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Platelet-derived growth factor-D modulates extracellular matrix homeostasis and remodeling through TIMP-1 induction and attenuation of MMP-2 and MMP-9 gelatinase activities

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ABSTRACT

Platelet-derived growth factor-D (PDGF-D) is a more recent recognized growth factor involved in the regulation of several cellular processes, including cell proliferation, transformation, invasion, and angiogenesis by binding to and activating its cognate receptor PDGFR-β. After bile duct ligation or in the carbon tetrachloride-induced hepatic fibrosis model, PDGF-D showed upregulation comparable to PDGF-B. Moreover, adenoviral PDGF-D gene transfer induced hepatic stellate cell proliferation and liver fibrosis. We here investigated the molecular mechanism of PDGF-D involvement in liver fibrogenesis. Therefore, the GRX mouse cell line was stimulated with PDGF-D and evaluated for fibrotic markers and PDGF-D signaling pathways in comparison to the other PDGF isoforms. We found that PDGF-D failed to enhance Col I and α-smooth muscle actin (α-SMA) production but has capacity to upregulate expression of the tissue inhibitor of metalloprotease 1 (TIMP-1) resulting in attenuation of MMP-2 and MMP-9 gelatinase activity as indicated by gelatinase zymography. This phenomenon was restored through application of a PDGF-D neutralizing antibody. Unexpectedly, PDGF-D incubation decreased both PDGFR-α and -β in mRNA and protein levels, and PDGF-D phosphorylated tyrosines specific for PDGFR-α and -β. We conclude that PDGF-D intensifies fibrogenesis by interfering with the fibrolytic activity of the TIMP-1/MMP system and that PDGF-D signaling is mediated through both PDGF-α and -β receptors.

1. Introduction

Platelet-derived growth factor (PDGF) belongs to a family of growth factors consisting of four secreted extracellular ligands encoded by four different genes and assembled into disulfide-bonded dimers via homo- or heterodimerization [1]. The PDGF ligands exert their biological effects through two structurally related tyrosine kinase receptors, the PDGFR-α and PDGFR-β. Ligand/receptor interactions *in vivo* are PDGF-AA and PDGF-CC which induce PDGFR-α dimerization while PDGF-BB and PDGF-DD induce PDGFR-β dimerization. Other interactions between PDGF ligands and receptors are demonstrated by cell culture experiments, whereas *in vivo* evidence is currently still lacking [2]. PDGF is a potent mitogen, chemo-attractant and survival factor for mesenchymal cells [3]. It plays a critical role in physiological repair mechanisms and in the pathogenesis of various proliferative diseases. It aggravates more severe pathological conditions such as fibrosis, atherosclerosis, cancer and others [4]. Over-activity of PDGF has been implicated in the development of fibrosis in various organ systems including liver fibrosis which is associated with induction of the PDGFR-β [5]. PDGF-B and -D have been shown to intensify liver fibrosis due to high affinity binding with PDGFR-β [6–11].

PDGF-D, a newly discovered isoform is mainly expressed in the cardiovascular system and provides both autocrine and paracrine signaling through PDGFR-β [12,13]. Transgenic PDGF-D stimulates proliferation of cardiac interstitial fibroblasts and arterial vascular smooth muscle cells, resulting in cardiac fibrosis followed by dilated cardiomyopathy and subsequent cardiac failure [14]. In kidney, PDGF-D plays a significant role in the pathogenesis of tubulointerstitial injury through binding to PDGFR-β in both human obstructive

Abbreviations used: α-SMA, α-smooth muscle actin; Col I, collagen type I; MMP, metalloproteinase; PDGF, platelet-derived growth factor; TIMP-1, tissue inhibitor of metalloproteinase 1.

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nephropathy and the corresponding murine model of ureteral obstruction [15]. Systemic infection with an adenoviral vector expressing PDGF-D induced prominent mesangioproliferative nephritis in mice, whereas antagonistic PDGF-D in a rat model of mesangioproliferative disease ameliorated renal changes [16]. This phenomenon was confirmed by podocyte-specific overexpression of PDGF-D causing mesangioproliferative disease, glomerulosclerosis, and crescentic glomerulonephritis [17].

Besides the kidney, also other organs are affected by circulating PDGF proteins. In mice receiving PDGF-B and PDGF-D adenoviruses significant histopathologically changes were observed in the liver, bone and lung, including hepatic stellate cell proliferation and liver fibrosis [16]. In a bile duct ligation model (BDL), PDGF-D was upregulated comparable to the level of PDGF-B and localized around the periportal and perisinusoidal areas of BDL livers in close correlation with periportal myofibroblasts and HSC. Actual localization of PDGF-D was identical to that of PDGF-B and PDGFR-β [11]. These findings seem to suggest that inactivation of PDGF-D/PDGFR signaling has a significant impact in liver fibrotic therapy.

We therefore investigated whether specific PDGF antagonists directed against PDGF-D are able to mitigate liver fibrosis. We analyzed the molecular mechanism of PDGF-D involvement in liver fibrogenesis using the murine GRX hepatic stellate cell line and found that PDGF-D is not capable to enhance Col I and α-SMA production but instead is able to significantly upregulate TIMP-1 expression in mRNA and protein levels, resulting in attenuation of MMP-2 and MMP-9 gelatinase activity as indicated by gelatinase zymography. This phenomenon was restored by application of a PDGF-D-specific neutralizing antibody. PDGF-D stimulation further did phosphorylate tyrosine specific for PDGFR-α and -β resulting in down regulated both PDGFR-α and -β, indicating that PDGF-D signals through both PDGFRs.

2. Material and methods

2.1. Cell culture and stimulation

The continuous GRX mouse cell line was obtained from livers of C3H/HeN mice infected by transcutaneous penetration of cercarias from the *Schistosoma mansoni* Belo Horizonte (BH) strain [18]. This cell line was obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, Brazil). Since its cellular morphology, vitamin A production, and matrix production is similar to that of hepatic stellate cells, we applied the GRX cell line to further explore the PDGF-D signaling biology. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. Cells were serum-starved for 24 h and stimulated with given doses of recombinant rat PDGF-A, -B, human PDGF-C or human PDGF-D (all from R&D Systems, Wiesbaden, Germany) for 10 min to evaluate the signaling pathways. For fibrotic marker proteins and gelatinase zymography cells were incubated for 24, 48 and 72 h. The sources and concentrations of growth factors, inhibitors and neutralizing antibodies that were used in the stimulation experiments are listed in Suppl. Table 1.

2.2. RNA isolation, cDNA synthesis, and qRT-PCR

GRX cell total RNA was isolated through QIAzol lysis reagent and RNeasy Mini kits (Qiagen, Hilden, Germany) according to manufacturer's instructions, followed by DNAse digestion and subsequent RNeasy clean up. Primers for amplification were selected from sequences deposited in the GenBank database (Suppl. Table 2) using the online ProbeFinder Software (Universal Probe Library Assay Design Center, Roche, Mannheim, Germany). First-strand cDNA was synthesized from 1 μg RNA in 20 μl volume using SuperScript™ II reverse transcriptase and random hexamer primers (Invitrogen, Life Technologies, Darmstadt, Germany). For quantitative real-time PCR, cDNA derived from 25 ng RNA was amplified in 25 μl volume using SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen). PCR conditions were 50 °C for 2 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All primers are given in Suppl. Table 2.

2.3. SDS-PAGE and Western blot analysis

Cell lysates were prepared using RIPA buffer containing 20 mM Tris–HCl (pH 7.2), 150 mM NaCl, 2% (w/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and the Complete™-mixture of proteinase inhibitors (Roche). Equal amounts of cellular protein extracts or supernatants were diluted with Nu-PAGE™ LDS electrophoresis sample buffer with DTT as reducing agent, heated at 95 °C for 10 min, and separated in 4–12% Bis-Tris gradient gels, using MOPS or MES running buffer (Invitrogen). Proteins were electroblotted onto nitrocellulose membranes, and equal loading was shown in Ponceau S stain. Subsequently, non-specific binding sites were blocked in TBS containing 5% (w/v) non-fat milk powder. All antibodies (Suppl. Table 3) were diluted in 2.5% (w/v) non-fat milk powder in Tris-buffered saline. Primary antibodies were visualized using horseradish peroxidase conjugated anti-mouse-, anti-rabbit- or anti-goat IgG (Santa Cruz Biotech, Santa Cruz, CA) and SuperSignal chemiluminescent substrate (Pierce, Bonn, Germany).

2.4. Gelatinase zymography

Cultured media from GRX cells stimulated with PDGF isoforms were analyzed for gelatin degradation activity through electrophoresis under non-reducing condition in 10% Tris-Glycine gelatin gel (Invitrogen). Gel was incubated in 1× zymogram renaturing buffer (Invitrogen) containing 2.5% (v/v) Triton® X-100 for 30 min at room temperature with gentle agitation, equilibrated 30 min at RT in 1× zymogram developing buffer (Invitrogen) containing 50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) Brij 35, followed by overnight incubation with fresh developing buffer at 37 °C. The zymogram gel was washed 3× with deionized water and stained with the SimplyBlue Safestain (Invitrogen) for 1 h at room temperature with gentle shaking and washed with deionized water for 1–3 h. The zymogram gel was scanned and the band intensities measured.

3. Results

3.1. PDGF-B and -D decrease collagen type I expression in GRX mouse cell line

GRX cells were incubated with PDGF isoforms A, B, C or D for 24, 48 and 72 h. PDGF-B and -D both showed markedly reduced Col I levels, while the levels of α-SMA was unaffected. PDGFR-β levels furthermore showed significant downregulation from PDGF-B and -D due to receptor binding following endocytosis and ubiquitination. Exceptionally, PDGF-D as the specific ligand for PDGFR-β in fact did downregulate PDGFR-α to the same levels as PDGF-B (Fig. 1A). In cultured media, TIMP-1 was upregulated by all PDGF isoforms, with 10% FCS showing the highest levels. PDGF-B and -D isoforms induced TIMP-1 expression much stronger than PDGF-A and -C. By contrast to TIMP-1 expression, the levels of MMP-2 was downregulation by PDGF-B and -D and 10% FCS as compared to 0.5% FCS, PDGF-A and -C (Fig. 1A). Col I and fibronectin did maintain their low levels upon PDGF-B and -D stimulation. Given foregoing results, we went on to investigate the mRNA levels of the

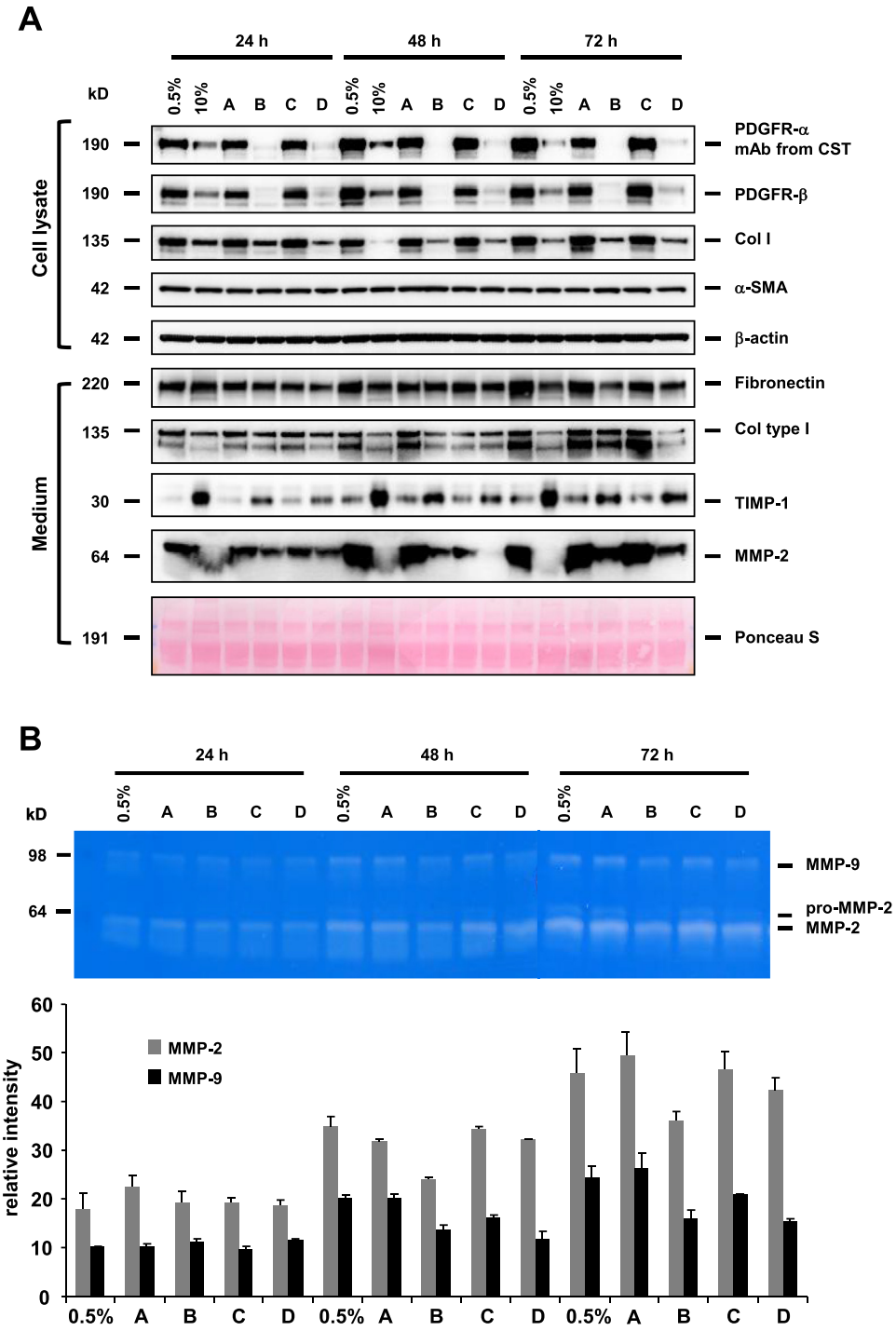

Fig. 1. PDGF-B and -D decrease collagen type I expression. (A) GRX cells were incubated in medium containing 0.5% FCS with the different PDGF isoforms (A, B, C or D) for 24, 48 and 72 h. Cells that received no PDGF served as a control. A Western blot analysis of cell lysate and culture media was performed and the expression of PDGFR-α, PDGFR-β, collagen type I (Col I) and α-SMA, Fibronectin, TIMP-1 and MMP-2 analyzed. β-actin and Ponceau S stain served as loading controls. The antibodies used in this set of experiments are listed in Suppl. Table 3. (B) Zymography of culture media showing MMP-2 and MMP-9 gelatinase activity (*upper panel*) with densitometric quantification (*lower panel*).

subject proteins and found PDGF-B and -D to decrease Col I, α-SMA, PDGFR-α and -β, MMP-2, MMP-9 and MMP-14 mRNA, but to increase TIMP-1 production, while TIMP-2 and TIMP-3 decreased significantly, thus confirming the findings in the protein levels (Suppl. Figs. 1 and 2). This PDGF-B and PDGF-D-induced alterations in TIMP and MMP gene expression also induced a decrease of MMP-9 and MMP-2 activities that became visible in zymography after 48 h (MMP-9) or 72 h (MMP-2) (Fig. 1B).

3.2. PDGF isoform signaling in mouse GRX cell line and efficacy of PDGF-D neutralizing Ab (MAB1159) in specific inhibition of PDGF-D signaling

In the next set of experiments, GRX cells expressing both relevant receptors PDGFR-α and -β served as a model for analyzing PDGF signaling. PDGF-B and PDGF-D proved the most significant activators of PDGFRs as evidenced by the strongest signal in

phospho-Tyr (p-Tyr or pY) specific for both PDGFR-α and -β including p-Crk, the specific adaptor protein that associates to activated PDGFR-α (Fig. 2A). PDGF-B and -D prominently activated the downstream signaling molecules such as p-PLCγ, p-Akt/PKB of PI3K, p-JNK, p-ERK1/2, and p-p38 of MAPK pathways (Fig. 2B). All PDGF isoforms that activate MAPK did also enhance linker Smad1 and linker Smad2 phosphorylation (Fig. 2C). We further demonstrated that the mouse monoclonal neutralizing antibody MAB1159 is highly effective in inhibition of PDGF-D activities (Fig. 2D) and specifically inhibits only PDGF-D signaling as evidenced by GRX cells stimulation with different PDGF isoform in the presence of MAB1159. The signaling of other PDGF isoforms was not interfered by MAB1159 (Fig. 2E).

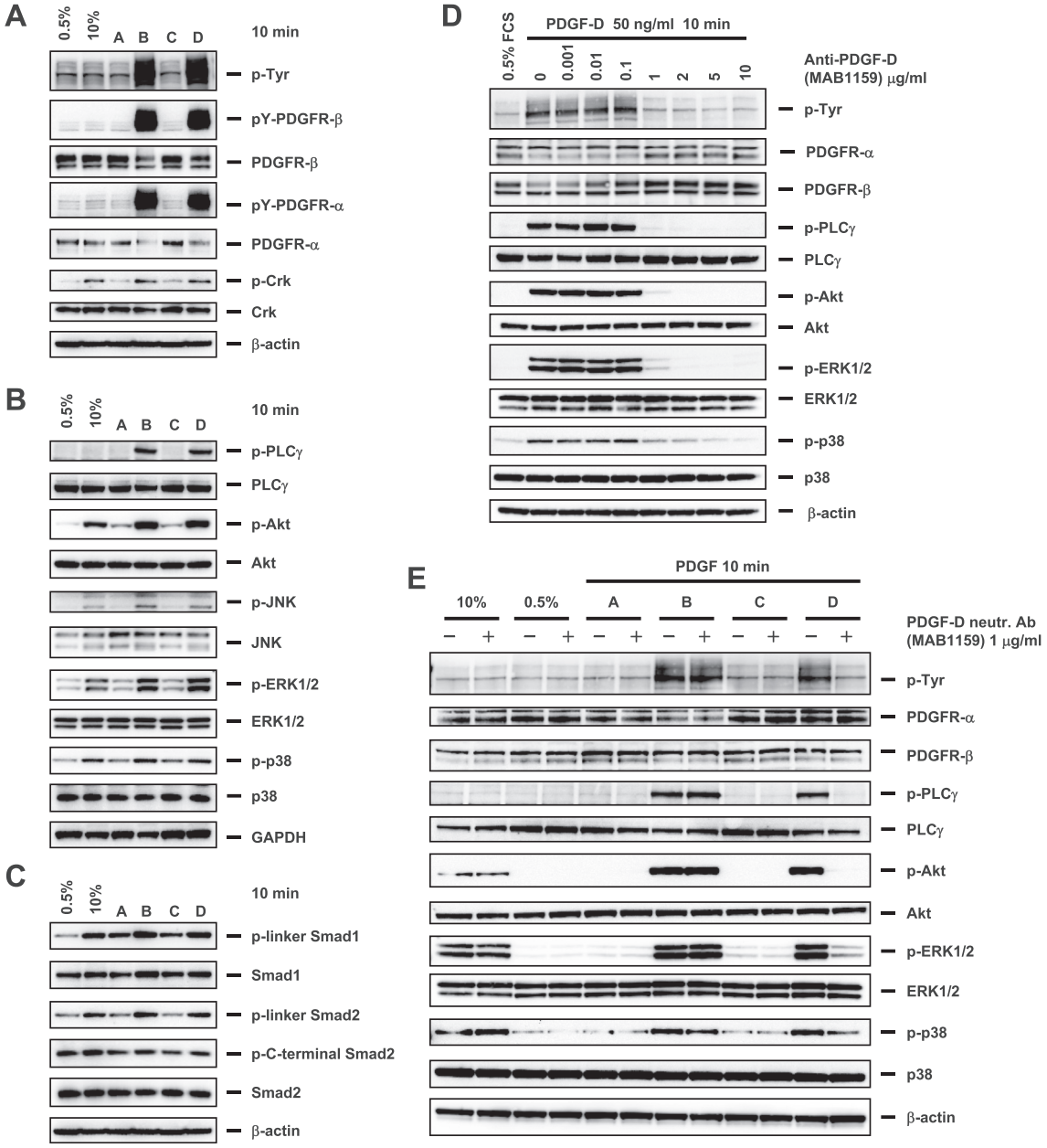

Fig. 2. PDGF isoform signaling in GRX mouse cell line and effects of PDGF-D neutralizing Ab (MAB1159) in inhibition of PDGF-D signaling. (A) GRX cells were stimulated with the different PDGF isoforms for 10 min. The phosphorylation of tyrosine and the tyrosine specific for PDGFR-β was analyzed by Western blot analysis. Strongest positivity was found in cells that were stimulated with PDGF-B or -D, corresponding to the decrease of PDGFR-β quantities. The phosphotyrosine specific for PDGFR-α were also significantly upregulated after PDGF-B and -D stimulation. Simultaneously, CrkII phosphorylation increased and PDGFR-α was down regulated. Probing with a β-actin specific antibody served as control in this set of experiment. (B) Downstream PDGFR signaling molecules were detected amongst which p-PLCγ, p-Akt PI3K, p-JNK, pERK1/2, and p-p38 MAPK, with GAPDH as loading control. (C) Linker Smad1 and Smad2 phosphorylation corresponding to MAPK activation are shown with β-actin as loading controls (D) Mouse monoclonal PDGF-D neutralizing antibody (MAB1159) upwards from 1 μg/ml showed effective inhibition of PDGF-D activities as evidenced by the blocking of PDGF-D-induced phosphotyrosine of PDGFRs, PLCγ, Akt of PI3K, JNK, ERK1/2, and p38 of MAPK phosphorylation. (E) MAB1159 specifically inhibits only PDGF-D signaling as indicated by GRX cell stimulation with different PDGF isoforms in the presence of MAB1159. While PDGF-D stimulation signals of pTyr, p-PLCγ, p-Akt PI3K, p-JNK, pERK1/2, and p-p38 MAPK were inhibited by this antibody, the signaling of other PDGF isoforms was not affected by MAB1159. β-actin served as a loading control. The antibodies used in this set of experiments are listed in Suppl. Table 3.

3.3. Neutralizing antibody (MAB1159) inhibits PDGF-D-induced TIMP-1 expression and restores MMP activity

We next applied the neutralizing antibody MAB1159 that is specific for PDGF-D. When this antibody was applied together with PDGF-D during stimulation, we observed that MAB1159 significantly inhibits PDGF-D-induced TIMP-1 production (Fig. 3A). The treatment with this antibody was also sufficient to restore MMP-2 and MMP-9 gelatinase activities in zymography (Fig. 3B).

3.4. PDGF-D induces TIMP-1 production through ERK and p38 MAPK

GRX cells were next incubated with PDGF-D in the presence of different signaling pathway inhibitors, including the recombinant soluble PDGFR-β-Fc chimera (sPDGFR-β-Fc) [9,19]. The sPDGFR-β-Fc, PD98059 MEK inhibitor, SB20358 p38 inhibitor and SB431542 ALK5 inhibitor attenuated PDGF-D-induced TIMP-1 expression as shown in both cell lysate and cultured media (Fig. 3C).

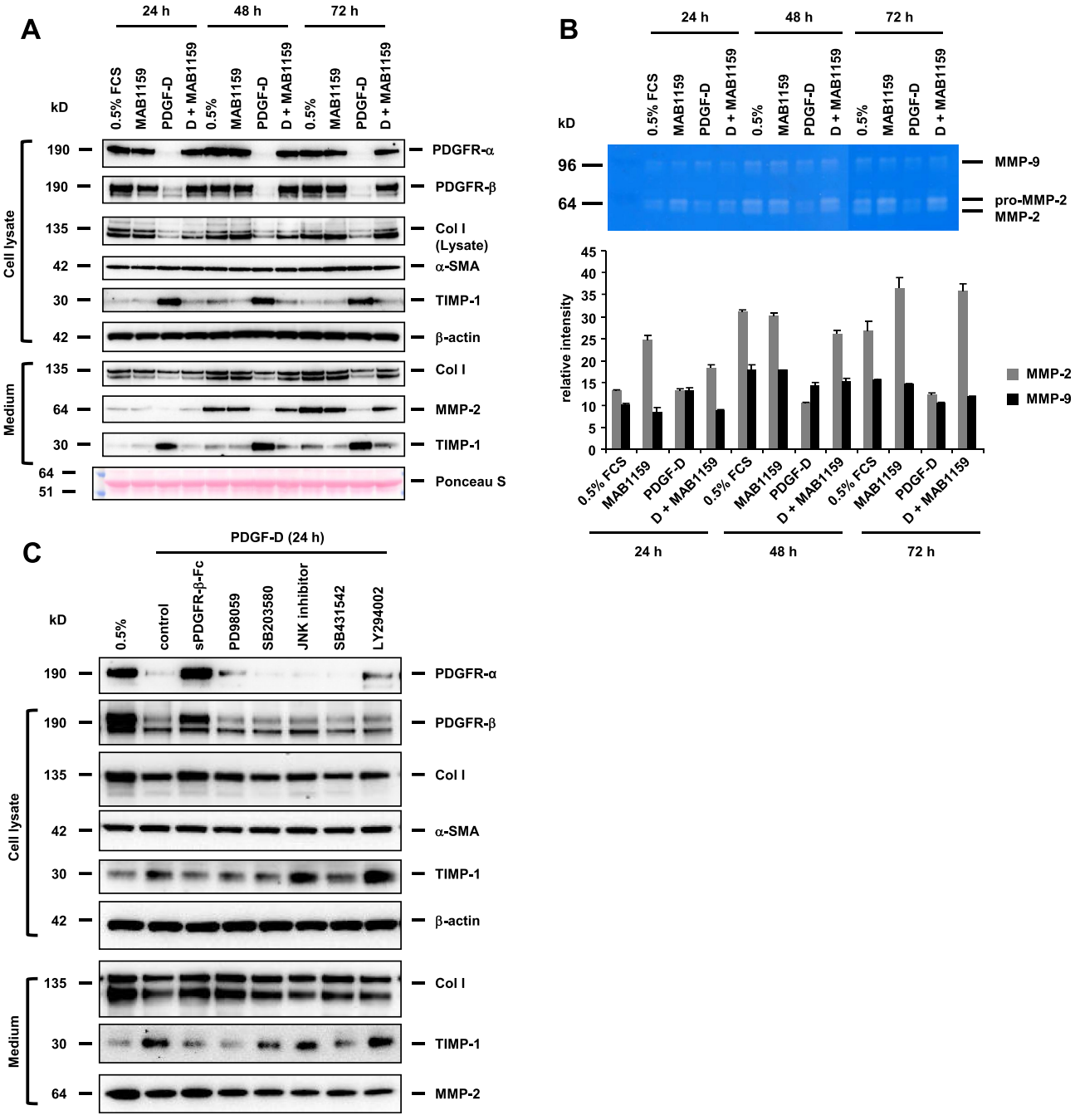

Fig. 3. PDGF-D neutralizing Ab (MAB1159) inhibits PDGF-D induced TIMP-1 expression and restores MMP activity. (A) GRK cells were incubated with MAB1159 1 h before PDGF-D stimulation. Western blot of cell lysate and culture media for expression of PDGFR-α and -β, Col I, α-SMA, TIMP-1 and MMP-2 was performed. β-actin served as a loading control. (B) Zymography of the culture media showing MAB1159 to restore MMP-2 and MMP-9 gelatinase activities. (C) PDGF-D induces TIMP-1 production through ERK and p38 MAPK. In this experiment, GRX cells were incubated with soluble PDGFR-β-Fc and indicated signaling pathway inhibitors for 1 h followed by PDGF-D stimulation for 24 h. Subsequently, Western blot analysis of cell lysate and culture media was performed showing that the sPDGFR-β-Fc, PD98059, SB20580 and SB431542 attenuated TIMP-1 expression. PDGFR-α and -β, Col I and MMP-2 were down regulated by PDGF-D except after sPDGFR-β-Fc pre-incubation, while α-SMA remained unchanged. β-actin served as loading control for equal protein loading. The antibodies and inhibitors used in this set of experiments are listed in Suppl. Tables 3 and 2.

4. Discussion

PDGFs play an important role in normal embryonic development but their abnormal expression also contributes to a variety of diseases. PDGFs and their receptors are currently under investigation as targets in numerous proliferative disorders, including cancer, cardiovascular and fibrotic diseases. We employed the GRX mouse cell line as a model to investigate PDGF signaling with strong emphasis on the molecular mechanism of PDGF-D in liver fibrogenesis and found PDGF-B and -D to reduce Col I production. This finding coincides with the reduction of both PDGFR-α and -β levels due to ligand binding, signaling, endocytosis and ubiquitination. Moreover, the production of both receptors was attenuated due to physiological feedback mechanisms to prevent over-activity of the growth factors. Under normal physiological conditions, PDGF signaling is controlled by a balancing feedback mechanism which derives at its ultimate regulatory responses by equilibrating stimulatory and inhibitory signals that arise in parallel [20]. Ligand binding of PDGFR promotes receptor internalization endocytosis and lysosomal degradation, thereby limiting the duration of PDGFR signaling [21–23]. Col I reduction was very significant upon PDGF-B and −D incubation compared to primary hepatic stellate cells that despite their scant collagen production showed significantly increased Col I accumulation upon long-term PDGF-B and -D incubation [11]. One possible cause for this observation might be intracellular collagen degradation that consists of up to 60% of newly synthesized collagen in GRX cells and higher numbers of PDGF-induced mitotic cells that produce low amounts of collagen [24].

PDGF-D signaling in GRX cells is similar to that of PDGF-B as shown by a strong induction of phosphotyrosine, conceivably specific for PDGFR-β (Fig. 2A). PDGF-B and -D were also strong stimulators of downstream PDGFR signaling pathways such as p-PLCγ, p-Akt of PI3K, p-ERK and p-p38 of MAPK. Beside the reduction of PDGFR-β, we surprisingly found that PDGFR-α was also down-regulated with 10% FCS in the presence of PDGF-B and −D, while no changes were induced by PDGF-A or -C. This finding indicates PDGF-B and -D to signal via both PDGFR-α and -β as confirmed by elevated levels of p-Crk, the PDGFR-α specific adaptor protein that is phosphorylated through PDGR-α activation [25,26]. The ligand/receptor interaction, most likely PDGF-B and -D, binds to the PDGFR-αβ heterodimer since GRX cells possess a high proportion of PDGFR-β compared to PDGFR-α as evidenced by qRT-PCR (Suppl. Fig. 1). PDGF-B and -D signaling through PDGFR-α/β heterodimer results in stronger endocytosis of PDGFR-α in GRX cells compared to that induced by PDGF-A and -C, the PDGFR-α specific ligands that bind PDGFR-α/α homodimer.

PDGF-D showed prominent stimulation of PDGFR and PDGF-D/PDGFR signaling, thus having a significant impact in liver fibrosis *in vivo* [11,16]. In our search for PDGF-D antagonists we found PDGF-D neutralizing antibody (MAB1159) to be effective and specific for PDGF-D (Fig. 2D and E). MAB1159 did inhibit PDGF-D-induced TIMP-1 expression and restored MMP-2 production and gelatinase activity of MMP-2 and MMP-9 (Fig. 3A and B), indicating that PDGF-D regulates the extracellular matrix components essential for tissue homeostasis and remodeling.

We next investigated the mechanisms of PDGF-B and -D induced TIMP-1 production and found that PD98059 and SB203580, the ERK and p38 of MAPK inhibitors attenuate PDGF-D-induced TIMP-1 production. Additionally, the TGF-β type I receptor (ALK5) inhibitor SB431542, did down regulated PDGF-D-induced TIMP-1 expression in both cell lysate and cultured media (Fig. 3C). This seems to point at ALK5-induced TIMP-1 production downstream of MAPK. We could further demonstrate that PDGF activation of MAPK correlates well with PDGF-induced linker Smad1 and Smad2 phosphorylation (Fig. 2C). The linker Smad phosphorylation from MAPK inhibits Smad nuclear translocation or prevents accumulation of Smad2 and Smad3 in the nucleus and inhibits TGF-β signaling in order to block mitotic inhibitory effects of TGF-β during PDGF-induced cellular mitosis and proliferation [27,28].

In summary, our data indicate that PDGF-D modulates extracellular matrix homeostasis and remodeling by influencing the fibrolytic activity of the TIMP-1/MMP system by stimulating both PDGF-α and -β receptors.

Conflict of interest

The authors have nothing to declare.

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Appendix. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2014.12.106.

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