Imatinib Mesylate and AMN107 Inhibit PDGF-Signaling in Orbital Fibroblasts: A Potential Treatment for Graves' Ophthalmopathy

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Purpose. Excessive orbital fibroblast proliferation and hyaluronan production are characteristic of Graves' ophthalmopathy (GO) and are driven by local mediators. Imatinib mesylate and AMN107 are tyrosine kinase inhibitors that inhibit fibroblast proliferation and collagen production in lungs and skin. This study was conducted to determine whether imatinib mesylate and AMN107 inhibit orbital fibroblast proliferation and hyaluronan production induced by PDGF-BB and TGF-β_1 and whether expression of the genes *PDGF-B* and *TGF-B*$_1$ (growth factors suggested to play a role in GO) are increased in GO orbital tissues.

Methods. *PDGF-B* and *TGF-B*$_1$ mRNA levels were determined in orbital tissues of 13 patients with GO and 5 control patients. Orbital fibroblasts were cultured from eight patients with GO and three control patients and the effect of imatinib mesylate and AMN107 on PDGF-BB and TGF-β_1–induced orbital fibroblast proliferation, signaling cascades, hyaluronan synthase (*HAS*) gene expression and hyaluronan production were determined.

Results. *PDGF-B* and *TGF-B*$_1$ mRNA levels were significantly increased in GO orbital tissues. Imatinib mesylate and AMN107 inhibited PDGF-BB–induced orbital fibroblast proliferation, *HAS* induction and hyaluronan production by blocking PDGF-receptor phosphorylation. TGF-β_1 induced *HAS* expression and hyaluronan production. This induction was not inhibited by imatinib mesylate or AMN107, due to the inability of TGF-β_1 to activate c-Abl kinase activity in orbital fibroblasts.

Conclusions. Imatinib mesylate and AMN107 inhibit orbital fibroblast proliferation and hyaluronan production induced by PDGF-BB; a factor highly expressed in orbital tissue from patients with GO. The drugs, however, had no effect on TGF-β_1–induced *HAS* expression and hyaluronan production. Nevertheless, imatinib mesylate and AMN107 should be considered as treatment candidates for GO. (*Invest Ophthalmol Vis Sci.* 2009;50:3091–3098) DOI:10.1167/iovs.08-2443

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

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

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

In this study, we demonstrate increased *PDGF-B* and *TGF-B*$_1$ mRNA levels in orbital tissues from patients with GO. In addition, we show that imatinib mesylate and AMN107 are potent inhibitors of PDGF-BB–induced orbital fibroblast prolif-

eration as well as hyaluronan synthesis through inhibition of PDGF-R phosphorylation. TGF-β_1–induced *HAS* expression and hyaluronan production was not inhibited by imatinib mesylate or AMN107, because of the inability of TGF-β_1 to activate c-Abl kinase activity in orbital fibroblasts. Smad signaling was activated by TGF-β_1. This study provides evidence that imatinib mesylate and AMN107 should be considered candidates for the treatment of patients with therapy-resistant GO, especially those with severe periocular edema and ocular motility dysfunction who are not responding to common therapies.

Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, and trypsin/EDTA were purchased from Cambrex BioWhittaker (Verviers, Belgium). The human fetal lung fibroblast cell line (HFL-1) was obtained from ATCC (Manassas, VA). Recombinant human PDGF-BB, recombinant human TGF-β_1, and a hyaluronan ELISA were obtained from R&D Systems (Abingdon, UK). Anti–PDGF-Rβ antibody (SC-339) was purchased from Santa Cruz Biotechnologies (Heidelberg, Germany). Anti–phospho-tyrosine (cat no. 9411), anti–c-Abl (cat no. 2862), anti-Smad3 (cat no. 9513), and anti–phospho-Smad3 (cat no. 9520S) antibodies were purchased from Cell Signaling (Danvers, MA). Anti–β-actin (AB6276) was purchased from Abcam (Cambridge, UK). Anti–c-Abl antibody (8E9) was kindly provided by Dynomics (Rotterdam, the Netherlands). Imatinib mesylate and AMN107 were kindly provided by Novartis Pharma (Basel, Switzerland). SB431542 was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). GST-CRK was a kind gift of Edward B. Leof (Mayo Clinic, Minneapolis, MN).

Patients and Control Subjects

The patients' characteristics are given in Tables 1A and 1B, and clinical scores were determined as described before.[18] Orbital tissue was obtained from 15 patients with GO undergoing orbital decompression surgery. Six of the orbital tissues were used for mRNA detection and yielded orbital fibroblast strains, seven of the tissues were only used for mRNA detection, and from two tissues only fibroblast strains were established. Of the patients, 3 had active and 12 inactive disease. All patients undergoing orbital surgery were euthyroid. Furthermore, orbital tissue was obtained from seven control patients without thyroid or inflammatory disease and undergoing orbital surgery for other reasons. One control orbital tissue was used for mRNA detection and yielded an orbital fibroblast strain, four control tissues were used only for mRNA detection and, from two control tissues, only fibroblast strains were established. All tissues were obtained in the Rotterdam Eye Hospital (Rotterdam, The Netherlands) after informed consent, 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).

Detection of *PDGF-B* and *TGF-B_1* mRNA Levels in Orbital Tissue

RNA was isolated (RNeasy columns; Qiagen, Hilden, Germany) and reverse transcribed into cDNA.[19] *PDGF-B* and *TGF-B_1* transcript levels were determined by real-time quantitative PCR (RQ-PCR; 7700 PCR system; Applied Biosystems [ABI], Foster City, CA). Transcript levels were normalized to the control gene abelson.[19] Primer–probe combinations used are listed in Table 2.

Orbital Fibroblast Culture

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

Table 1. Characteristics of Patients and Controls from Whom Tissues and Strains Were Obtained for the Study

A. Orbital Tissues Used for mRNA Determination

	Patients with GO (n = 13)	Control (n = 5)
Mean age, y (range)	51 (27–78)	53 (37–80)
Sex (m/f)	1/12	0/5
Smoking (yes)	7/13	0/5
Graves' disease	13/13	0/5
RAI	12/13	—
Surgery	1/13	—
Strumazol	8/13	—
Treatment GO	13/13	0/5
Surgery	13/13	—
Prednisone	13/13	—
Radiation	4/13	—
Euthyroid	13/13	5/5
TSH-receptor antibodies	13/13	0/5
TPO antibodies	6/13	0/5
Clinical activity score (range)	2 (1–6)	—
NO-SPECS (range)*	3 (1–7)	—

B. Orbital Fibroblast Strains

	Patients with GO (n = 8)	Control (n = 3)
Mean age, y (range)	54 (42–78)	59 (49–70)
Sex (m/f)	1/7	1/2
Smoking (yes)	5/8	0/0
Graves' disease	8/8	0/0
RAI	7/8	—
Surgery	0/8	—
Strumazol	8/8	—
Treatment GO	8/8	0/0
Surgery	8/8	—
Prednisone	8/8	—
Radiation	3/8	—
Euthyroid	8/8	3/3
TSH-receptor antibodies	8/8	0/3
TPO antibodies	7/8	—
Clinical activity score (range)	4 (1–7)	—
NO-SPECS (range)*	4 (1–7)	—

* NO-SPECS score: as described elsewhere.[18]

Orbital Fibroblast Proliferation Assay

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

TABLE 2. RQ-PCR Primer-Probe Combinations

Gene	Forward Primer	Reverse Primer	Probe	Product Length (bp)
PDGF-B	Fw_Hu_PDGFB_EMC: TCCCGAGGAGCTTTATGAGATG	Rv_Hu_PDGFB_EMC: CGGGTCATGTTCAGGTCCAAC	T_Hu_PDGFB_EMC: AGTGACCACTCGATCCGCTCCTTTG	123
TGF-B$_1$	Fw_Hu_TGFB1_EMC: CGCGTGCTAATGGTGGAA	Rv_Hu_TGFB1_EMC: AGAGCAACACGGGTTCAGGT	T_Hu_TGFB1_EMC: CCACAACGAAATCTATGACAAGTTCAAGCAGA	124
HAS1	Fw_Hu_HAS1_EMC: GCAAGCGCGAGGTCATGT	Rv_Hu_HAS1_EMC: CGGGGGTCCTCGTCCA	T_Hu_HAS1_EMC: ACTACGTGCAGGTCTGTGACTCGGACAC	136
HAS2	Fw_Hu_HAS2_EMC: AATGGGGTGGAAAAGAGAAGTC	Rv_Hu_HAS2_EMC: CAACCATGGGATCTTCTTCTAAAAC	Tr_Hu_HAS2_EMC: TCCACACTTCGTCCCAGTGCTCTGA	150
HAS3	Fw_Hu_HAS3_EMC: AAGGCCCTCGGCGATTC	Rv_Hu_HAS3_EMC: CCCCCGACTCCCCCCTACT	T_Hu_HAS3_EMC: ACATCCAGGTGTGCGACTCTGACACTGTG	125

tion was expressed as the percentage change in mean absorbance from that of cells exposed to DMEM 1.0% FCS alone.

HAS mRNA Expression in Orbital Fibroblasts

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

Hyaluronan Production by Orbital Fibroblasts

Fibroblasts were seeded at 1.5×10^5 cells/well into 24-well plates in DMEM 10% FCS and allowed to adhere. They were then incubated in DMEM 1.0% FCS in the presence or absence of imatinib mesylate or AMN107 for 16 hours. Subsequently, the cells were cultured in DMEM 1.0% FCS with or without TGF-β_1 (10 ng/mL), PDGF-BB (50 ng/mL), or a combination of TGF-β_1 and PDGF-BB for 24 hours. The supernatants were harvested, and hyaluronan levels were determined by ELISA. Because of variability in basal production levels, the results are expressed as *x*-fold induction relative to the basal hyaluronan production.

Detection of PDGF-R Phosphorylation

To assess PDGF-R activation, the cultures were stimulated with PDGF-BB (50 ng/mL) for the indicated times (see Fig. 3) and lysed (20 mM Tris [pH 8.0], 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol, and protease inhibitors). Equivalent amounts of protein (~30 μg) were loaded onto gels for SDS-PAGE. Western blots were stained with either anti–PDGF-R or anti-phosphotyrosine antibodies. To determine the effect of imatinib mesylate and AMN107 on PDGF-BB–induced PDGF-R phosphorylation, immunoprecipitation using an anti–PDGF-R antibody was performed on cell lysates (~500 μg protein). Immune complexes were collected with a mix of protein A- and G-Sepharose (Sigma-Aldrich, Zwijndrecht, The Netherlands) and the level of PDGF-R phosphorylation was determined with an anti-phosphotyrosine antibody, as described.[6]

Detection of c-Abl Kinase Activity

c-Abl kinase assays were essentially performed as described.[6] Briefly, cultures were stimulated for the indicated times (see Fig. 6) and lysed at 4°C. Immunoprecipitation was performed with an anti–c-Abl antibody (cat no. 2862; Cell Signaling). Immune complexes were collected with a mix of protein A- and G-Sepharose and washed in lysis buffer and three times in kinase buffer (10 mM HEPES [pH 7.4], 50 mM $NaCl_2$, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM DTT, and protease inhibitors). Kinase reaction was performed in 10 μL kinase mix containing 2 μg GST-CRK, 100 μM ATP, and 0.5 μCi γ-^{32}P-dATP for 15 minutes at 37°C. The reaction was stopped by adding loading buffer, and samples were loaded on gels for SDS-PAGE. Total c-Abl and actin protein were detected using anti–c-Abl (8E9) and anti–β-actin antibodies.

Detection of Smad Activation

To assess Smad activation, the cultures were treated with TGF-β_1 for the indicated times (see Fig. 7) and lysed. Equivalent amounts of protein were loaded on gels for SDS-PAGE, and Western blots were subsequently stained with antibodies to phosphorylated-Smad3, Smad-3, and β-actin.

Statistical Analysis

PDGF-B and *TGF-B$_1$* mRNA levels in orbital tissue were analyzed with the Mann-Whitney test. Data concerning the effect of imatinib mesylate

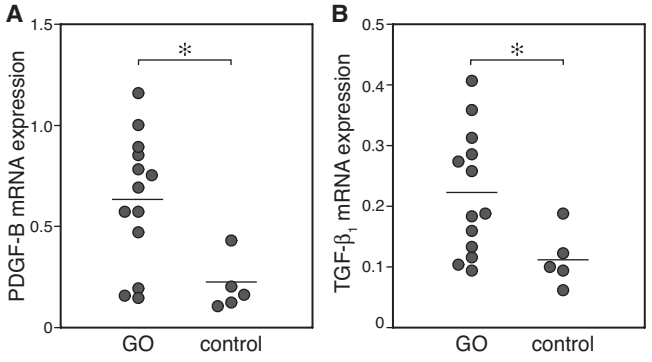

FIGURE 1. Relative mRNA expression for *PDGF-B* (**A**) and *TGF-B1* (**B**) in orbital tissue from patients with GO and control subjects. mRNA expression levels were determined by RQ-PCR and normalized to the expression levels of the abelson gene. Each data point represents a single individual. *Horizontal bars*: mean values. Data were analyzed with the Mann-Whitney test. $^*P < 0.05$.

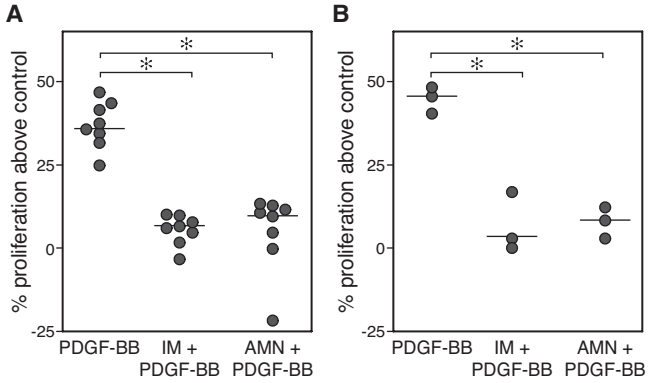

FIGURE 2. The effect of imatinib mesylate (IM; 2.5 μg/mL) and AMN107 (AMN; 2.5 μg/mL) on PDGF-BB (50 ng/mL)–induced proliferation of GO (**A**) and control (**B**) orbital fibroblasts. Each data point represents a fibroblast strain obtained from a single individual. *Horizontal bars*: mean values. Data were analyzed using the Student's *t*-test. $^*P < 0.001$.

and AMN107 on orbital fibroblast proliferation, *HAS* expression levels and hyaluronan production were analyzed with the paired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

PDGF-B and TGF-β_1 mRNA Levels in GO Orbital Tissue

PDGF-B mRNA levels were significantly higher (approximately threefold: $P < 0.05$; Fig. 1) in GO compared with control orbital tissue. Also, *TGF-B1* mRNA levels were significantly higher (approximately twofold: $P < 0.05$; Fig. 1) in GO compared with control orbital tissue. No difference in *PDGF-B* and *TGF-B1* mRNA expression was observed between patients with active or inactive GO, and no correlations were observed between expression levels and clinical activity scores (CAS).[18]

Effect of Imatinib Mesylate and AMN107 on PDGF-BB–Induced Orbital Fibroblast Proliferation and PDGF-R Phosphorylation, and of TGF-β_1 on Orbital Fibroblast Proliferation

PDGF-BB equally stimulated proliferation of orbital fibroblasts derived from patients with GO and control patients with a mean induction of 37% and 45% proliferation above control, respectively (Fig. 2). No difference in PDGF-BB–induced proliferation was observed between fibroblasts from patients with active or inactive GO. Initial studies revealed that imatinib mesylate and AMN107 dose-dependently (range, 0.16–2.5 μg/mL) inhibited PDGF-BB–induced orbital fibroblast proliferation (data not shown). At the dose of 2.5 μg/mL, imatinib mesylate and AMN107 inhibited PDGF-BB–induced proliferation of GO and control orbital fibroblasts up to basal proliferation levels (GO: imatinib and AMN107 both 5% proliferation above control; $P < 0.05$, controls: imatinib 6% and AMN107 8% proliferation above control, $P < 0.05$; Fig. 2).

With regard to the mechanisms involved, PDGF-BB stimulated PDGF-R phosphorylation in a time-dependent manner (Fig. 3A). Imatinib mesylate and AMN107 both reduced PDGF-BB–induced PDGF-R phosphorylation (Fig. 3B), indicating efficient inhibition of PDGF-R tyrosine kinase activity in orbital fibroblasts by both tyrosine kinase inhibitors.

In contrast, stimulation with TGF-β_1 (range, 2.5–40 ng/mL) did not induce orbital fibroblast proliferation and was not further explored.

Effect of PDGF-BB and TGF-β_1 on *HAS* Expression

Hyaluronan synthesis is regulated by three hyaluronan synthases, encoded by individual genes; *HAS1*, *-2*, and *-3*.[22] We determined whether PDGF-BB and TGF-β_1 influence the expression of these *HAS* genes in orbital fibroblasts. PDGF-BB stimulation increased *HAS1* and *-2* expression by approximately sixfold in GO orbital fibroblasts ($P < 0.01$), but did not influence *HAS3* expression (Fig. 4). In control orbital fibroblasts PDGF-BB stimulation increased *HAS2* expression approximately threefold, but did not affect *HAS1* and *-3* expression. TGF-β_1 stimulation increased *HAS1* expression ~60-fold ($P < 0.05$) in both GO and control fibroblasts, but did not affect *HAS2* and *-3* transcript levels (Fig. 4). No differences were observed between GO orbital fibroblasts from active or inactive disease with regard to PDGF-BB and TGF-β_1–induced *HAS* expression.

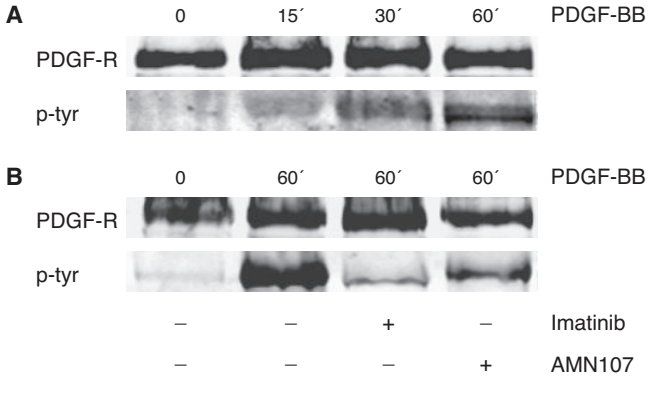

FIGURE 3. Orbital fibroblasts were stimulated with PDGF-BB (50 ng/mL), with or without Imatinib mesylate (2.5 μg/mL) and AMN107 (2.5 μg/mL), for the indicated times. (**A**) Western blot analysis was performed on lysates with antibodies against PDGF-R (*top row*) and phosphorylated tyrosine residues (*bottom row*). (**B**) *Top row:* Western blot analysis was performed with PDGF-R antibody on an aliquot before immunoprecipitation. *Bottom row:* after PDGF-R immunoprecipitation, phosphorylated PDGF-R was determined with an antibody against phosphorylated tyrosine residues. Data depicted are from a representative experiment.

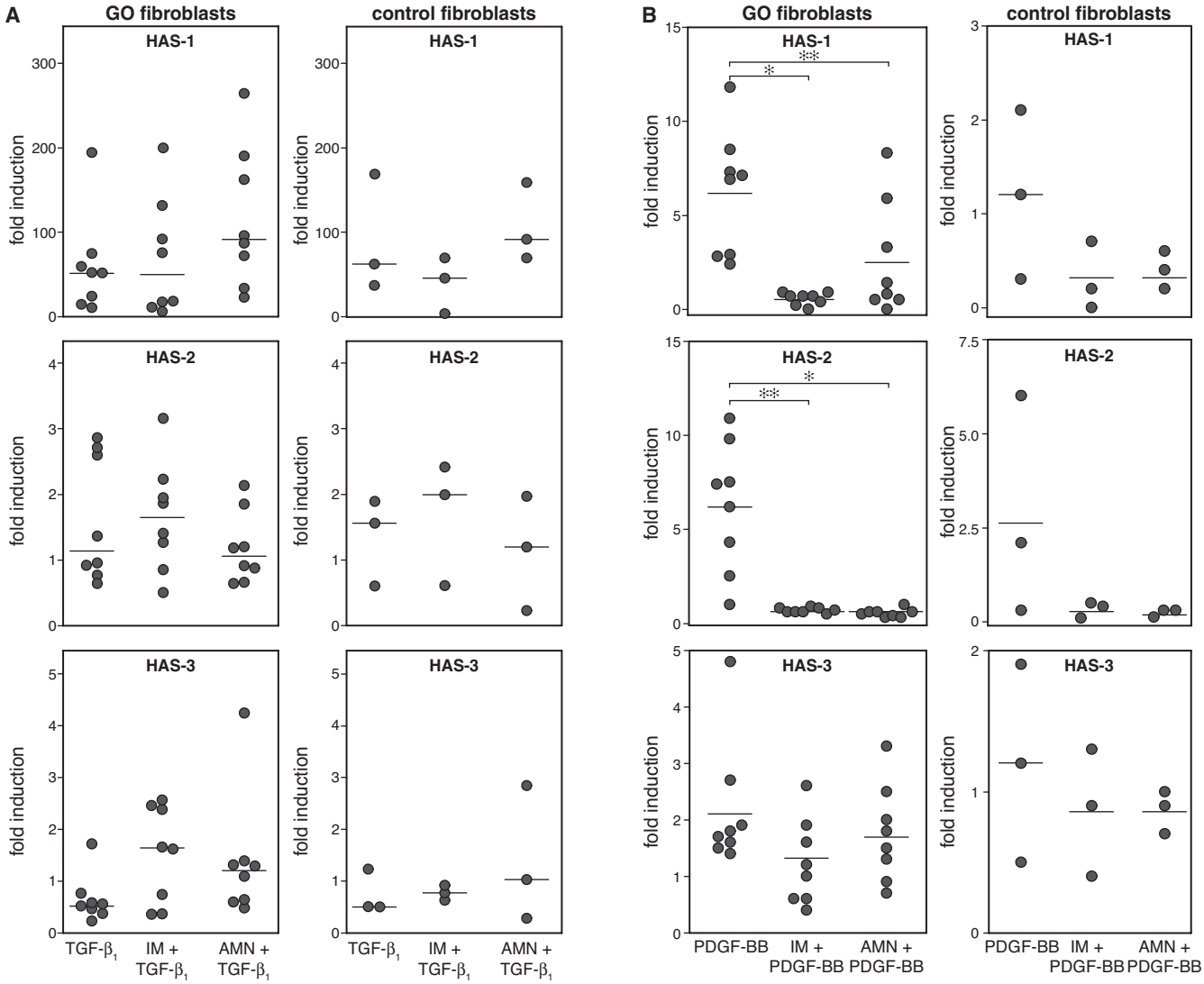

FIGURE 4. The effect of imatinib mesylate (IM; 2.5 μg/mL) and AMN107 (AMN; 2.5 μg/mL) on (**A**) TGF-β_1 (10 ng/mL)– and (**B**) PDGF-BB (50 ng/mL)–induced *HAS* expression in orbital fibroblasts. mRNA levels of *HAS1*, *-2* and *-3* were determined by RQ-PCR and normalized to the expression levels of abelson. The *first row* depicts *HAS1* mRNA levels, the *second row* depicts *HAS2* mRNA levels, and the *third row HAS3* mRNA levels in GO and control orbital fibroblasts. Each data point represents an orbital fibroblast strain obtained from a single individual. Data are presented as *x*-fold induction relative to the unstimulated control. *Horizontal bars*: mean values. Data were analyzed with Student's *t*-test. $^*P < 0.001$, $^{**}P < 0.05$.

Effect of Imatinib Mesylate and AMN107 on PDGF-BB–Induced *HAS1* and *-2* Expression and Hyaluronan Production

Imatinib mesylate reduced PDGF-BB–induced expression of *HAS1* and *-2* in GO orbital fibroblasts up to basal expression levels ($P < 0.05$; Fig. 4). AMN107 reduced PDGF-BB–induced *HAS1* expression in GO orbital fibroblasts by approximately threefold ($P < 0.05$) and *HAS2* expression up to basal expression levels ($P < 0.05$; Fig. 4). Both imatinib mesylate and AMN107 inhibited the PDGF-BB–induced *HAS2* expression in control orbital fibroblasts (Fig. 4).

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

Effect of Imatinib Mesylate and AMN107 on TGF-β_1–Induced *HAS1* Expression and Hyaluronan Production and of TGF-β_1 on c-Abl Kinase Activity in Orbital Fibroblasts

Although TGF-β_1 clearly upregulated *HAS1* expression in orbital fibroblasts and although it also stimulated hyaluronan production by both GO (~4-fold induction; Fig. 5) and control (~14-fold induction; Fig. 5) orbital fibroblasts, imatinib mesylate and AMN107 neither affected the TGF-β_1–induced increase of *HAS1* expression (Fig. 4) nor reduced the TGF-β_1–induced hyaluronan production (Fig. 5). It must be noted that TGF-β_1–induced *HAS1* expression was blocked by the selective TGF-β_1 receptor I kinase inhibitor SB431542 in a dose-

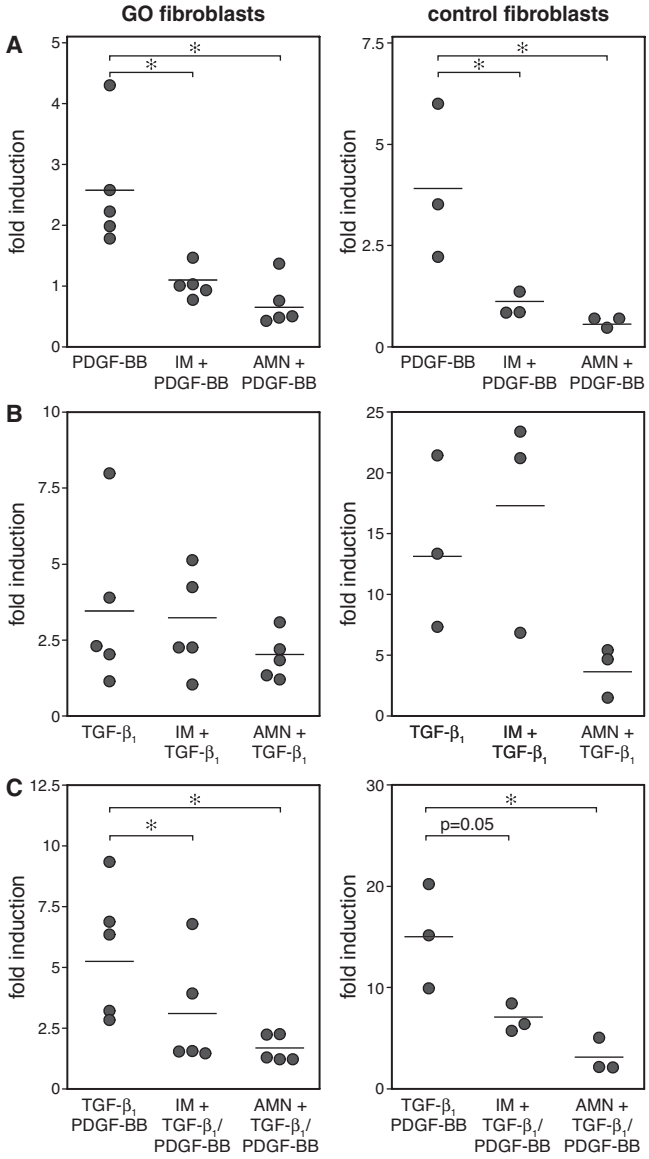

FIGURE 5. The effect of imatinib mesylate (IM; 2.5 μg/mL) and AMN107 (AMN; 2.5 μg/mL) on (**A**) PDGF-BB (50 ng/mL)–, (**B**) TGF-β_1 (10 ng/mL)–, and (**C**) PDGF-BB/TGF-β_1–induced hyaluronan production by orbital fibroblasts. Hyaluronan levels in culture supernatants were determined by ELISA. The *first row* depicts hyaluronan production in GO and control fibroblasts after PDGF-BB stimulation, the *second row* after TGF-β_1 stimulation and the *third row* after stimulation with both PDGF-BB and TGF-β_1. Each data point represents an orbital fibroblast strain obtained from a single individual. Data are presented as *x*-fold induction relative to the unstimulated control. *Horizontal bars*: mean values. Data were analyzed with Student's *t*-test. $^*P < 0.05$.

dependent manner (data not shown), showing the system's ability to respond. Also imatinib mesylate and AMN107 reduced the hyaluronan production in orbital fibroblasts after costimulation by PDGF-BB and TGF-β_1.

To examine this inability of the tyrosine kinase inhibitors regarding TGF-β_1 effects in orbital fibroblasts further, we studied the effects of TGF-β_1 stimulation on c-Abl kinase activity. It appeared that TGF-β_1 stimulation did not increase c-Abl kinase activity in orbital fibroblasts but was associated with a decrease in total c-Abl protein, in contrast to the effect of TGF-β_1 on lung fibroblasts, where it induced c-Abl kinase activity without

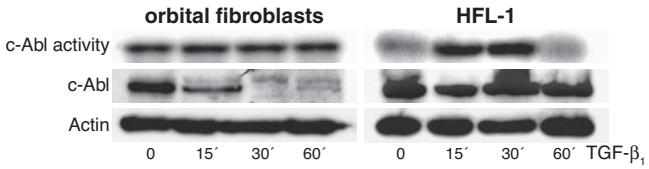

FIGURE 6. Orbital fibroblasts (*left*) and HFL-1 cells (*right*) were stimulated with TGF-β_1 (10 ng/mL) for indicated times. *Top row:* after c-Abl immunoprecipitation, c-Abl kinase activity was determined by using GST-CRK as a substrate. *Remaining rows:* Western blot analysis was performed with antibodies against c-Abl and β-actin on an aliquot before immunoprecipitation. Data are from a representative experiment.

affecting total c-Abl levels (Fig. 6). TGF-β_1 did induce Smad3 phosphorylation in orbital fibroblasts (Fig. 7A), and the induction was not inhibited by imatinib mesylate and AMN107 (Fig. 7B). These data indicate that in orbital fibroblasts the Smad pathway is activated by TGF-β_1, whereas the c-Abl pathway is not.

DISCUSSION

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

PDGF-BB, a homodimeric protein consisting of two PDGF-B chains, is regarded as one of the most potent mitogens for fibroblasts and increased PDGF-BB production plays a key role in fibrosis of a variety of organ systems.[9] We report, for the first time to our knowledge, increased levels of *PDGF-B* mRNA in orbital tissues from patients with GO. In line with previous

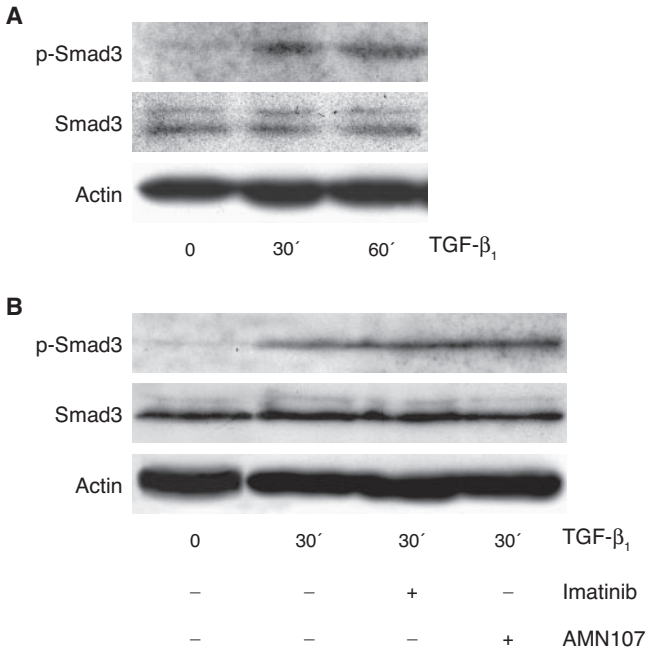

FIGURE 7. Orbital fibroblasts were stimulated with TGF-β_1 (10 ng/mL) for the indicated times (**A**). Western blot analysis was performed on lysates with antibodies against phosphorylated-Smad3, Smad3, and β-actin. Data depicted are from a representative experiment. Orbital fibroblasts were stimulated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

observations,[15] we also demonstrate that PDGF-BB is capable of inducing proliferation of orbital fibroblasts. We also found elevated levels of *TGF-B1* mRNA in GO orbital tissue. However, in our study, TGF-β_1 did not induce orbital fibroblast proliferation, which is opposed to the study by Heufelder and Bahn,[15] who found TGF-β_1 to be mitogenic for GO orbital fibroblasts. Unfortunately, we were unable to examine the actual orbital PDGF-BB and TGF-β_1 protein levels because of a lack of tissue. However, several studies in fibrotic diseases showed clear correlations between PDGF-B and TGF-β_1 mRNA and protein levels.[27–30] PDGF-B and TGF-β_1 mRNA levels did not correlate with disease activity. Although the reason and underlying mechanism for this are unclear so far, the data suggest that PDGF-BB and TGF-β_1 are involved in both active and inactive GO.

Next to proliferation, excessive hyaluronan production by orbital fibroblasts plays an important role in GO.[1,5] Both TGF-β_1 and PDGF-BB are powerful stimulators of hyaluronan production by fibroblasts from a variety of organs and diseases characterized by tissue remodelling.[16,31,32] In our study, PDGF-BB enhanced *HAS1* and *-2* mRNA expression in GO orbital fibroblasts, whereas TGF-β_1 increased *HAS1* mRNA expression, in contrast with previous reports demonstrating that PDGF-BB does not induce *HAS* expression in orbital fibroblasts.[33] We take this discrepancy to be due to differences in methodologic approaches and consider the PDGF-BB–induced *HAS* expression to be genuine, since we found it to co-exist with an increased hyaluronan production. In sum, our combined findings of increased orbital expression of *PDGF-B* and *TGF-B1* mRNA and in vitro stimulation data strengthen the case for the involvement of PDGF-BB and TGF-β_1 in the increased hyaluronan synthesis in GO.

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

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

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

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

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

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