

Platelet-Derived Growth Factor-BB: A Stimulus for Cytokine Production by Orbital Fibroblasts in Graves' Ophthalmopathy

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

METHODS. Orbital, lung, and skin fibroblasts were stimulated with PDGF-BB, and cytokine (IL-1 β , IL-6, IL-8, IL-16, CCL2, CCL5, CCL7, TNF- α) production was measured by ELISA. Involvement of NF- κ B activation through PDGF signaling was investigated by electrophoretic mobility shift assay, specific NF- κ B inhibitors, and the PDGF-receptor kinase inhibitor imatinib mesylate.

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

CONCLUSIONS. PDGF-BB is a potent inducer of proinflammatory cytokines via the NF- κ B pathway in orbital fibroblasts, whereas cytokine production by fibroblasts from other anatomic locations showed a moderate response. These data suggest a possible role for PDGF-BB in regulating orbital inflammation in GO and identify the PDGF signaling cascade as a therapeutic target

in GO. (*Invest Ophthalmol Vis Sci.* 2010;51:1002-1007) DOI: 10.1167/iovs.09-4338

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

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

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

Platelet-derived growth factor (PDGF)-BB is a homodimeric protein centrally involved in wound healing and fibrotic diseases by stimulating chemotaxis, proliferation, and extracellular matrix production by mesenchymal cells, such as fibroblasts.^{17,18} PDGF has also been described to activate NF- κ B signaling, a pathway involved in the production of several proinflammatory cytokines.¹⁷ Although the latter suggests that PDGF can be involved in the regulation of inflammation, only sparse data supporting this is available. So far, PDGF-BB has been reported to induce expression of the *JE* and *KC* genes, which encode CCL2 and CXCL1, by murine fibroblasts.¹⁹⁻²¹ In humans, PDGF-BB induces CCL2 and IL-6 production by skin and lung fibroblasts and IL-8 production by human corneal fibroblasts.²²⁻²⁴

Recently, we demonstrated elevated PDGF-B mRNA expression in orbital tissue from patients with GO.²⁵ For the present study, we hypothesize that PDGF-BB stimulates cytokine pro-

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Supported by the Stichting Wetenschappelijk Onderzoek Oogzickenhuis Grant SWOO 2007-01 (LvS) and the Nederlandse vereniging van Graves' patiënten.

Submitted for publication July 20, 2009; revised September 1, 2009; accepted September 4, 2009.

Disclosure: L. van Steensel, None; D. Paridaens, None; G.M. Dingjan, None; P.L.A. van Daele, None; P.M. van Hagen, Novartis Pharma (C); R.W.A.M. Kuijpers, None; W.A. van den Bosch, None; H.A. Drexhage, None; H. Hooijkaas, None; W.A. Dik, None

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TABLE 1. Patients and Controls

	Active GO (n = 5)	Inactive GO (n = 5)	Controls (n = 5)
Age, y (range)	63.4 (32-79)	56.2 (43-69)	64.4 (49-78)
Sex (M/F)	0/5	0/5	1/4
Smoking	4/5	3/5	1/5
Graves' disease	5/5	5/5	0/5
RAI	4/5	5/5	—
Surgery	1/5	0/5	—
Strumazol	5/5	5/5	—
Treatment GO	5/5	5/5	0/5
Surgery	5/5	5/5	—
Prednisone	5/5	5/5	—
Radiation	1/5	2/5	—
Euthyroid	5/5	5/5	5/5
TSH-receptor antibodies	5/5	5/5	0/5
TPO antibodies	3/5	4/5	0/5
Clinical Activity Score (range)	5 (4-6)	2 (1-3)	—
NO-SPECS (range)*	4 (2-7)	3 (1-7)	—

TPO, thyroid peroxidase.

* NO-SPECS score has been described previously.²⁶

duction by orbital fibroblasts, thereby driving orbital inflammation in GO. We examined the effect of PDGF-BB on IL-1 β , IL-6, IL-8, IL-16, CCL2, CCL5, CCL7, and TNF- α production by orbital fibroblasts from patients with GO and controls as well as involvement of NF- κ B activation. Given that orbital fibroblasts are known to respond differently to stimuli than fibroblasts from other anatomic sites,^{13,25} lung and skin fibroblasts also were investigated.

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

PATIENTS, MATERIALS, AND METHODS

Reagents

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

Patients and Controls

Orbital tissue was obtained from 10 patients with GO undergoing orbital decompression surgery and from five control patients without known thyroid disease and undergoing orbital surgery for other reasons (Table 1). All patients were euthyroid at the time of the orbital surgery. All tissues were obtained, after informed consent, in the Rotterdam Eye Hospital (Rotterdam, The Netherlands) in accordance with the principles of the Declaration of Helsinki and after approval by

the institutional review board at the Erasmus MC, University Medical Center (Rotterdam, The Netherlands).

Orbital Fibroblast Culture

Orbital fibroblast strains were established as described previously.²⁵ Briefly, orbital tissue was cut into small pieces and cultured in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (DMEM 10% FCS) in a humidified atmosphere of 5% CO₂ at 37°C. Once fibroblast monolayers were obtained, cultures were serially passaged after gentle treatment with trypsin/EDTA. Fibroblast strains used for experiments were between the 6th and 12th passages.

Cytokine Production by Fibroblast Cultures

Fibroblasts were seeded at 3×10^5 cells/well into six-well plates in DMEM 10% FCS and allowed to adhere. Then the fibroblasts were incubated overnight in DMEM supplemented with 1% FCS and antibiotics (DMEM 1% FCS). Subsequently, cells were washed and cultured in DMEM 1% FCS with or without PDGF-BB (50 ng/mL) for 24 hours. To examine NF- κ B involvement, fibroblasts were preincubated with SC-514 (100 μ M) 1 hour before PDGF-BB stimulation or with SN50 (15 μ M) or SN50M (15 μ M) for 15 minutes before PDGF-BB stimulation. To demonstrate the involvement of PDGF signaling, the PDGF receptor tyrosine kinase inhibitor imatinib mesylate (2.5 μ g/mL) was supplemented overnight before PDGF-BB stimulation, as described previously.²⁵ Supernatants were harvested and cytokine levels were determined by ELISA according to the manufacturer's instructions. IL-16 ELISA was performed as previously described.²⁷

NF- κ B Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared by washing the cells twice with ice-cold PBS and subsequent scraping in 2 mL ice-cold PBS containing protease inhibitors. Cells were centrifuged and lysed with ice-cold lysis buffer containing 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and a protease inhibitor cocktail. After incubation and centrifugation, nuclei were lysed in ice-cold lysis buffer containing 20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and a protease inhibitor cocktail. Protein concentration was determined using the Bradford method. EMSA was performed as described previously.²⁸ Double-stranded γ -ATP-³²P-labeled oligonucleotide probes specific for either NF- κ B or OCT-1 were prepared using the following oligonucleotides: NF- κ B sense, 5'-AGTTGAGGGGACTTCCAGGC-3'; NF- κ B antisense, 5'-GCCTGGGAAAGTC-CCCTCAACT-3'; OCT-1 sense, 5'-TGTCGAATGCAAATCACTAGAA-3'; OCT-1 antisense, 5'-TTCTAGTGATTTGCATTGACA-3'. To determine binding, equal amounts of nuclear extracts (10 μ g) were incubated with labeled double-stranded oligonucleotide. Poly-dIdC was added to prevent unspecific binding of proteins to the probe. Specificity of the binding reaction was confirmed by a competition assay with a fourfold excess of unlabeled double-stranded oligonucleotide probe. Supershift analysis was performed by preincubating nuclear protein extracts with antibodies specific for the p65 or the p50 NF- κ B subunit for 30 minutes before incubation with labeled probe. Complexes were separated on a non-denaturing PAGE gel, and bands were detected by a biomolecular imager (Typhoon scanner; GE Healthcare, Piscataway, NJ). Densitometric analysis was then performed (ImageQuant 5.2; Molecular Dynamics/GE Healthcare, Diegen, Belgium).

Statistical Analysis

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

RESULTS

Effect of PDGF-BB on Cytokine Production by Orbital Fibroblasts

PDGF-BB significantly induced IL-6, IL-8, CCL2, CCL5, and CCL7 production by orbital fibroblasts from both patients

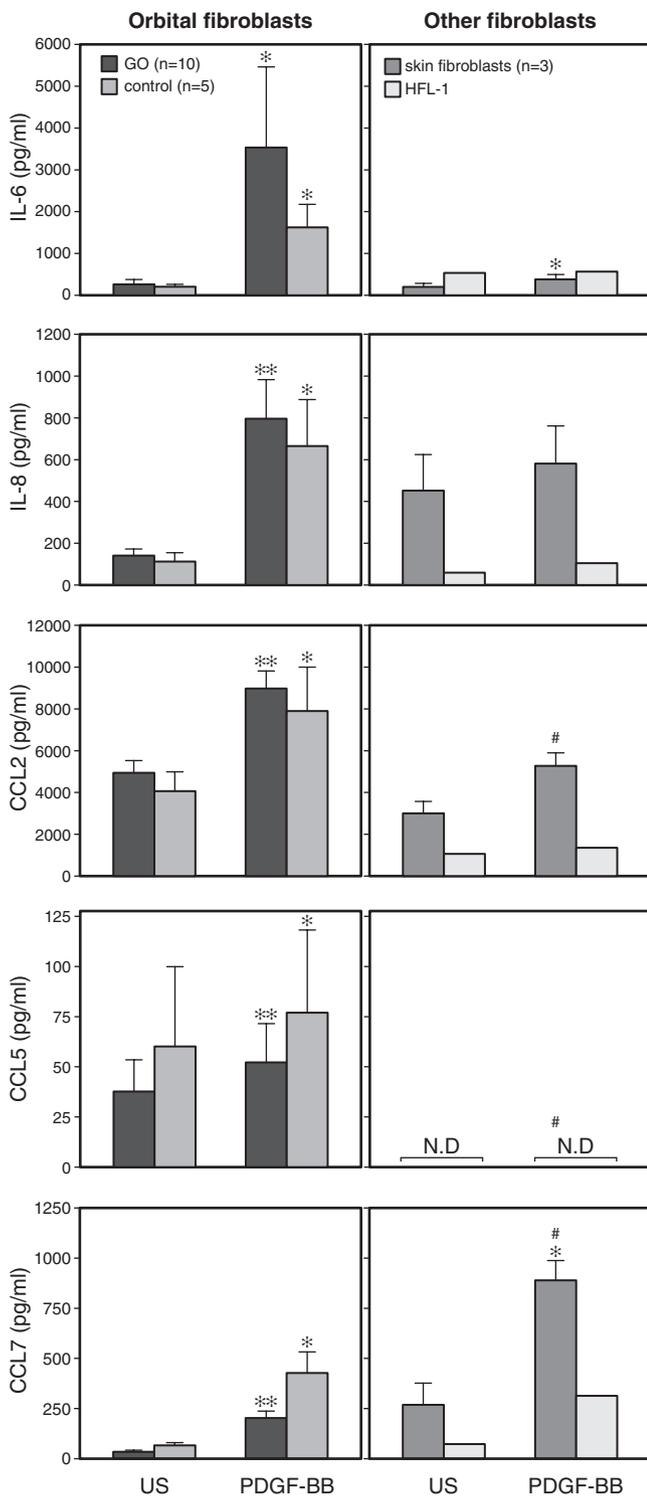


FIGURE 1. The production of IL-6, IL-8, CCL2, CCL5, and CCL7 by orbital ($n = 15$), skin ($n = 3$), and lung ($n = 1$) fibroblasts on 24 hours of stimulation with PDGF-BB (50 ng/mL). Each bar represents the mean \pm SEM. Data were analyzed using the paired Student's *t*-test or the Mann-Whitney *U* test. * $P < 0.05$; ** $P < 0.01$ versus unstimulated; # $P < 0.05$ orbital versus skin fibroblasts. ND, not detectable; US, unstimulated.

with GO and control subjects (all $P < 0.05$; Fig. 1) but did not induce IL-1 β , IL-16, or TNF- α production (data not shown). No differences in PDGF-BB-induced cytokine production were observed between orbital fibroblasts obtained

from patients with GO with active or inactive disease or control subjects.

Effect of PDGF-BB on Cytokine Production by Lung and Skin Fibroblasts

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

Effect of PDGF-BB on NF- κ B Activity in Fibroblasts

The production of many cytokines is controlled by the NF- κ B signaling pathway; therefore, we determined whether PDGF-BB induced the nuclear translocation of active NF- κ B in fibroblasts. Nuclear NF- κ B activity was detectable between 15 to 45 minutes after PDGF-BB stimulation and was induced to comparable levels in orbital fibroblasts, lung fibroblasts, and skin fibroblasts (Fig. 2). Analysis for OCT-1, a constitutive active transcription factor, revealed equal loading between all tested samples (Fig. 2). Addition of excess unlabeled probe (CP; either NF- κ B or OCT-1) to the reaction mixture was associated with loss of signal, demonstrating specificity of the DNA-binding proteins (Fig. 2). To further characterize the nuclear proteins binding to the NF- κ B motif, nuclear extracts were preincubated with antibodies to p65 or p50, two protein subunits of the active NF- κ B heterodimer. Incubation of nuclear extracts with these antibodies retarded mobility of the labeled NF- κ B oligonucleotide probe, thereby supershifting activity to the top of the gel (Fig. 2). This suggests that the PDGF-BB-induced nuclear NF- κ B contained both the p65 and the p50 subunits in all tested fibroblasts.

NF- κ B Involvement in PDGF-BB-Induced Cytokine Production by Orbital Fibroblasts

To investigate whether PDGF-BB-induced NF- κ B activity was involved in cytokine production by orbital fibroblasts, we randomly selected orbital fibroblasts from seven patients with GO and four control subjects. These fibroblasts were stimulated with PDGF-BB after preincubation with SC-514 (which blocks NF- κ B activation) or SN50 (which blocks nuclear translocation of the activated NF- κ B complex), and cytokine levels were determined in culture supernatants.

SC-514 (Fig. 3A) and SN50 (Fig. 3B) significantly (all $P < 0.05$) inhibited PDGF-BB-induced IL-6, IL-8, and CCL5 production to basal levels. In addition, CCL2 and CCL7 levels were significantly (all $P < 0.05$) inhibited by these NF- κ B inhibitors, although this never reached basal production levels. The nonsense control peptide SN50M did not inhibit PDGF-BB-induced cytokine production by orbital fibroblasts (Fig. 3B).

Effect of Imatinib Mesylate on PDGF-BB-Induced Cytokine Production by Orbital Fibroblasts

Previously, we demonstrated that imatinib mesylate blocks PDGF receptor phosphorylation and subsequent signaling in orbital fibroblasts.²⁵ Therefore, to confirm that PDGF receptor

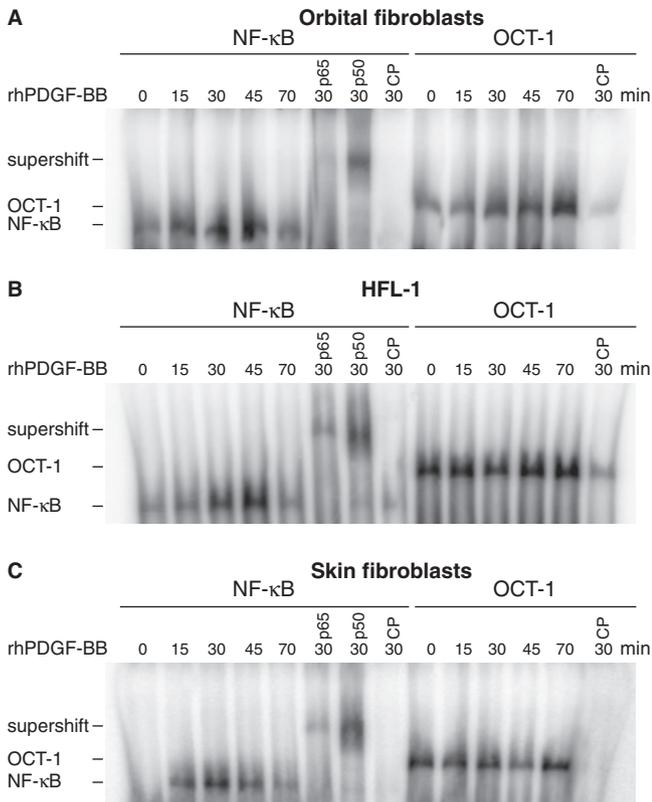


FIGURE 2. Induction of NF-κB activity after PDGF-BB (50 ng/mL) stimulation. Binding activity to an oligonucleotide containing an NF-κB consensus motif was assayed in nuclear extracts from orbital (A), lung (HFL-1; B) and skin (C) fibroblasts. As loading control, the same nuclear extracts were analyzed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

activation was required, we tested whether imatinib mesylate blocked PDGF-BB-induced NF-κB activation and cytokine production. As expected, inhibition of PDGF receptor activation by imatinib mesylate prevented PDGF-BB induced NF-κB activation (Fig. 4A) and subsequent cytokine production (all $P < 0.05$; Fig. 4B).

DISCUSSION

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

Recently, we found increased PDGF-B mRNA expression in GO orbital tissue and demonstrated enhanced proliferation and hyaluronan production by orbital fibroblasts on PDGF-BB stimulation, suggesting a role of the homodimeric growth factor PDGF-BB in the pathophysiology of GO.²⁵ Here we demonstrate for the first time that PDGF-BB induces the production of IL-6, IL-8, CCL2, CCL5, and CCL7, cytokines relevant to the pathophysiology of GO, by orbital fibroblasts. IL-6 recruits and activates B cells and stimulates plasma cell differentiation and immunoglobulin production.²⁹ Furthermore, increased IL-6 mRNA levels have been described in orbital tissue from patients with GO.⁹ IL-6 increases thyroid-stimulating hormone receptor expression on orbital fibroblasts³⁰ and is considered a determinant of orbital adipogenesis, another important component of the pathology of GO.³¹ IL-8 is a powerful attractant for neutrophils.³² Increased IL-8 mRNA levels have been detected in orbital tissue from patients with GO,⁹ and elevated serum IL-8 levels have been associated with hyperthyroidism.¹⁴ CCL2 and CCL7 are attractants for monocytes and macrophages,³³ which are both abundantly present in the infiltrate in GO orbital tissue.^{14,34} In addition, increased CCL2 mRNA expression levels in GO orbital tissue correlate positively with macrophage infiltration into the adipose tissue.¹⁴ CCL5 is well known for its ability to attract T-lymphocytes.³⁵ Although expression of CCL5 in GO orbital tissue has so far never been

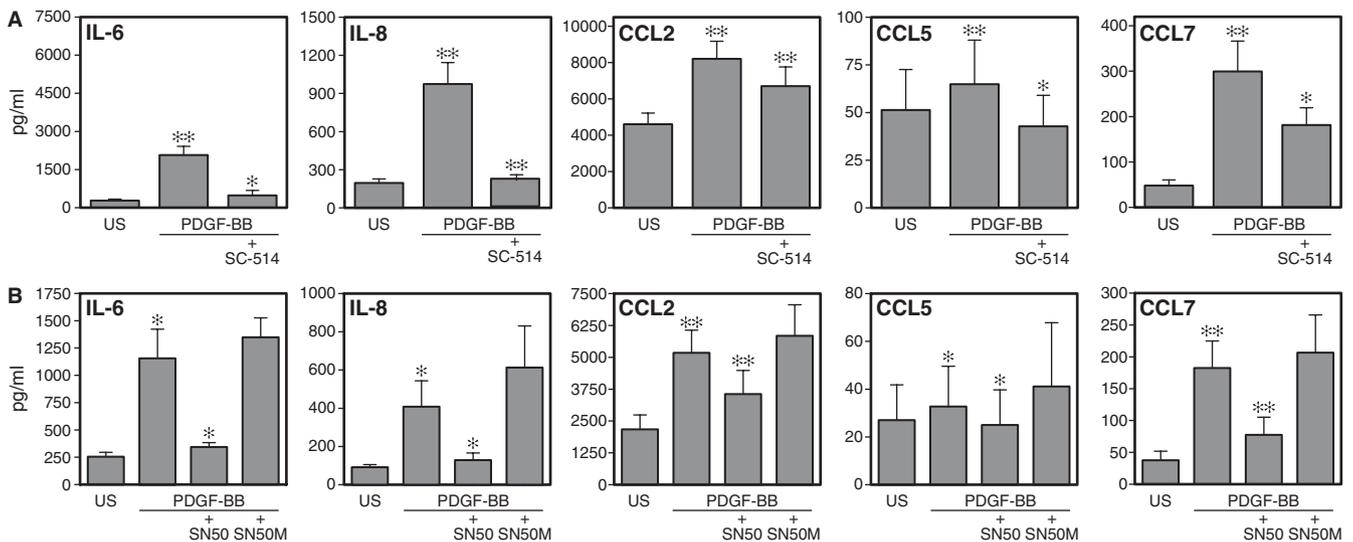


FIGURE 3. IL-6, IL-8, CCL2, CCL5, and CCL7 production by GO ($n = 7$) and control ($n = 4$) orbital fibroblasts is inhibited by blockade of the NF-κB pathway. Fibroblasts were stimulated for 24 hours with PDGF-BB (50 ng/mL) after (A) 60 minutes of preincubation with SC-514 (100 μM) or (B) 15 minutes of preincubation with SN50 (15 μM) and SN50M (15 μM). Each bar represents the mean ± SEM. Data were analyzed using the paired Student's *t*-test. * $P < 0.05$ and ** $P < 0.01$ PDGF-BB versus unstimulated and PDGF-BB versus PDGF-BB + SC514 or PDGF-BB + SN50. US, unstimulated.

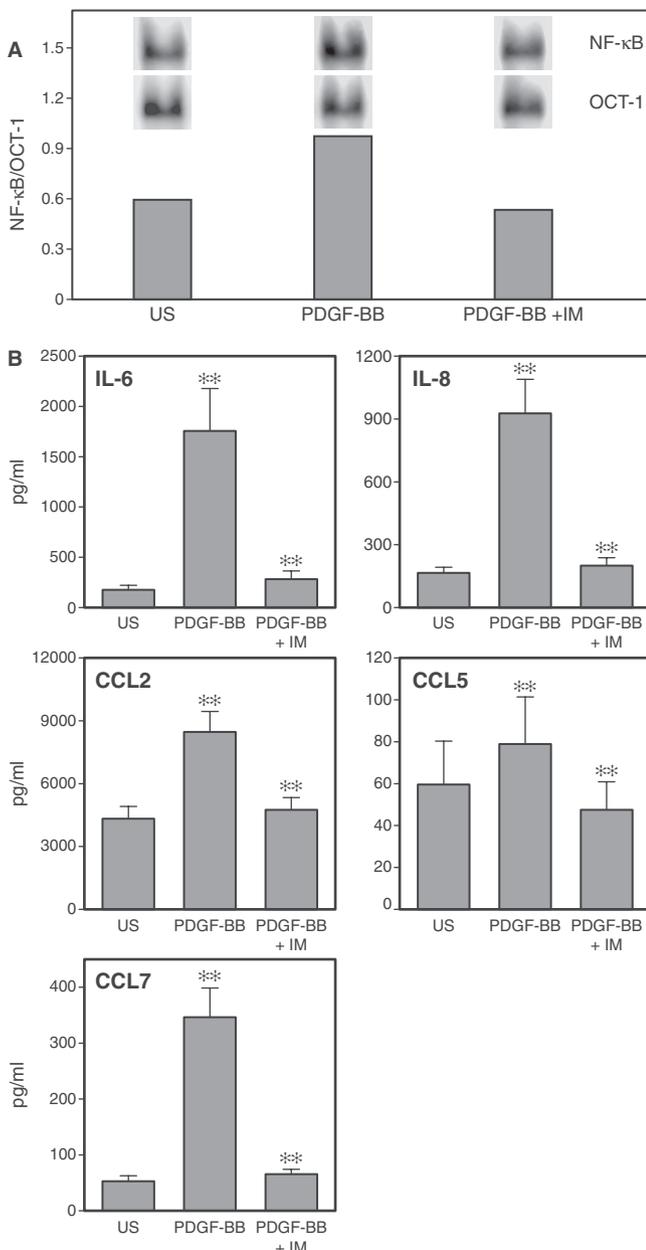


FIGURE 4. Imatinib mesylate (IM; 2.5 $\mu\text{g}/\text{mL}$) inhibits PDGF-BB (50 ng/mL)-induced NF- κB activation and subsequent proinflammatory cytokine production. **(A)** Orbital fibroblasts were stimulated for 30 minutes with PDGF-BB with or without preincubation with imatinib mesylate. Subsequently, binding activity of nuclear extracts to an oligonucleotide containing a NF- κB or OCT-1 consensus motif was assayed, and by densitometry the NF- κB /OCT-1 ratio was calculated. Data are from a representative experiment. **(B)** GO ($n = 7$) and control ($n = 4$) orbital fibroblasts were stimulated for 24 hours with PDGF-BB with or without preincubation with imatinib mesylate, and subsequently IL-6, IL-8, CCL2, CCL5, and CCL7 levels were determined. Each bar represents the mean \pm SEM. Data were analyzed using the paired Student's *t*-test. * $P < 0.05$ and ** $P < 0.01$ PDGF-BB versus unstimulated and PDGF-BB versus PDGF-BB + IM. US, unstimulated.

demonstrated, a role for CCL5 in orbital T-lymphocyte recruitment in GO has been suggested based on its production by orbital fibroblasts on stimulation with immunoglobulins from patients with Graves' disease.^{7,36} Our current data suggest that PDGF-BB, through the activation of orbital fibroblasts, induces the production of several cytokines in orbital tissue, thereby regulating immune cell infiltration into the orbit in GO. Such a

role for PDGF-BB is supported by studies in which pulmonary overexpression of the *PDGF-B* gene resulted in marked alveolitis, comprising mainly mononuclear cells and macrophages.^{19,37}

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

Our current data underscore the unique functional nature that has been attributed to the orbital fibroblast,^{16,38} which may be a critical determinant for the development of GO.¹³ Orbital fibroblasts produced more IL-6, IL-8, CCL2, and CCL5 on PDGF-BB stimulation than skin and lung fibroblasts. This was not related to the inability of skin and lung fibroblasts to activate NF- κB DNA binding activity on PDGF-BB stimulation. Differences in NF- κB dimer induction are also unlikely to have contributed because PDGF-BB induced NF- κB p65/p50 heterodimer activity equally in orbital, skin, and lung fibroblasts. Induction of p65/p50 activity is in line with previous observations of PDGF-BB-induced NF- κB activation in fibroblasts.³⁹ We cannot exclude that specific nuclear modifications of NF- κB did enhance transcriptional activity^{40,41} in orbital fibroblasts. In addition, the expression level and activity of other transcription factors might have influenced the transactivation potential of NF- κB .^{41,42} In contrast to skin and lung fibroblasts, orbital fibroblasts had some basal expression of CCL5 that was enhanced on PDGF-BB stimulation, contrary to previous studies demonstrating the absence of basal CCL5 production by orbital fibroblasts.⁷ Possibly this discrepancy is related to low basal production levels and the sensitivity of the assays used.

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

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