasts from GO patients during active and inactive stages of disease and comparison with healthy control orbital fibroblasts should be performed. Such studies might provide novel candidate genes related to disease activity and disease remission upon treatment and may provide novel treatment targets.

❖ **Combined therapy in Graves' ophthalmopathy**

Although considerable new information has been garnered regarding the pathogenesis of fibrotic diseases, therapeutic options are limited. Currently, only two drugs, pirfenidone [146] and nintedanib [147], have been approved for the treatment of IPF and the clinical effects of nintedanib in SSc associated lung fibrosis are under current investigation (ClinicalTrials.gov NCT02597933). In this thesis it was demonstrated that various pro-fibrotic mediators are involved in GO pathogenesis and it could be hypothesized that other, still uncharacterized, mediators may be involved. Because of this complexity with many factors involved, it is very unlikely that any single drug will be successful in adequately modifying a major fibrotic disease [148]. Therefore, it is suggested that combination treatments should be explored in fibrotic diseases. In recent studies in a murine pneumoconiosis (pulmonary fibrosis) model, combination therapy consisting of imatinib mesylate and the TKI lapatinib was found to be more effective when compared to administration of the drugs individually [148]. The rationale behind this combined treatment is that it blocks c-Abl, PDGF-R and epidermal growth factor receptor (EGF-R). As patients with GO may also not respond to monotherapy with immunosuppressants or TKI, it would be of interest to investigate the potential use of treatment with a combination of drugs or with drugs directed at multiple targets in GO.

As already suggested, leukotriene receptor antagonist montelukast in combination with the HRH1 antagonist cetirizine would be of interest to investigate in a larger group of patients. In addition, combined treatment of phosphatase activity of SS with TKI in targeting RTK activity might show more beneficial effects in disease activity than introducing SS or TKI alone. As has been shown in the murine pulmonary fibrosis model, combinations of TKIs could be considered in GO, to target multiple pathways. Moreover, prostaglandin F2-alpha (PGF2α) significantly reduced proliferation and adipogenesis in orbital fibroblasts from GO patients [149]. The use of PGF2α eye drops in targeting proptosis (ClinicalTrials.gov NCT02059655) in combination with the TKIs dasatinib or nintedanib would be of interest to follow the clinical outcome from the combination of systemic administration of TKI and local administration of these non-invasive eye drops in GO patients. Future studies will more and more address the topic of combination treatment, based on the complex processes involved in the pathogenesis of fibrotic diseases and will potentially establish the most effective combinations for clinical use in GO.

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Chapter 10

Appendix

Abbreviations

ABC	avidin–biotin complex
AEC	3-amino-9-ethyl-carbazole
BAFF	B-cell activating factor
bFGF	basic fibroblast growth factor
CAS	clinical activity score
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CML	chronic myeloid leukemia
Col1α1	collagen type 1 α 1
CXCL	chemokine (C-X-C motif) ligand
DMEM	Dulbecco's modified Eagle's medium
dSSc	diffuse SSc
ECM	extracellular matrix
EGF-R	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FGF-R	fibroblast growth factor receptor
fT4	free thyroxine
G-CSF	granulocyte colony-stimulating factor
GD	Graves' disease
GO	Graves' ophthalmopathy

HAS	hyaluronan synthase
HRH	histamine receptor
HRP	horse-radish-peroxidase
HYAL	hyaluronidase
ICAM-1	Intercellular adhesion molecule-1
IFN	interferon
IGF-1	insulin-like growth factor-1
IL	interleukin
IL-1RA	interleukin-1 receptor antagonist
IM	Imatinib mesylate
IPF	idiopathic pulmonary fibrosis
IRF	interferon regulatory factor
LDH	lactate dehydrogenase
lSSc	limited SSc
MRSS	Modified Rodnan Skin Score
NF-kB	nuclear factor kappa-B
PAR	protease-activated receptor
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGF-R	platelet-derived growth factor receptor
PGD_2	prostaglandin D2
PGE_2	prostaglandin E2
PGF2α	prostaglandin F2-alpha
PI3K	phosphoinositide 3-kinase
PIIINP	N-terminal propeptide of type III collagen

PKC	protein kinase C
PPAR-γ	peroxisome proliferator-activated receptor gamma
RQ-PCR	real-time quantitative PCR
SCF	stem cell factor
SEM	standard error of the mean
SS	somatostatin
SSc	Systemic sclerosis
SSTR	somatostatin receptor
TBX5	T-box transcription factor 5
Tg	thyroglobulin
TGF-β	transforming growth factor-β
Th	T-helper lymphocyte
TKI	tyrosine kinase inhibitor
TLC	total lung capacity
TNF	tumor necrosis factor
TPO	thyroid peroxidase
TSH	thyroid stimulating hormone
TSHR	thyrotropin receptor
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial derived growth factor receptor

English Summary

Graves' ophthalmopathy (GO), also referred to as thyroid eye disease, is an extra-thyroidal complication that develops in ~25-50% of patients with Graves' disease (GD; an autoimmune disease of the thyroid gland that results in hyperthyroidism). Clinical symptoms of GO result from the increase in orbital tissue volume within the non-compliant space limited bony orbital cavity. These clinical symptoms comprise amongst others upper eyelid retraction, edema, erythema of the periorbital tissues and conjunctivae, and proptosis. The clinical course of GO consists of an initial active phase with inflammation and edema. This active phase may persist several months, then activity subsides and progresses to a chronic inactive phase with extensive orbital tissue remodeling and fibrosis. Immunologically active GO is characterized by infiltration of the extraocular muscles and adipose/connective tissue with mononuclear cells, primarily CD4+ T lymphocytes, some CD8+ T lymphocytes, monocytes, macrophages, B lymphocytes and plasma cells. Mast cells are more abundant in the chronic fibrotic disease phase. These inflammatory cells activate orbital fibroblasts via the secretion of inflammatory mediators (e.g. cytokines), autoantibodies (directed against thyrotropin receptor (TSH-receptor; TSHR) and insulin-like growth factor 1 receptor (IGF-1R) or by physical cellular interaction with the orbital fibroblasts. The activated orbital fibroblasts increase their proliferative activity, produce inflammatory mediators, differentiate into adipocytes and pro-fibrotic myofibroblasts and produce excess amounts of extracellular matrix (ECM) components, especially the hydrophilic glycosaminoglycan hyaluronan. Thereby, orbital fibroblasts fulfill central roles in orbital inflammation and tissue remodeling in GO.

Previous studies showed elevated platelet-derived growth factor (PDGF)-BB levels in GO orbital tissue and demonstrated that PDGF-BB stimulates proliferation, production of hyaluronan, cytokines/chemokines and enhances TSHR expression by orbital fibroblasts. In this thesis the contribution of PDGF-BB to GO was further explored as well as the inhibition of PDGF signaling by clinically available tyrosine kinase inhibitors (TKI) that prevent PDGF receptor (PDGF-R) auto-phosphorylation.

In **chapter 4** PDGF-BB was demonstrated to enhance adipogenesis of orbital fibroblasts cultured in a proadipogenic environment. This suggests that in GO PDGF can contribute to orbital tissue expansion by stimulating adipogenesis. However, this might be hugely dependent on the local environment that orbital fibroblasts encounter as preliminary studies in this thesis revealed that transforming growth factor (TGF)-β_1, which is also elevated in GO, may inhibit the pro-adipogenic effects of PDGF-BB. These data do however illustrate the need to examine the effects that combinations of stimuli, e.g. growth factors and cytokines, exert on orbital fibroblasts. Along this line, basic

fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been proposed to contribute to GO as increased levels were found in orbital tissue and serum from GO patients. However, so far the effects of bFGF and VEGF, either alone or in combination with other growth factors, on orbital fibroblasts have hardly or not been studied. In **chapter 5** it was demonstrated that VEGF marginally stimulated hyaluronan production by orbital fibroblasts, while it did not affect proliferation and interleukin (IL)-6 production. bFGF stimulated proliferation and hyaluronan production by orbital fibroblasts, but not IL-6 production. VEGF did not modify the effects induced by bFGF and/or PDGF-BB. In contrast, bFGF synergistically amplified the effects of PDGF-BB on IL-6 and hyaluronan production by orbital fibroblasts and additively enhanced PDGF-BB-induced orbital fibroblast proliferation. These data suggest that bFGF may represent an important contributor to GO pathogenesis, especially in conjunction with PDGF-BB. Further studies in **chapter 6** showed that PDGF-BB and bFGF prolonged the duration of *PDGF-B* mRNA induction in the orbital fibroblasts compared to stimulation with PDGF-BB or bFGF alone. Studies with a PDGF-BB neutralizing antibody suggested that the synergistic effects of PDGF-BB and bFGF on hyaluronan and IL-6 production are mediated by induction of autocrine PDGF-BB signaling.

During the chronic phase of GO mast cells are abundantly present within the orbital tissue. However, the contribution of mast cells and their products to GO are generally unknown. In **chapter 7** increased mast cell numbers were observed in orbital tissues from patients with GO and they are located closely to orbital fibroblasts and adipocytes. Furthermore, it was found that the mast cell mediator histamine stimulated the production of the nuclear factor kappa-B (NF-κB) controlled-cytokines IL-6, IL-8 and chemokine (C-C motif) ligand (CCL)-2 by orbital fibroblasts, while the cytokines CCL5, CCL7, chemokine (C-X-C motif) ligand (CXCL)-10 and CXCL11 that are controlled by other transcription factors were not induced by histamine. This effect of histamine was found to be mediated by the histamine receptor subtype-1 (HRH1) which was the histamine receptor subtype most abundantly expressed by orbital fibroblasts. From these data it can be proposed that mast cell derived histamine is involved in regulating inflammation and monocyte recruitment in GO orbital tissue. Mast cell inhibition or interference with histamine or histamine receptor activity may thus represent therapeutic targets for GO treatment. Remarkably, mast cells have previously been identified as source of PDGF-BB in GO and preliminary studies performed in this thesis suggest that PDGF-BB stimulates orbital fibroblasts to produce stem cell factor (SCF), an important growth factor for mast cells. These data support existence of an intricate interplay between mast cells and orbital fibroblasts in GO.

Collectively, the *in vitro* data generated in this thesis have refined our understanding of the pathogenesis of GO (Figure 1). This can be of importance for optimization and development of future therapies, as will be further discussed hereunder.

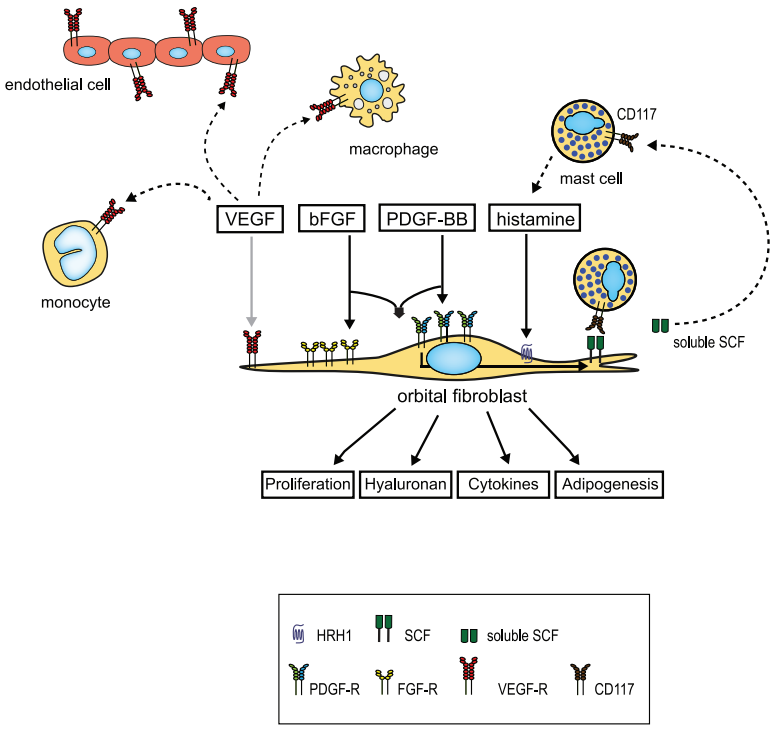


Figure 1. PDGF-BB, bFGF, VEGF and histamine in the pathogenesis of Graves' ophthalmopathy.

Proliferation, cytokine production, hyaluronan production and adipogenesis by orbital fibroblasts are central features of GO pathogenesis. PDGF-BB stimulates proliferation, the production of hyaluronan, cytokines and adipogenesis by orbital fibroblasts. In addition PDGF-BB enhances SCF expression by orbital fibroblasts. SCF is a cytokine that acts as survival factor for mast cells. SCF is mostly expressed at the cell membrane and it can be hypothesized that it provides survival signals to mast cells upon cellular interaction between orbital fibroblasts and mast cells via activation of SCF receptor (CD117/c-Kit). Alternatively this survival signal might also be generated by the soluble form of SCF (indicated by dashed line). bFGF induces orbital fibroblast proliferation and hyaluronan production, but not IL-6 production while VEGF hardly affects orbital fibroblast activation (indicated by grey line), presumably due to low VEGF-receptor (VEGF-R) expression in orbital fibroblasts. Monocytes, macrophages and endothelial cells express VEGF-R abundantly and therefore it is hypothesized that VEGF affects these cell types in GO (indicated by dashed lines). PDGF-BB and bFGF act synergistically on IL-6 and especially hyaluronan production by orbital fibroblasts, as indicated by the combined arrow. Orbital fibroblasts express histamine receptor subtype-1 (HRH1) and via this receptor histamine stimulates the

production of NF-κB controlled cytokines, while it does not affect hyaluronan production.

The PDGF-Rs and FGF receptors (FGF-Rs) belong to the receptor tyrosine kinase (RTK) family and can be targeted with TKI that specifically inhibit the tyrosine kinase activity of these receptors and thus their activation. The studies performed in **chapter 3** illustrated that the TKI dasatinib effectively suppressed PDGF-BB-induced proliferation, hyaluronan, CCL2, IL-6 and IL-8 production by orbital fibroblasts at low concentrations. Moreover, dasatinib was found to exhibit stronger anti-inflammatory, anti-proliferative, and hyaluronan synthesis-suppressing effects than the TKI imatinib mesylate. Dasatinib also inhibited PDGF-BB enhanced adipogenic differentiation of orbital fibroblasts (**Chapter 4**) and was found to suppress mRNA expression of *CCL2*, *IL6*, *IL8*, hyaluronan synthase 2 (*HAS2*) and the fat predominant transcription factor peroxisome proliferator-activated receptor (*PPAR*)-γ in whole orbital tissue from GO patients (**Chapters 3 and 4).** Together these data showed that dasatinib can interfere with pathways that are involved in the pathophysiology of GO and based on these findings the potential clinical implications of dasatinib in GO should be further studied.

In **chapter 9** of this thesis we described for the first time the treatment of a patient with therapy-refractory GO with dasatinib. This female patient suffered from severe and progressive GO (clinical activity score (CAS) 6/7), despite treatment with high doses of glucocorticoids. In whole orbital tissue cultures from this patient treatment with dasatinib resulted in reduction in cytokine (*IL6*, *IL8*, *CCL2*, *TNFα* and *IL10*), *HAS1-3*, adhesion molecule (intercellular adhesion molecule 1 (*ICAM-1*)), and growth factor (*TGF-β1* and *PDGF-B*) mRNA expression. Based on the *in vitro* data, patient was subsequently treated with dasatinib once daily 100 mg. After 6 weeks of treatment clinical improvement was found with a CAS of 4/7. Four months after start of dasatinib, the patient seemed to become resistant to the treatment resulting in sight-threatening GO. Interestingly, pretibial myxedema (also characterized by excessive hyaluronan accumulation) completely disappeared upon dasatinib treatment and did not relapse until now. Collectively, these data support the potential clinical implication of the TKI dasatinib in patients with very severe and/or sight-threatening GO, in which conventional therapies are ineffective. However, optimal implementation might require further studies as is also clear from the trial described in **chapter 8**. Patients with therapy-refractory systemic sclerosis (SSc), as a clinical model for fibrotic diseases, were treated with imatinib mesylate. Of the ten patients described in this study, only two showed a significant reduction in modified Rodnan skin score (mRSS). The patients that responded to treatment with the TKI imatinib mesylate were those with biochemical evidence of excessive collagen synthesis (high serum levels of the N-terminal propeptide of collagen type-III (PIIINP) and active disease as reflected by high B-cell-

activating factor (*BAFF*) mRNA levels in monocytes. These data suggest that serum PIIINP levels and monocyte *BAFF* mRNA levels could potentially be used as biomarkers to identify those SSc patients that might be eligible for therapy with imatinib mesylate or other TKI. Further studies will have to reveal whether these or other biomarkers could also be used in determining therapeutic approaches in GO patients.

Moreover, therapies with other TKI that simultaneously target multiple key molecules in the pathogenesis of GO should be further explored. In **chapter 5** it was demonstrated that nintedanib, a TKI that targets PDGF-Rs, FGF-Rs and VEGF-Rs, was more effective in inhibiting the combined effects of PDGF-BB and bFGF on orbital fibroblast activation than dasatinib, that targets PDGF-Rs but not FGFRs. Nintedanib was previously found to be effective in the treatment of patients suffering from idiopathic pulmonary fibrosis and based on the growth factors involved it could be hypothesized that nintedanib is a drug with potential clinical implications in the treatment of GO.

Nederlandse samenvatting

De ziekte van Graves is een auto-immuunaandoening van de schildklier die leidt tot hyperthyreoïdie. Graves orbitopathie (GO), ook wel oogziekte van Graves genoemd, is een complicatie van de ziekte van Graves die optreedt in 25 tot 50% van de patiënten. De symptomen van GO zijn het gevolg van volumetoename van het orbitale weefsel in de oogkas. De oogkas bestaat uit botweefsel, waardoor er bij volumetoename van orbitaal weefsel druk uitgeoefend wordt op de structuren in deze oogkas. Het oog kan daardoor alleen naar voren verplaatst worden, wat leidt tot proptosis (uitpuilende, wijd opengesperde ogen), het typische klinische beeld. De verdere klinische symptomen van GO bestaan uit teruggetrokken onder- en/of bovenoogleden, oedeem, roodheid van de periorbitale weefsels en conjunctiva.

Het beloop van GO wordt initieel gekenmerkt door een actieve fase met ontstekingsactiviteit en ontwikkeling van oedeem. Deze actieve fase kan maanden duren, waarna de ontstekingsactiviteit zal afnemen en het beeld zich zal ontwikkelen richting een chronische inactieve fase, gekenmerkt door een verstoorde weefselstructuur. Immunologisch wordt de actieve fase van GO gekenmerkt door infiltratie van extra-oculair spierweefsel en het orbitale vet- en bindweefsel met mononucleaire cellen, voornamelijk CD4+ T lymfocyten, en in mindere mate CD8+ T lymfocyten, monocyten, macrofagen, B lymfocyten en plasmacellen. Mestcellen zijn vaker aanwezig in de chronische fase, waarin fibrosering (overmatige bindweefselproductie) van weefsel optreedt. De infiltrerende ontstekingscellen activeren orbitale fibroblasten door de uitscheiding van ontstekingsmediatoren (zoals cytokinen en groeifactoren), auto-antistoffen (o.a. gericht tegen de thyroid stimulating hormone receptor (TSHR) en insulin-like growth factor 1 receptor (IGF-1R)) of via direct cel-cel contact tussen ontstekingscellen en orbitale fibroblasten.

Geactiveerde orbitale fibroblasten verhogen hun celdeling, produceren ontstekingsmediatoren, differentiëren tot adipocyten (vetcellen) en pro-fibrotische myofibroblasten en produceren grote hoeveelheden extracellulaire matrix componenten, voornamelijk het hydrofiele glycosaminoglycaan hyaluronan. Op basis van deze processen wordt aan de orbitale fibroblasten een centrale rol in het ontstekingproces en de weefselverandering in GO toegekend.

Voorgaande studies hebben aangetoond dat platelet-derived growth factor (PDGF)-BB verhoogd tot expressie komt in oogweefsel in GO en dat PDGF-BB de celdeling, productie van hyaluronan en cytokinen/chemokinen stimuleert. Bovendien zorgt PDGF-BB voor een verhoogde expressie van TSHR op orbitale fibroblasten. In de studies beschreven in dit proefschrift wordt de rol van PDGF-BB in de pathogenese van GO

nader onderzocht. Bovendien werd onderzocht wat het effect is van het blokkeren van de signalering van PDGF-BB door zogenaamde tyrosine kinase remmers, die auto-phosphorylatie van de PDGF receptor (PDGF-R) voorkomen, op diverse parameters van ziekteactiviteit in GO.

In **hoofdstuk 4** werd beschreven dat PDGF-BB de adipogenese van orbitale fibroblasten stimuleert. Dit suggereert dat PDGF een bijdrage levert aan de expansie van orbitaal (vet)weefsel in GO. Deze effecten zijn echter sterk afhankelijk van het micro-milieu waarin de orbitale fibroblasten verblijven. Preliminaire studies in dit proefschrift hebben namelijk aangetoond dat transforming growth factor (TGF)-β_1, een groeifactor die ook verhoogd tot expressie komt in GO, de pro-adipogene effecten van PDGF-BB mogelijk weer remt. Deze resultaten illustreren het bestaan van complexe interacties tussen verschillende ontstekingsmediatoren met betrekking tot de effecten op orbitale fibroblasten. Dit geeft bovendien aan dat het essentieel is om ook de effecten van combinaties van verschillende groeifactoren en cytokinen op orbitale fibroblasten te onderzoeken. Analoog hieraan werd op basis van de verhoogde expressie van basic fibroblast growth factor (bFGF) en vascular endothelial growth factor (VEGF) in orbitaal weefsel en serum van patiënten met GO een mogelijke rol aan bFGF en VEGF toebedeeld in de pathogenese van GO. Echter, tot op heden zijn de effecten van bFGF en VEGF, alleen of in combinatie met andere groeifactoren, op orbitale fibroblasten niet tot nauwelijks onderzocht.

In **hoofdstuk 5** werd aangetoond dat VEGF slechts een minimale bijdrage levert aan de stimulatie van hyaluronan productie door orbitale fibroblasten en VEGF had geen effect op de proliferatie en interleukine (IL)-6 productie. bFGF daarentegen stimuleerde de proliferatie van orbitale fibroblasten alsmede hyaluronan productie, maar had eveneens geen effect op de productie van IL-6. VEGF was niet in staat de door bFGF en/of PDGF-BB geïnduceerde effecten te beïnvloeden, terwijl bFGF de effecten van PDGF-BB op IL-6 en hyaluronan productie door orbitale fibroblasten alsmede de proliferatie capaciteit versterkte.

Deze data suggereren dat bFGF een belangrijke rol speelt in de pathogenese van GO, voornamelijk in combinatie met PDGF-BB. Aanvullende studies beschreven in **hoofdstuk 6** toonden aan dat PDGF-BB en bFGF de duur van inductie van PDGF-B mRNA in orbitale fibroblasten verlengde, in vergelijking met de stimulatie met alleen PDGF-BB of bFGF. Studies met neutraliserende antistoffen tegen PDGF-BB suggereerden dat de synergistische effecten van PDGF-BB en bFGF op de productie van hyaluronan en IL-6 gemedieerd worden door de inductie van autocriene PDGF-BB signalering.

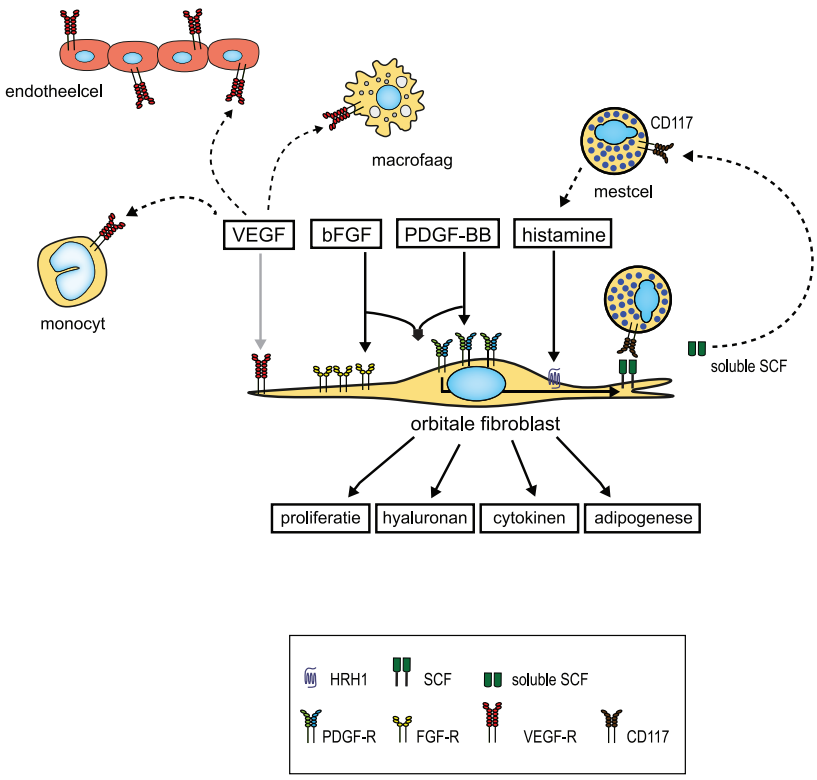
Tijdens de chronische fase van GO zijn mestcellen in overmaat aanwezig in het orbitale weefsel. De bijdrage van mestcellen en door mestcellen uitgescheiden mediatoren in de

pathogenese van GO is echter nog niet opgehelderd. In de studies beschreven in **hoofdstuk 7** werden verhoogde aantallen mestcellen gevonden in orbitaal weefsel van patiënten met GO en deze mestcellen werden aangetroffen in de directe omgeving van orbitale fibroblasten en adipocyten. Bovendien werd aangetoond dat de mestcel mediator histamine de productie van de door de transcriptiefactor nuclear factor kappa-B (NF-κB) gecontroleerde cytokinen IL-6, IL-8 en chemokine (C-C motif) ligand (CCL)-2 door orbitale fibroblasten stimuleerde. Histamine had daarentegen geen invloed op de productie van CCL5, CCL7, chemokine (C-X-C motif) ligand (CXCL)-10 en CXCL11 die onder controle staan van andere transcriptiefactoren dan NF-κB. De effecten van histamine bleken te worden gemedieerd via histamine receptor subtype-1 (HRH1), het histamine receptor subtype dat voornamelijk tot expressie kwam op orbitale fibroblasten.

Deze data suggereren dat histamine afkomstig uit mestcellen betrokken is bij de regulatie van ontsteking en het aantrekken van monocyten in orbitaal weefsel in GO. Remming van mestcel activiteit of interferentie met histamine of de histamine receptor kan derhalve een mogelijke therapeutische benadering zijn in de behandeling van GO.

Opvallend in dit kader is het gegeven dat mestcellen in GO tevens een bron kunnen zijn van PDGF-BB en preliminaire studies zoals beschreven in dit proefschrift toonden aan dat PDGF-BB de orbitale fibroblasten aanzet tot de productie van stem cell factor (SCF), een belangrijke groeifactor voor mestcellen. Deze data ondersteunen de hypothese dat er in GO een belangrijke rol is weggelegd voor de interactie tussen mestcellen en orbitale fibroblasten.

Samenvattend hebben de *in vitro* data die werden verkregen door de studies uit dit proefschrift meer inzicht gegenereerd in de processen betrokken in de pathogenese van GO (zie figuur 1). Deze nieuwe inzichten kunnen van groot belang zijn voor ontwikkeling en verbetering van de behandeling van GO, zoals hieronder verder wordt besproken.



Figuur 1. PDGF-BB, bFGF, VEGF en histamine in de pathogenese van Graves orbitopathie.

Proliferatie, cytokine productie, hyaluronan productie en adipogenese door orbitale fibroblasten zijn centrale processen in de pathogenese van GO. PDGF-BB stimuleert proliferatie, de productie van hyaluronan, cytokinen en adipogenese door orbitale fibroblasten. Bovendien stimuleert PDGF-BB de expressie van SCF in orbitale fibroblasten. SCF is een cytokine en een belangrijke overlevingsfactor voor mestcellen. SCF komt voornamelijk tot expressie op de celmembraan en door cellulaire interactie tussen orbitale fibroblasten en mest cellen via de activatie van de SCF receptor (CD117/c-Kit) levert SCF mogelijk een bijdrage in de overleving van mestcellen in het orbitale weefsel. Anderzijds kan dit overlevingssignaal ook gegeneerd worden door soluble SCF (onderbroken lijn). bFGF stimuleert proliferatie van orbitale fibroblasten en hyaluronan productie door deze cellen, maar heeft geen effect op IL-6 productie, terwijl VEGF nauwelijks activatie van orbitale fibroblasten en productie van hyaluronan

beïnvloedt (grijze lijn), mogelijk als gevolg van lage VEGF-receptor (VEGF-R) expressie in orbitale fibroblasten. Daarentegen is de VEGF-R expressie op monocyten, macrofagen en endotheelcellen hoog en VEGF kan daardoor een mogelijk effect hebben op deze cellen in GO (onderbroken lijnen). PDGF-BB en bFGF hebben een synergistisch effect op IL-6 en vooral hyaluronan productie door orbitale fibroblasten (gecombineerde pijl). Orbitale fibroblasten brengen de histamine receptor subtype-1 (HRH1) tot expressie en via deze receptor stimuleert histamine de productie van door NF-κB gecontroleerde cytokinen, terwijl het de hyaluronan productie niet beïnvloedt.

De PDGF-Rs en FGF receptoren (FGF-Rs) behoren tot de receptor tyrosine kinase (RTK) familie. Deze receptoren kunnen beïnvloed worden door tyrosine kinase remmers (TKI), welke de tyrosine kinase activiteit en daardoor de activatie van deze receptoren remmen. De studies beschreven in **hoofdstuk 3** hebben aangetoond dat de TKI dasatinib in lage concentraties in staat is de PDGF-BB geïnduceerde proliferatie, hyaluronan, CCL2, IL-6 en IL-8 productie door orbitale fibroblasten te onderdrukken. Bovendien heeft dasatinib een sterker anti-inflammatoir en antiproliferatief effect alsmede een sterker onderdrukkend effect op de productie van hylarunon door orbitale fibroblasten dan de TKI imatinib mesylaat.
Dasatinib remde eveneens de door PDGF-BB gestimuleerde adipogenese van orbitale fibroblasten (**hoofdstuk 4**) en onderdrukte de mRNA expressie van *CCL2*, *IL6*, *IL8*, hyaluronan synthase 2 (*HAS2*) en peroxisome proliferator-activated receptor (*PPAR*)-γ (een transcriptiefactor betrokken in adipogenese) in totaal orbitaal weefsel van patiënten met GO (**hoofdstukken 3 en 4**).
Deze data hebben geleerd dat dasatinib kan interfereren met diverse processen die een belangrijke rol spelen in de pathogenese van GO en op basis van deze bevindingen zullen de mogelijke therapeutische opties van dasatinib in GO verder onderzocht moeten worden.

In **hoofdstuk 9** van dit proefschrift wordt voor de eerste keer de behandeling van een patiënte met een therapieresistente GO met dasatinib beschreven. Deze vrouwelijke patiënt leed aan een ernstige en progressieve GO (klinische activiteit score (CAS) 6/7), ondanks behandeling met hoge dosering glucocorticoïden. In kweken van totaal orbitaal weefsel van deze patiënte resulteerde incubatie met dasatinib tot afname van cytokine (*IL6*, *IL8*, *CCL2*, *TNFα* en *IL10*), *HAS1-3*, adhesiemolecuul (intercellular adhesion molecule 1 (*ICAM-1*)), en groeifactor (*TGF-β1* and *PDGF-B*) mRNA expressie in het weefsel. Op basis van deze *in vitro* data werd patiënte vervolgens behandeld met dasatinib 1 dd 100 mg. Na 6 weken behandeling was er een duidelijke afname van GO (CAS 4/7). Vier maanden na starten van de behandeling trad er echter progressieve ziekteactiviteit op, hetgeen leidde tot acute chirurgische interventie, vanwege een visusbedreigende situatie. Het pretibiaal myxoedeem (eveneens

gekarakteriseerd door excessieve accumulatie van hyaluronan) wat eveneens verdween na 6 weken behandeling bleef opvallend genoeg afwezig tot op heden.
De *in vitro* en *in vivo* data ondersteunen de hypothese dat de TKI dasatinib een potentiele nieuwe behandeling biedt voor patiënten met ernstige en/of visusbedreigende GO, waar de conventionele behandelingen falen. Aanvullende studies, alvorens TKI op groter schaal kunnen worden ingezet in GO, zijn nodig zoals ook blijkt uit de studie beschreven in **hoofdstuk 8**.
Patiënten met therapieresistente systemische sclerose (SSc), als een klinisch model voor fibroserende aandoeningen, werden behandeld met imatinib mesylaat. Van de 10 patiënten beschreven in de studie, werd in twee gevallen een significante verbetering van het huidbeeld gevonden als gemeten middels de 'modified Rodnan skin score' (mRSS).
De patiënten die gunstig reageerden op de behandeling waren de patiënten met biochemische aanwijzingen voor excessieve collageen synthese (hoge serumwaarden voor het N-terminal propeptide of collagen type-III (PIIINP) en actieve ziekte, gemeten aan de hand van hoge B-cell-activating factor (*BAFF*) mRNA levels in monocyten.
Deze data suggereren dat serum PIIINP waarden en monocyt BAFF mRNA levels gebruikt zouden kunnen worden als biomarkers om de patiënten met SSc te selecteren die in aanmerking komen voor een behandeling met imatinib mesylaat of andere TKIs. Vervolgstudies zullen moeten uitwijzen of deze of andere biomarkers ook gebruikt kunnen worden in het effectief inzetten van (nieuwe) behandelstrategieën in GO.
Anderzijds zal verder onderzocht moeten worden of behandelingen met andere TKIs, die tegelijkertijd meerdere processen beïnvloeden die van belang zijn in de pathogenese van GO, zinvol kunnen zijn. In **hoofdstuk 5** werden studies beschreven die aantoonden dat nintedanib, een TKI die zowel de PDGF-Rs, FGF-Rs en VEGF-Rs remt, effectiever was in het remmen van de gecombineerde effecten van PDGF-BB en bFGF op orbitale fibroblast activatie in vergelijking met dasatinib, dat wel de PDGF-Rs beïnvloedt maar niet de FGF-Rs. Nintedanib was in klinische studies effectief in de behandeling van idiopathische longfibrose en op basis van de bekende factoren die betrokken zijn in de pathogenese van GO kan verondersteld worden dat nintedanib in de toekomst een mogelijke therapeutische rol kan spelen in de behandeling van GO.

Acknowledgements

I would like to start writing my acknowledgements by telling you about how I came to the Netherlands. Dating back to 2012, I met Martin, Prof. Dr. Martin van Hagen, for the first time via the connection between Erasmus MC, Rotterdam, the Netherlands and Chulalongkorn University, Bangkok, Thailand. It was one afternoon in Chulalongkorn University, that Martin was having an informal talk with Nattiya, Prof. Dr. Nattiya Hirankarn. Knowing that I was looking for the possibility to do my Ph.D. abroad, he offered me an internship for 6 months as he wrote an email right away to his team in Erasmus MC. Weeks and months afterwards, my application was turned down by many universities as it was a difficult period for financing a Ph.D. scholarship. Then I decided not to wait any longer for other opportunities but to start an internship with Martin. Six months turned into 3 years and 4 months in the Netherlands…

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Dear Martin, how can I find enough words to say to you about all the things you have done for me? You were my promotor on the weekdays and my foster parent, together with Ning, outside (or sometimes during) working hours. Thank you very much for both of your efforts to keep me in a normal stage of heath and mind during the whole stay in the Netherlands. I could not imagine how my life abroad would be without both of you around. I have to then quote your own words in which I will do the same. "Whether you like it or not, I will be around for the rest of your life!"

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Dear AJ, Prof. Dr. A.J. van der Lelij, thank you very much for your contribution as a secretary of my reading committee by guiding me through the process of this promotion. Thank you very much also for all your participation in the research meetings we had. It is always nice to learn what is better about endocrinology compared to immunology. As of today I am becoming an immunologist, let's see how far I can go in this career! Thank you also for all the birthday celebrations and all the great times at all the conferences!

Dear Robin, Prof. Dr. Robin Peeters, thank you for your contribution to my research. It was very nice working with you in one of my last projects at Erasmus MC. Although I missed your big day, big congratulations to you on your professorship! In addition, thank you also for all the great times at all the conferences!

Dear Leo, Prof. Dr. Leo Hofland, thank you very much for all your time and your contribution to the somatostatin project. I learned a lot from you. Thank you also for the nice tea and the nice time in the train to Antwerp. In addition, I would also like to pass on my special thanks to Fadime Dogan for all the technical help on this project.

Dear Peter, Prof. Dr. Peter J. van der Spek, thank you very much for fighting along with me on the methylation project! Special thanks also to Sigrid for all the help with the analysis.

Dear Hemmo, Prof. Dr. Hemmo Drexhage, thank you very much for raising interesting remarks in all the research meetings we had. Thank you very much also for arranging the trip to Amsterdam for us. It was really nice and we all enjoyed it!

Dear Pieter and Marjan, Dr. Pieter Leenen and Dr. Marjan Versnel, thank you very much for both of your contributions to our unit meeting. It was always nice to stimulate some thinking processes on Monday mornings. I had a lot of nice feedback about my work and it was always a great place to practice my presentation skills. Thank you again for arranging that.

Dear Monique, Dr. Monique Mulder, thank you very much for helping us with the

triglyceride measurements. Special thanks to Leonie van der Zee - van Vark for all the technical support.

Dear Jeroen, Dr. H.J. van de Peppel, thank you very much for teaching me how to perform a microarray! Special thanks also to Xander, A.T. den Dekker, for your help and for all the technical support.

Dear Dr. Jeroen Demmers, thank you very much for your help on the proteomics consultation. Special thanks also to Karel Bezstarosti for all the technical support.

Dear Bart, Axel, Roy, Bing, Dennis, Sebastian, Jan, Paul and Gertjan, thank you very much for all the great times we had in Asia! Let's continue our collaboration!

Dear Rina & Noor, thank you for accepting to be my paranimfen especially when we are all new to this. Rina, we met on my birthday. How special was that? I have always had you ever since I came to the Netherlands and that makes me miss Rotterdam. Thanks for all your support so that I can go strongly through these many things. I wish you great success in completing your PhD and all the happiness to you and your family. Like we used to say, more fun is waiting for us on the other side of the world! Keep in touch!! Noor, I don't know when I became so attached to you ☺, but thanks for always being there for me and for all the advice. Thanks also for all the great times we had and also for helping me clean the apartment. The picture of you with the bike and all my stuff on it was quite memorable. ☺ If you miss me, you know where to find me!

Dear Christopher, I will never forget how good life was in Rotterdam to have a friend like you. Thanks for all the nice food you cooked, lunch/dinner/drinks/tea/sweets we had, and for the ride to here and there. ☺ Thanks also for listening to all of my whatever stories. One word of advice, try to be more like Rina and Noor, they know better about how to handle me, otherwise I will be too spoiled! ☺ Although I am avoiding meeting you in America, never ever forget me Chris!

Willem Jan, you took my blood!!! How can I forget and forgive you? ☺ Thanks for all the great fun we had. Thanks also for the nice time in the lab and for helping me out with this and that. Wishing you a lot of success with your future!

Dear Naomi & Marieke, thanks for all the smiles and laughter we shared. That was priceless. We are now living in three different continents in the world. Who knows? We might soon enough reunite again! Keep in touch!

Bastiaans! How did we manage to survive with each other in the past 3 years? Jeroen, I am thankful for all your help and support in many ways. I am also happy to see you achieve what you worked hard for. Stay strong and don't stop moving forward! Hope to see you somewhere again in the coming year. ☺

Britt, I am sorry that you have to join Jeroen and me for the PhD committee for the whole year. I am sure that must have been tough for you. ☺ Thanks for taking care of me when I was sick during the PhD retreat because that meant more work for you. But, I do think that we made a nice year for everybody, didn't we? ☺ Thanks team! Sorry that I did not make your gossip come true, but that was a nice preparation for yourself! Congratulations!!

Kaizer! Dear Fabian, my life is very quiet now without you. ☺ Thanks for the great friendship we shared. Thanks also for the nice food, nice eating-out, many nice weekends, nice suggestions and for all the great fun. Keep your promise! I will see you in Thailand, but definitely not at Pang Chang Elephant Camp! Good luck with everything!

Dear Vilma & Judith. Vilma ☺, thanks for your effort in working on the project. Research does not always bring nice results and that's one thing I can teach you. I am really glad to hear that you are still not giving up on research and looking for a Ph.D. position. Thanks also for the nice Italian treat/lunch in the sun/drinks outside of work. Judith ☺, thanks also for the work you helped me with on the project. Thanks for all the nice talk/drink/dinner – we did eat and drink a lot for our size! I had a really nice time in Nijmegen with you and Chris. Last but not least, I would also like to thank both of you for helping each other out. What is better than having nice lab partners, right? I wish you both great success in what you hope for. Let me know if you plan to visit Asia!

Dew and Keng, you are the next generation of Thai Ph.D. students at Erasmus MC. Stay strong, it will be very fast when you look back. Good luck with your studies and do it better than I did! ☺

My fellow Ph.D.'s; Alice, Anna, Chris, Christina, Diana, Jenna, Jorn, Karin, Liza, Magda, Malou, Marjolein, Marlieke, Martine, Prisca, Sandra, Simar, Wendy, Wida, Wouter B, Wouter vdB and Zana, thanks for all the great memories. ☺

Dear Carlotta, I wouldn't be able to start a life here without you. Thanks for all the great time we had in Thailand and also for helping me around in Rotterdam. I am glad to see how successful you are today! Greetings to your mom and your family as well. Keep in touch!

Nick & Simon, Marloes and Simon, thanks for all the great fun! Our memories cannot be complete without reminding you of the great stories from the Efteling and Locus Publicus. ☺ Thanks for all the nice talks and nice dinner (with allergy) at your place. ☺ Let's meet next time in Thailand!

Dear Judy, thank you very much for all your help from the beginning until the end of my study. Without you I could not manage to arrange everything for my promotion. I really

appreciate it. Also, thanks for helping me adjust to Rotterdam life from day 1 by introducing me to Chinese places for the food that I am used to. ☺

Dear Conny, Nicole and Benjamin. I have a small question. Would you ever forget me? ☺ Thank you very much for all of your help and support. Thanks for teaching me about ELISA's, Conny, and thanks for teaching me about flow cytometry, Nicole. Benjamin, although it was short, thanks for all the support you put on my project. Dear Marion, Marja, Corrien, Dicky and Nicoline. Thank you very much also for all your support and all the nice times in the lab. Thank all of you for all the nice talks/teas/treats/lunches/dinners/borrels!

Dear Marco, Diana, Jac, Jacolien, Cindy, Elham, Sanae, thanks for all the nice lunches, borrels, lab days and BBQ's at Diana's and Wim's place. Thanks also for the nice Carnival at your place, Diana! ☺

Dear A3, Corine, Annemarie, Harm, Angelique, Anne, Wouter, Annet, Marie-Jose, Marjan, Inge, Stefan and Yvonne thanks for the great times in the lab on the 11th floor. Rianne, Maaike, Patricia, Monique and Gellof, thanks for all your support.

Dear Pep, my beloved neighbor. ☺ Thanks for everything. It was as if I had my brother with me in Rotterdam. Thanks for helping me out with the house/bike/all the gadgets and also for listening about my stuff at work. It was great fun with Gift, my long-lost sister, in Germany. ☺ P'Gap, P'Pui, Tridti, Bally, Oat, P'Aey, N'Jub and P'Noi. Thanks also for sharing great times together in Europe and for making me feel less homesick. Thanks also for staying in touch in Thailand!

Dear Nueng and Noina, my beloved friends. Thanks for always arranging nice holiday trips for me every year when I was back to Thailand and also thanks for coming all the way from Thailand to visit me. It was great fun, always, when we are all together. How can we ever forget what happened in Paris? ☺

Dear Cousins, I am blessed to be born into this family. Thank you all for the love and care and I realize now how much I missed it.

Dear brothers; Poom, Root and Mos. I know that it is not easy to have a sister like me but I am grateful and thankful to have you all. Thanks for taking care of mom when I was away and thanks for taking care of me. Please continue to do so. ☺

Dear papa, thank you for raising me to be who I have become today. Dear mom, Khun Arunsri, thank you for everything. I will never be as good a mother as you. I know it was difficult to raise me up but thank you for never giving up on me.

Dear Boat, thanks for bearing with me for all of these years. Thanks for your support in doing this Ph.D. and your effort with the long-distance relationship. We made it!

Biography

Sita Virakul was born in Bangkok, Thailand, the 22nd of September 1985. She obtained her bachelor degree (second class honor) in Microbiology from Chulalongkorn University, Bangkok, Thailand in 2007. Thereafter, she continued with her study and graduated with Master of Science degree in Medical Microbiology in 2010 from the same University with the thesis entitled 'Development in detection of HLA-B*1502 and –B*5801 by SSP-PCR and LAMP with PNA probe'. From 2010 till 2012 she worked as a research assistant at the Department of Microbiology, Immunology Unit, Chulalongkorn University under the supervision of Prof. Dr. Nattiya Hirankarn on a project of pharmacogenetics in HLA allele and life-threatening drug hypersensitivity. In 2012 she started with her PhD project entitled 'The role of the fibroblast in inflammatory diseases' focusing on Graves' ophthalmopathy and systemic sclerosis under supervision of prof.dr. P.M. van Hagen, dr. W.A. Dik and dr. V.A.S.H. Dalm.

List of publications

Published:

Current perspectives on the role of orbital fibroblasts in the pathogenesis of Graves' ophthalmopathy.

Dik WA, **Virakul S** and van Steensel L. Exp Eye Res. 2016 Jan;142:83-91.

Platelet-derived growth factor-BB enhances adipogenesis in orbital fibroblasts.

Virakul S, Dalm VA, Paridaens D, van den Bosch WA, Mulder MT, Hirankarn N, van Hagen PM and Dik WA. IOVS. 2015 Aug;56, 5457-5464.

Platelet-derived growth factor: a key factor in the pathogenesis of graves' ophthalmopathy and potential target for treatment.

Virakul S, van Steensel L, Dalm VA, Paridaens D, van Hagen PM, Dik WA. Eur Thyroid J. 2014 Dec;3(4):217-26.

The tyrosine kinase inhibitor dasatinib effectively blocks PDGF-induced orbital fibroblast activation.

Virakul S, Dalm VA, Paridaens D, van den Bosch WA, Hirankarn N, van Hagen PM, Dik WA. Graefes Arch Clin Exp Ophthalmol. 2014 Jul;252(7):1101-9.

Manuscript submitted:

Histamine induces NF-κB controlled cytokine secretion by orbital fibroblasts via histamine receptor type-1.

Virakul S, Phetsuksiri T, van Holten – Neelen C, Schrijver B, van Steensel L, Dalm VA, Paridaens D, van den Bosch WA, van Hagen PM and Dik WA.

Basic FGF and PDGF-BB synergistically stimulate hyaluronan and IL-6 production by orbital fibroblasts: a rationale for multitarget therapy in Graves' ophthalmopathy?

Virakul S, Heutz JW, Dalm VA, Peeters RP, Paridaens D, van den Bosch WA, Hirankarn N, van Hagen PM and Dik WA.

Autocrine PDGF-BB signaling is involved in IL-6 and hyaluronan production by orbital fibroblasts co-stimulated with basic FGF and PDGF-BB.

Virakul S, Dalm VA, Peeters RP, Paridaens D, van den Bosch WA, Hirankarn N, van Hagen PM and Dik WA.

Limited, but potentially predictable effect of imatinib mesylate in systemic sclerosis using Interferon type I activation and type III procollagen N-terminal propeptide.

Brkic Z, **Virakul S**, Dik WA, Dalm VA, Maria NI, van Helden-Meeuwsen CG, Versnel MA, van Hagen PM, van Laar JA, Joosse ME, Thio HB, van Daele PL.

PhD portfolio

Name PhD candidate:	Sita Virakul
Erasmus MC Department:	Internal Medicine
Research School:	Molecular Medicine (MolMed)
PhD period:	July 2012 – April 2016
Promotor:	Prof. dr. P.M. van Hagen
Co-promotores:	Dr. W.A. Dik, Dr. V.A.S.H. Dalm

PhD training

Courses and workshops

2013	Advanced course Molecular Immunology (MolMed: 3.0 ECTS)
2013	Biomedical English writing (MolMed: 2.0 ECTS)
2014	Research management for PhD students (MolMed1.0 ECTS)
2015	The course on R (MolMed: 1.4 ECTS)

(Inter)national Scientific meetings and presentations

Oral presentations

1. Platelet-derived growth factor enhances adipogenesis by orbital fibroblasts. Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium, 8th and 9th January 2014.
2. Low Dose Dasatinib Efficiently Blocks PDGF-induced Orbital Fibroblast Activation: a Potential Novel Therapeutic Agent in Fibrotic Disease?
    - The 12th International Ocular Inflammation Society Congress, Valencia, Spain, 27th February – 1st March 2014.
    - Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium, 9th and 10th January 2014.

3. New approaches to the treatment of fibrotic disease. Chulalongkorn University and Erasmus University Medical Center International Symposium, Bangkok, Thailand, 13th November 2013.

4. Understanding the pathophysiology of Graves' Ophthalmopathy leads to target therapy?

    - Special seminar, Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, 16th June 2015.
    - Khon Kaen University and Erasmus University Medical Center International Symposium, Khon Kaen, Thailand, 26th January 2016.

Poster Presentations

1. Platelet-derived growth factor enhances adipogenesis by orbital fibroblasts.

    - The 9th European Workshop on Immune-Mediated Inflammatory Diseases. Amsterdam, the Netherlands, 2nd – 4th September 2015.
    - The Dutch Society for Immunology (NVVI Winter school) 2014, Kaatsheuvel, the Netherlands, 17th - 19th December 2014.

2. Targeting fibrosis with somatostatin analogues. The Dutch Society for Immunology (NVVI Winter school) 2014, Kaatsheuvel, the Netherlands, 17th - 19th December 2014.
3. The tyrosine kinase inhibitor dasatinib efficiently blocks PDGF-induced orbital fibroblast activation: a potential novel therapeutic agent in fibrotic disease? The 3rd Systemic Sclerosis World Congress, Rome, Italy, 6th - 8th February 2014.
4. Low dose dasatinib efficiently blocks PDGF-induced orbital fibroblast activation: a potential novel therapeutic agent in fibrotic disease? The Dutch Society for Immunology (NVVI Winter school) 2013, Noordwijkerhout, the Netherlands, 18th – 19th December 2013.
5. Macrophage-fibroblast interplay: a target for neuropeptide-based treatment of fibrotic disease?.

    - Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium, 10th and 11th January 2013.
    - The Dutch Society for Immunology (NVVI Winter school) 2012, Noordwijkerhout, the Netherlands, 19th December 2012.
    - The 7th Immune-Mediated Inflammatory Diseases. Noordwijk, the Netherlands, 28th – 30th November 2012.

At the department of Immunology

2012 – 2015 Attending Journal clubs

2012 – 2015 Attending department and research meetings

2012 – 2015 Attending seminars and mini-symposia

2013 – 2014 PhD committee

Teaching

2014 – 2015 Supervising MSc thesis (research internship)

Membership

2012 – 2015 NVVI