

Platelet-Derived Growth Factor-BB Enhances Adipogenesis in Orbital Fibroblasts

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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.

Keywords: Graves' ophthalmopathy, platelet-derived growth factor-BB, adipogenesis, orbital fibroblast

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 differenti-

ate 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 μ M biotin (Sigma-Aldrich Corp., St. Louis, MO, USA), 17 μ M pantothenic acid (Sigma-Aldrich Corp.), 1 μ M insulin (Sigma-Aldrich Corp.), 10 μ g/mL transferrin (Merck, Darmstadt, Germany), 0.2 nM Triiodothyronine (T3; Sigma-Aldrich Corp.), 0.2 μ M carbaprostaglandin (PGI₂; Cayman Chemical Company, Ann Arbor, MI, USA), and 10 μ M rosiglitazone (Sigma-Aldrich Corp.) for 14 days. In the first 3 days the differentiation medium also was supplemented with 10 μ M dexamethasone (Sigma-Aldrich Corp.) and 0.1 mM isobutylmethylxanthine (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 PGI₂ 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- μ m 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 $\times 200$ 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 μ L 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.

Proliferation 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 IgG₁ 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

TABLE. RQ-PCR Primer-Probe Combination

Gene	Forward Primer, 5'–3'	Reverse Primer, 5'–3'	Probe, 5' FAM–3' TAMRA
<i>ABL</i>	TGGAGATAACATCTAAAGCATAACTAAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTGGTTTGGGCTTCACACCCATT
<i>PPAR-γ</i>	TGACAGCGACTTGGCAATATTT	TCTTCAATGGGCTTCACATTCA	CAAACTGGGCGGTCTCCACTGAG

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).

The adipogenesis enhancing effect of PDGF-BB was confirmed by triglyceride measurement, which showed significantly ($P < 0.05$) higher triglyceride accumulation in

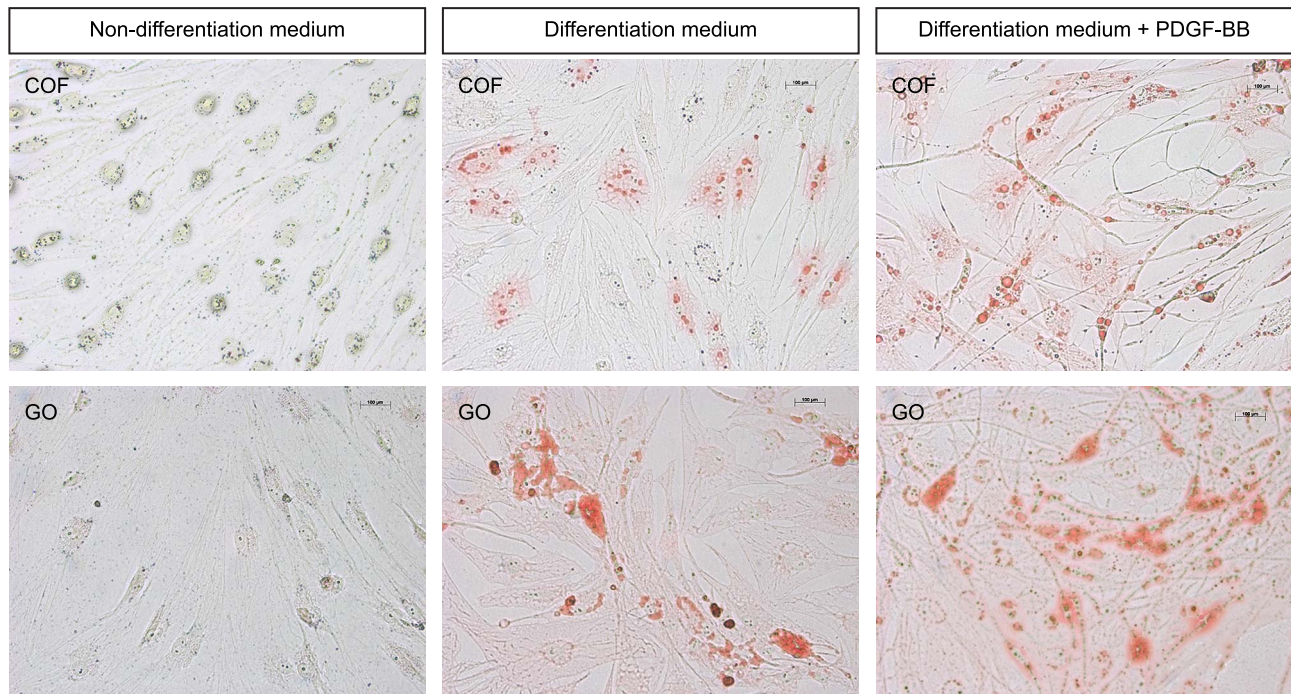


FIGURE 1. Oil-Red-O staining on orbital fibroblasts. Control orbital fibroblasts (COF, upper panels) and GO orbital fibroblasts (GO, lower panels) were cultured in nondifferentiation medium (left column), adipocyte differentiation medium (middle column), and adipocyte differentiation medium in the presence 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.

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).

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).

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 $\mu\text{g}/\text{mL}$) 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).

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.

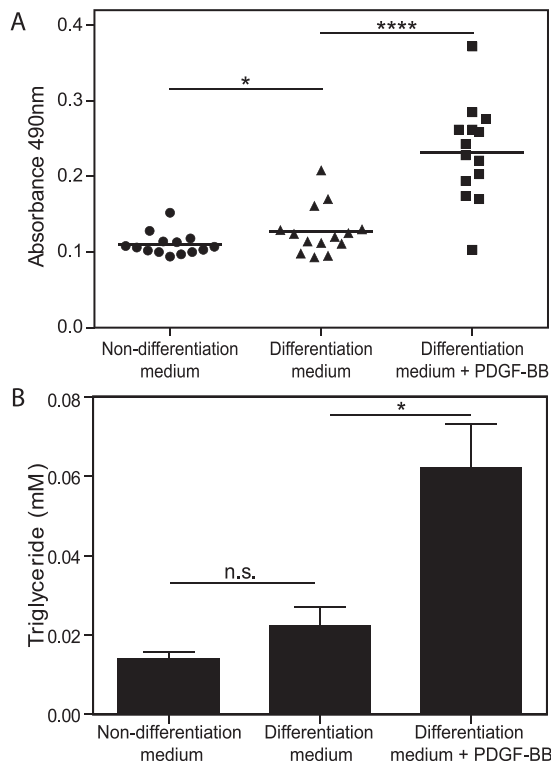


FIGURE 2. Quantification of adipogenesis. **(A)** Oil-Red-O staining was quantified in eluate after 14 days of differentiation from orbital fibroblasts from controls ($n = 5$) and GO patients ($n = 9$) using a spectrophotometer at 490 nm. Each dot represents the orbital fibroblast strain from one individual 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 \pm 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.

Dasatinib reduced the expression of PPAR- γ mRNA in all 3 orbital tissues compared to untreated tissues (Fig. 6).

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

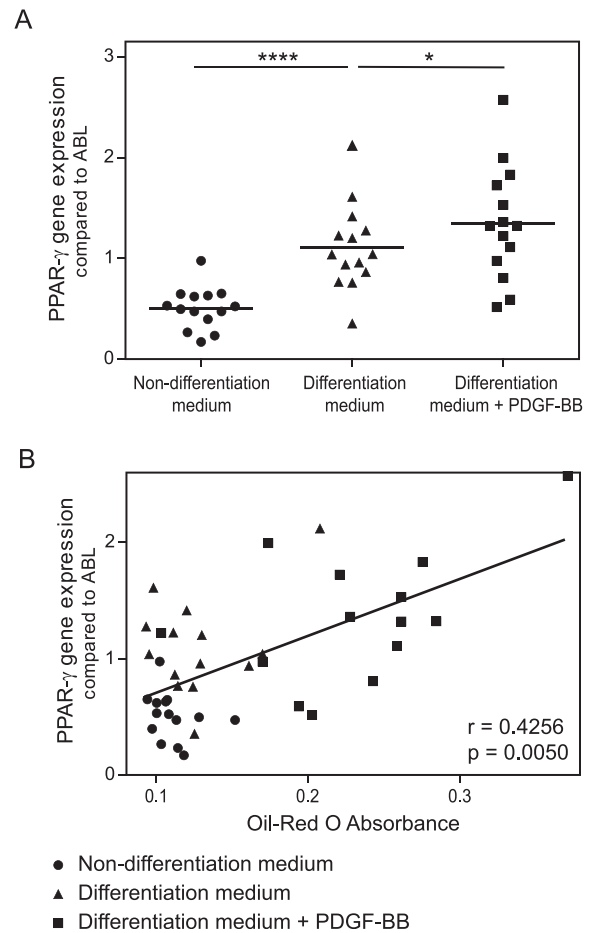


FIGURE 3. Expression of PPAR- γ mRNA in orbital fibroblasts upon adipocyte differentiation. **(A)** Expression of PPAR- γ mRNA was determined by RQ-PCR in orbital fibroblasts from controls ($n = 5$) and GO patients ($n = 9$) after 14 days of adipocyte differentiation. Each dot represents the orbital 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.

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-1 β or PGD₂.²²⁻²⁴ 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

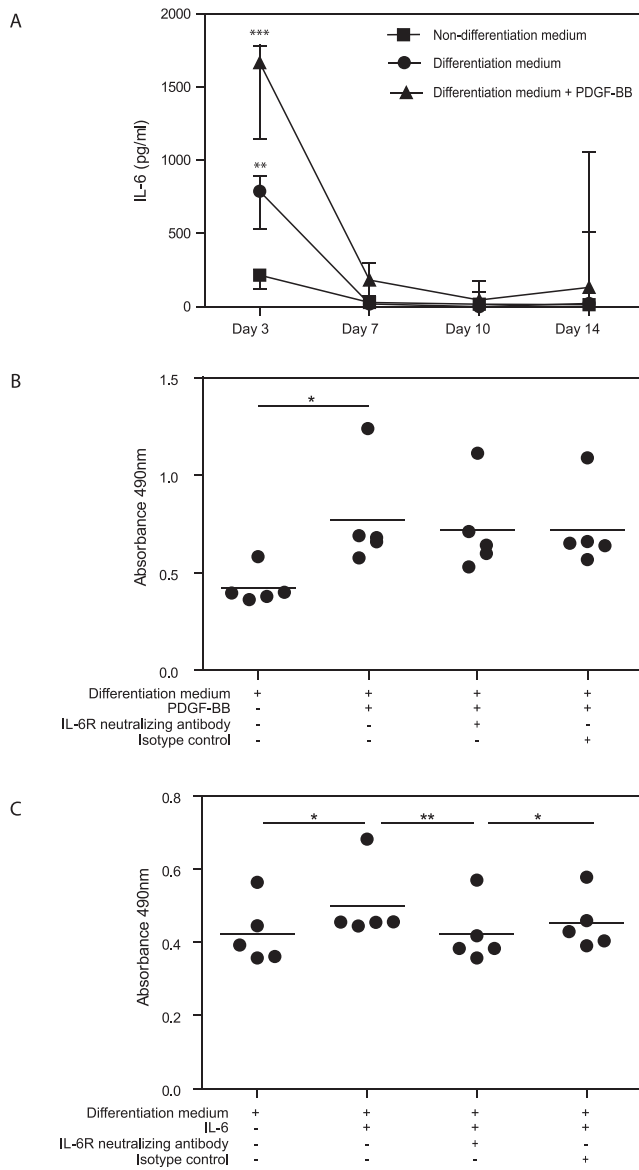


FIGURE 4. Production of IL-6 by orbital fibroblasts during adipocyte differentiation. **(A)** Concentration of IL-6 was determined by ELISA in culture supernatant obtained following 3, 7, 10, and 14 days of differentiation from controls ($n = 2$) and GO patients ($n = 3$) orbital fibroblasts. Data are presented as mean \pm 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 μ g/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.

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.

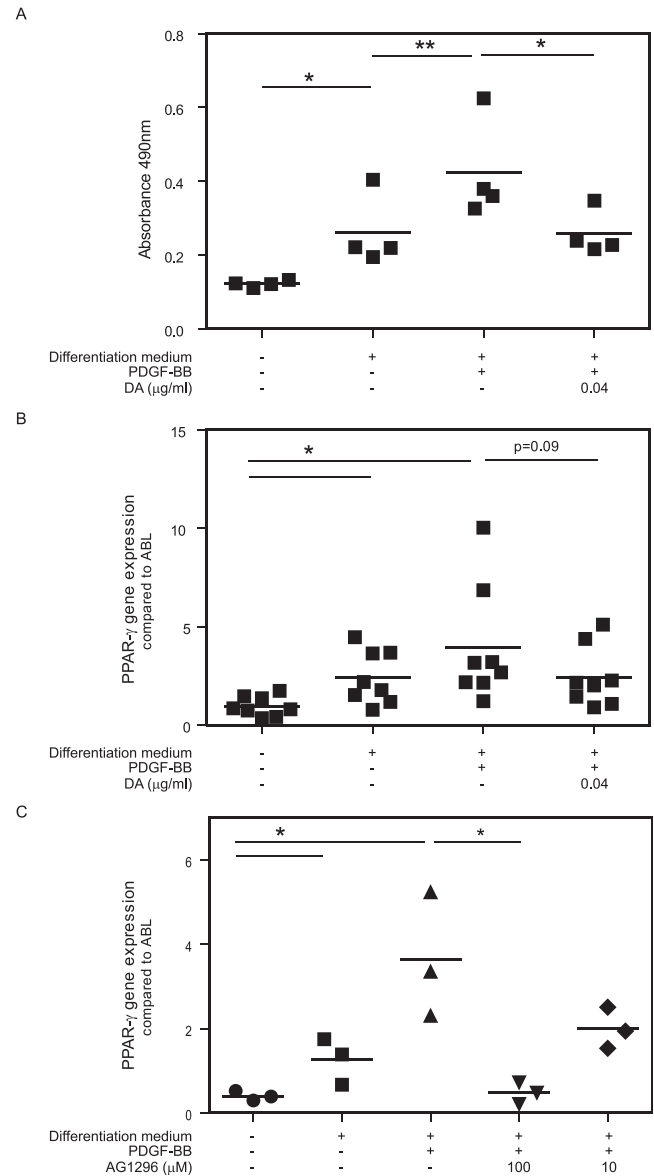


FIGURE 5. Effect of dasatinib on adipogenesis in orbital fibroblasts. **(A)** Oil-Red-O staining was quantified in eluate from orbital fibroblasts (controls, $n = 2$; GO patients, $n = 2$) cultured for 14 days in differentiation medium with/without PDGF-BB and either in the presence or absence of the PDGF-receptor TKI dasatinib (0.04 μ g/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 μ M) 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.

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

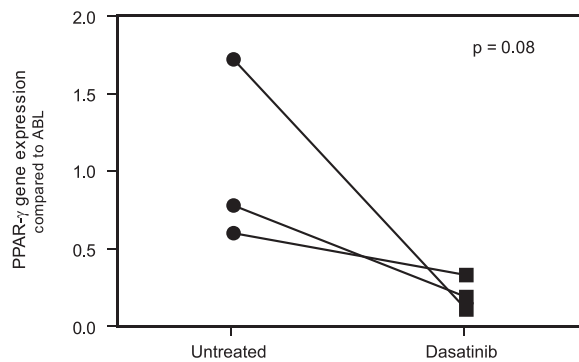


FIGURE 6. Effect of dasatinib on PPAR- γ mRNA expression in cultured orbital tissue from GO patients. Orbital tissue from three GO patients was cultured in the presence or absence of dasatinib (2.5 μ g/mL) overnight. Expression of PPAR- γ mRNA was determined by RQ-PCR. Data were analyzed using the paired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

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 μ g/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 μ g/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 μ g/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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