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Basic FGF and PDGF-BB synergistically stimulate hyaluronan and IL-6 production by orbital fibroblasts

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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.

1. Introduction

Graves' Ophthalmopathy (GO) is the most devastating extra thyroidal manifestation of Graves' disease (GD), which occurs in up to 50% of GD patients and significantly impairs quality of life (Wickwar et al., 2015; Taieb et al., 2016). GO is clinically characterized by upper and lower eyelid retraction, proptosis, edema and erythema of the conjunctivae and expansion of periorbital tissues resulting from adipose/connective tissue and extraocular muscle inflammation (Weetman, 2000; Bahn, 2010). Up to now, medical treatment strategies for GO are limited and far from optimal.

On a pathophysiological level, GO is an inflammatory fibroproliferative disease in which orbital fibroblasts represent a central role (van Steensel and Dik, 2010; Dik et al., 2016). 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 (Bahn, 2010; Dik et al., 2016). 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 (Salvi and Campi, 2015). However, the net biological effect of orbital fibroblast activating factors in the pathophysiology of GO can be complex and is far from being completely understood. This is for instance illustrated by the divergent effects of interleukin (IL)-4 and interferon (IFN)-γ on IL-

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1β-induced hyaluronan and prostaglandin E2 production (Han and Smith, 2006). Interplay between different orbital fibroblast activating factors can thus clearly impact pathogenesis, indicating 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 subsequently resulting in conformational changes, recruitment and activation of other downstream signalling molecules thereby initiating specific cellular responses (Hubbard and Miller, 2007). 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 (Hubbard and Miller, 2007; Hojjat-Farsangi, 2014). 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 (Nowak et al., 2014; van Steensel et al., 2009, 2012a). 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 (Virakul et al., 2014a, 2015; van Steensel et al., 2012b). *In vitro* studies demonstrated that the TKIs 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 (van Steensel et al., 2009, 2011, 2012a; Virakul et al., 2014a,b, 2015). These data suggest that TKIs 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 (Nowak et al., 2014; Ye et al., 2014; Figueroa-Vega et al., 2009). Immunohistochemical analysis of orbital tissues from GO patients revealed bFGF expression by fibroblasts, adipocytes and endothelial cells (Pawlowski et al., 2014; Matos et al., 2008). Orbital bFGF expression as well as bFGF serum levels were found to correlate positively with the clinical activity score (CAS) (Pawlowski et al., 2014; Matos et al., 2008). Also a positive correlation between orbital VEGF levels and CAS has been reported (Matos et al., 2008). 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 has hardly been studied (Kang and Lee, 2013; Kim et al., 2010), 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 have not yet been explored. However, considering that PDGFR targeting TKIs have been proposed as potential treatment options for GO, insight into this combined effect is crucial (Virakul et al., 2014a). The TKI nintedanib, targeting besides PDGFRs also FGFRs and VEGFRs, has been shown to be effective in the treatment of idiopathic pulmonary fibrosis. In contrast, other TKIs with RTK specificity limited to PDGFRs were less efficient in this disease (Daniels et al., 2010; Richeldi et al., 2014). 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.

2. Material and methods

2.1. 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 (van Steensel et al., 2009). GO patients were euthyroid and had not received steroid or other immunosuppressive treatments for at least three months prior to orbital decompression surgery. Further patient details are indicated in Table 1. 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 Bio-Whittaker, Verviers, Belgium) (van Steensel et al., 2009). Orbital fibroblasts were serially passaged with gentle treatment of trypsin/EDTA and used for experiments between the 4th and 12th passage.

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

Orbital fibroblasts were harvested from culture to determine growth factor receptor mRNA 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 (van Steensel et al., 2009). 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* (van Steensel et al., 2009). Primer-probe combinations used are listed in supplementary table 1.

2.3. Orbital fibroblast proliferation assay

Orbital fibroblasts were seeded at 6.0×10^3 cells/well in 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) in

Table 1
Characteristics of included GO patients.

Patient characteristics	
Age (range)	44 (26–54)
Sex (M/F)	1/3
Smoking	2/4
Graves' disease	4/4
- RAI	2/4
- Surgery	0/4
- Strumazol	4/4
Treatment GO	4/4
- Surgery	4/4
- Prednisone	1/4
- Radiation	0/4
Euthyroid	4/4
TSHR autoantibodies	4/4
TPO autoantibodies	0/4
Clinical activity Score (CAS) (range)	1 (0–1)