Dominant-negative soluble PDGF-β receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis

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Hepatic fibrogenesis is a consequence of hepatic stellate cells that become activated and transdifferentiate into a myofibroblastic phenotype with the ability to proliferate and synthesize large quantities of extracellular matrix components. In this process, platelet-derived growth factor (PDGF) is the most potent stimulus for hepatic stellate cell proliferation and migration, and is overexpressed during active hepatic fibrogenesis. This cytokine binds to the PDGF receptor type β, activates Ras and sequentially propagates the stimulatory signal sequentially via phosphorylation of Raf-1, MEK and the extracellular-signal regulated kinases ERK1/ERK2. Hepatic injury is associated with both increased autocrine PDGF signaling and upregulation of PDGF receptor. In this study, we report that a dominant-negative soluble PDGF-β receptor consisting of a chimeric IgG containing the extracellular portion of the PDGF receptor type β blocks HSC activation and attenuates fibrogenesis induced by ligation of the common bile duct in rats. In culture-activated hepatic stellate cells, the soluble receptor blocks phosphorylation of endogenous PDGF receptor, phosphorylation of the ERK1/EKR2 signal and reduces proliferative activities of HSC. *In vivo*, both the delivery of the purified soluble PDGF antagonist and the administration of adenoviruses expressing the artificial transgene were able to reduce significantly the expression of collagen and α-smooth muscle actin. Our results demonstrate that PDGF plays a critical role in the progression and initiation of experimental liver fibrogenesis, and suggest that early anti-PDGF intervention should have a therapeutic impact on the treatment of liver fibrogenesis.
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Hepatic fibrosis is a proliferative disease that may be initiated by a variety of factors including chronic viral hepatitis, alcoholic abuse, drug-induced lesions, heritable metabolic alterations, or exceptional functional disorders. Following liver injury of any etiology, hepatic stellate cells (HSC) become activated and transdifferentiate into proliferative, fibrogenic, and contractile myofibroblasts (MFB). The perpetuation of the activated phenotype involves key phenotypic responses mediated by increased cytokine effects and remodeling of extracelluar matrix (ECM).[1,2] Enhanced cytokine responses occur through multiple mechanisms; among these, increased expression of cell membrane receptors and enhanced signaling are essential. In particular,

receptor tyrosine kinases, for example, receptors for platelet-derived growth factor (PDGF), which mediate many of the stellate cell's responses to cytokines, are broadly upregulated during liver injury.[3] PDGFs form a family of dimeric isoforms inducing growth stimulation, chemotaxis, cell shape changes, and intracellular signaling in HSC from mouse, rat, or humans.[3] The PDGF receptor type α (PDGFRα) is constitutively expressed in quiescent HSC, whereas PDGF receptor type β (PDGFRβ) is acquired as the cells undergo myofibroblastic phenotypic changes.[4] Moreover, in liver tissue obtained from patients with chronic liver diseases, the expression of PDGF and its receptor subunits appears closely correlated to the extent of necroinflammation and fibrosis.[5] The cellular effects of PDGF isoforms are exerted through binding to two structurally related tyrosine kinase receptors. Ligand binding induces receptor dimerization and phosphorylation of tyrosine residues within the cytoplasmic domain, enabling a number of src-homology 2 (SH2) or phosphotyrosine-binding

(PTB) domains to bind to the phosphorylated receptors. This induces recruitment and activation of receptor-associated proteins following sequential activation of Ras, Raf-1, MEK and in turn the extracellular-signal regulated kinases ERK1 and ERK2 (also referred to as p44 and p42), thereby initiating various signaling pathways.[6–8] Subsequently, ERKs translocate into the nucleus and phosphorylate several transcription factors triggering a proliferative response.[9] In both cultured HSC and HSC of injured liver, there is a strong activation of the ERK pathway.[10,11] Increasing evidence indicates that the enhanced activity of PDGFR plays a pivotal role in liver fibrosis. Importantly, codistribution of PDGF with cells expressing its receptors has been clearly demonstrated after both acute and chronic liver tissue damage,[5,12] thus confirming an active role of this growth factor in the liver tissue repair process and in the development of hepatic fibrosis. Therefore, targeting the PDGF system by drugs that inhibit the tyrosine kinase receptor, or its intracellular signal transduction pathway, is attractive for therapeutic intervention in hepatic fibrosis. Some of these components were shown to prevent cellular activation and are beneficial in experimental models of liver fibrosis.[10,13–15] However, most of these agents will likely not be suitable for therapeutic applications in humans, because most of the main signaling pathways involved in PDGF-induced biological effects are common to other growth factors possibly acting with positive effects on other cell types within the same tissue.[3] Therefore, direct scavenging of PDGF or application of methods allowing targeting to HSC undergoing cellular activation and subsequent transdifferentiation would offer a strategy for the treatment of fibrotic liver diseases. Inspired by the overwhelming literature describing the highly beneficial impact of local or systemic expression of a dominant-negative TGF-β receptor (TβRII) resulting in the reduction of liver fibrogenesis, improved liver function, and inhibition of cellular activation,[16–18] we examined the effects of a dominant-negative soluble PDGFRβ (sPDGFRβ) on hepatic fibrogenesis in culture-activated HSC and in a biliary obstruction model induced by ligature of the common bile duct (BD) in rats.

Materials and methods

Cell Isolation and Culture

HSC were isolated from male Sprague–Dawley rats by the pronase–collagenase method followed by a density centrifugation on a Nycodenz gradient and cultured as described before.[19,20]

Construction of Ad5-CMV-EGFP, Ad5-CMV-sTβRII, and Ad5-CMV-sPDGFRβ

The construction of the recombinant replication-deficient reporter Ad5-CMV-EGFP expressing the enhanced green fluorescent protein (EGFP) was described previously.[21] For cloning of pΔE1sp1A-CMV-sTβRII, the NcoI fragment of the plasmid pIg-hTβRII [22] was blunted and cloned into the EcoRI site of pΔE1sp1A.[23] The extracellular domain (ECD) of rat PDGFRβ was isolated by RT–PCR from total rat MFB RNA using primers 5′-d(CAT GGG GCT TCC AGG AGT GAT)-3′ and 5′-d(GGG CAA GGA ATG TGG GAC CAC)-3′, corresponding to nt138–nt158 or complementary to nt1702–nt1722 of murine PDGFRβ mRNA (Access. No. NM_008809). The resulting cDNA fragment was cloned into pGEM-T Easy (Promega, Mannheim, Germany) generating vector pGEM-rPDGFRβ, followed by a second amplification using primer 5′-d(GAA TTC GGA CAC CAT GGG GCT TCC AGG AGT GAT)-3′ and 5′-d(GGA TCC ACT TAC CTG TCA GGG AAT GTG GGA CCA)-3′ introducing novel restriction sites for EcoRI and BamHI (underlined) and a downstream 3′-splice donor site (in bold). Subsequently, the ECD of PDGFRβ was incorporated into vector pIg harboring the Fc region of human IgG1, a CMV promoter, and SV40-polyadenylation signal.[22] Then, the ∼4.2 kbp-fragment containing the PDGFRβ:Fc fusion (sPDGFRβ), along with the CMV promoter and SV40-polyadenylation signal was transferred into the EcoRI and HindIII sites of adenoviral shuttle vector pΔE1sp1A. The integrity of all cloning boundaries was verified by sequencing, and the integration of the expression cassette from vector pΔE1sp1A-CMV-PDGFRβ into the adenoviral backbone vector pJM17[24] was achieved by in vitro homologous recombination as described.[21]

RNA Isolation and Northern Blot Analysis

Isolation, gel electrophoresis, and Northern blot analysis of RNA was carried out as described previously.[20,25] Hybridization probes were the 975-bp EcoRI fragment of pGEM-rColα1(I) (a kind gift from K Knoch), and the ∼1.6-kbp fragment of pGEM-rPDGFRβ, respectively. For internal standardization, the blots were rehybridized with a cDNA specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Polymerase Chain Reaction (PCR) for sPDGFRβ Expression Analysis

Total RNA (2 μg) isolated from liver samples was reverse transcribed in 20 μl using Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and random hexamer primers according to the manufacturer's instructions. Aliquots (2 μl) of the first strand cDNA samples were subjected to standard PCR using primer 5′-d(GCC TGC GTC ACG TGG ACC A)-3′, corresponding to the ECD of rat PDGFRβ (nt1383-1401; Acc. No. Z14119), and primer 5′-d(GGA AGA GGA AGA CTG ACG GT)-3′, specific for the Fc region of human IgG1. To verify

768

the integrity of each cDNA, a 251-bp fragment of *GAPDH* was amplified. Primers, according to Access. No. M32599, were 5′-d(TCG TGG ATC TGA CGT GCC GCC TG)-3′ and 5′-d(CAC CAC CCT GTT GCT GTA GCC GTA T)-3′.

SDS-PAGE and Immunoblotting

Whole cell or liver extracts were prepared as described before.[26–28] Equal amounts of proteins were resolved in NuPAGE™ Tris-Glycine gels (Novex, Groningen, The Netherlands) and electro-blotted onto a Protran membrane (Schleicher & Schuell, Dassel, Germany) according to standard procedures. The primary antibodies used were: α-SMA (clone asm-1 obtained from Roche, Mannheim, Germany or from Cymbos Biotech Ltd., Chandlers Ford, UK), PDGFRβ (sc-432, Santa Cruz Biotech, Santa Cruz, CA, USA), p-PDGFRβ (sc-12907, Santa Cruz), PDGFRβ (ECD) (Ab-1#PC17, Oncogene, San Diego, CA, USA), p44/p42 (#9102, Cell Signaling Technology, Inc., New England Biolabs GmbH, Frankfurt, Germany), and phospho-p44/42 (Thr202/Tyr204) (#9101, Cell Signaling Technology). Primary antibodies were visualized using horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz) and the supersignal chemiluminescent substrate (Pierce, Rockford, IL, USA).

ELISA for sPDGFRβ

For quantification of sPDGFRβ in rat serum, 96-well plates (C8 Maxisorp 445101, Nunc, Wiesbaden, Germany) were coated overnight at 4°C in 50mM NaHCO₃ (pH 9.1) containing 5 μg/ml rabbit anti-human IgG specific for the C_H2 domain (A 0089, DAKO, Hamburg, Germany). The plates were washed three times (0.05% Tween 20, 154 mM NaCl) and blocked with 200 μl 0.5% BSA (RIA grade, Sigma, Taufkirchen, Germany) in blocking buffer (50 mM Tris pH 7.7, 150 mM NaCl) at 4°C overnight. Upon washing, 100 μl diluted rat serum was applied to duplicate wells after being diluted two times with assay buffer (0.05% Tween 20 in blocking buffer). A standard curve was set using 100 μl of the purified sPDGFRβ (0–100 ng/ml) in 50% pooled-rat serum. The plates were incubated for 2 h at RT followed by washing and application of 100 μl of 0.5–1 μg/ml polyclonal goat anti-mouse PDGFRβ antibody (AF1042, R&D Systems, Wiesbaden, Germany) for 1 h at RT. After the next washing, 100 μl of 1:2000 diluted polyclonal horseradish peroxidase-conjugated donkey antigoat IgG antibody (sc-2056, Santa Cruz) was added, and further incubated for 1 h at RT. BM blue POD substrate solution containing 3,3′-5,5′-tetramethylbenzidine (Roche) was applied after washing and plates were incubated at RT. The reaction was interrupted after 10–30 min with 50 μl of 1 M H₂SO₄ and absorbance was measured in a Victor Multilabor microplate

reader (EG&G Wallac, Turku, Finland) at 450 nm against a reference wavelength of 690 nm.

Expression, Purification, and Sequencing of Soluble Receptors

COS-7 cells were infected with Ad5-CMV-sTβRII or Ad5-CMV-sPDGFβ in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. After 1 day, the medium was changed (0.5% FCS) and cells were cultured for additional 48 h. Thereafter, the supernatants were taken, cleared by a high-speed centrifugation, and proteins (sTβRII or sPDGFRβ) were isolated on a Hi Trap G column (Amersham Pharmacia Biotech, Freiburg, Germany) according to the instructions of the manufacturer. For protein sequencing purified samples were dialyzed extensively against deionized water and five (sPDGFRβ) or six (sTβRII) aminoterminal aminoacids were sequenced by Edman degradation at TOPLAB (Martinsried, Germany).

Adenoviral Expression Technology

Purification, infection of cultured liver cells, and administration of adenoviruses to animals were performed as described previously.[21,26–28]

Proliferation Assay

HSC were cultured in DMEM containing 10% FCS. On the 2nd day after seeding the serum content was reduced to 0.5% FCS for 24 h and cells were stimulated with 20 ng/ml PDGF-BB (R&D) and treated with indicated concentrations of purified sPDGFRβ. After a 24 h incubation period, cells were then exposed to 75 kBq/ml [6-³H]-thymidine (NEN Life Science Products, Dreieich, Germany) for a 24 h labeling period. Radioactivity incorporated into DNA was measured as described before.[29]

Experimental *In Vivo* Model of Liver Fibrogenesis

Male Sprague–Dawley rats were infected with recombinant adenoviruses (2×10^{10} pfu/animal), or normal saline solution via the tail vein. After 2 days the common BD was double ligated and excised under halothane anesthesia. Rats were killed after 9 days, and pieces of the livers were fixed in 4% paraformaldehyde for histological examination or were frozen immediately in liquid nitrogen and stored at −80°C for protein and RNA isolation. The study as presented was approved by the local committee for care and use of laboratory animals, and was performed according to strict governmental and international guidelines on animal experimentation. For application of purified soluble receptor proteins (sTβRII, sPDGFRβ), 25 μg/animal (0.1 mg/kg

body weight) was administered intravenously upon bile duct ligature (BDL) according to established protocols.[30] Injections into the peritoneum were repeated twice a week until the animals were killed 14 days after surgery.

Histological Examination of Liver Tissues

Sirius Red staining was performed as described previously.[31] Briefly, liver sections were deparaffinized and the slides were incubated for 1 h in a solution of saturated picric acid containing 0.1% Sirius Red 80. Stained slides were then incubated in 0.01 N HCl for 2 min and washed in running distilled water, dehydrated, mounted, and examined by light microscopy.

Immunohistochemistry

Tissue sections (1.5 μm) were treated with xylene, rehydrated with decreasing graded ethanol, and blocked against endogenous peroxidase using 3% H_2O_2. For α-SMA staining, the slides were treated with a monoclonal anti α-SMA (asm-1, Roche) followed by incubation with a biotinylated anti-mouse secondary antibody (BA-9200, Vector Laboratories, Burlingame, CA, USA). Thereafter, the secondary antibodies were complexed with an avidin-conjugated peroxidase (Vectastain ABC-Elite reagent, Vector Laboratories) and peroxidase activity was detected with diaminobenzidine (DAKO). For better documentation the tissue sections were briefly counterstained with methyl green.

Statistics

Results are presented as the mean of three independent experiments ($\pm$ s.d.). Statistical analysis was performed with an unpaired Student's t-test and differences were considered as significant (*) or highly significant (**) at $P < 0.05$ or < 0.01, respectively.

Results

Cloning, Expression and Purification of sPDGFRβ

The spontaneous activation of HSC on plastic and the induction of fibrogenesis by ligature of the common BD are established model systems for liver fibrosis in rat. In general, cultured HSC and MFB are nearly refractory to gene transfer by physical methods but vector systems based on adenoviruses (Ad) are suitable for delivery of transgenes into this hepatic subpopulation.[21] We generated adenovirus serotype 5 (Ad5) for constitutive expression of a chimeric cDNA, termed sPDGFRβ, encoding the ECD of the PDGF receptor type β fused to the IgG-Fc domain of human immunoglobulin. In this construct, both domains are separated by an intron of 379 bp in size (Figure 1a). In the translated protein the ECD of PDGFRβ is linked to the Fc domain via a

two-amino-acid spacer. A similar construct harboring the ECD of the TGF-β receptor type II (TβRII) fused to the Fc domain was cloned previously.[32] The expression of the chimeric sPDGFRβ was assessed by Northern blot and Western blot. In COS-7 cells, Ad5-CMV-sPDGFRβ infection resulted in a viral dose-dependent synthesis of two mRNAs approximately 4000 and 3600 nt in length, which were compatible with the expected sizes for the mature mRNA of the PDGFRβ-Fc fusion and its progenitor RNA harboring an intron of 379 nt (Figure 1b). The synthesis of the chimeric protein product was demonstrated in Western blot using a polyclonal antibody directed against the ECD of PDGFRβ (Figure 1c). To confirm that the synthesized protein is bivalent and has the proposed antibody-like structure, sPDGFRβ was purified from culture supernatants of Ad5-CMV-sPDGFRβ infected COS-7 cells and subjected to SDS-PAGE under nonreducing and reducing conditions (Figure 1d, left). In this analysis, the purified protein migrated as a dimer of approximately 250 kDa under nonreducing conditions (−DTT), and as a monomer with a molecular mass around 125 kDa under reducing conditions (+DTT). These results indicate that the infected cells secrete the PDGFRβ-Fc fusion as a dimer, consistent with the predicted structure of the chimeric protein harboring two cysteine residues in the hinge region of the Fc portion able to form two intermolecular disulfide bonds between two sPDGFRβ molecules. Likewise, the electrophoretic mobility of the purified sTβRII is redox-dependent (Figure 1d, right). The precise cleavage between signal sequences and mature exported soluble receptors (sPDGFRβ, sTβRII) was confirmed by amino-acid sequence analysis revealing that sPDGFRβ and sTβRII were processed at MGLPGVMPASVLRGGLL LFVLLLLGPQISQG*L (sPDGFRβ) and MGRGLLRGL WPLHIVLWTRIAST*I (sTβRII), respectively. The determined cleavage sites (marked by asterisks) are in agreement with the von Heijne prediction[33] and are identical to the signal sequence processing sites proposed for the endogenous proteins.[34,35]

sPDGFRβ Inhibits PDGF Signaling *In Vitro*

PDGF is the most potent stimulus for HSC proliferation and induces a marked increase in cell proliferation, as measured by ^{3}H-thymidine incorporation (Figure 2a, control). Preincubation with sPDGFRβ was associated with a statistically high significant inhibition of this action (Figure 2a). We next evaluated the effect of PDGF scavenging on phosphorylation of endogenous PDGFRβ. Exposure of HSC to PDGF-BB resulted in tyrosine phosphorylation of a protein of ~190 kDa, compatible with the size of endogenous PDGFRβ (Figure 2b). Pretreatment with sPDGFRβ prevented the phosphorylation of this band, indicating that the soluble receptor is able to interfere with early PDGF signaling. Likewise, the phosphorylation of the downstream mediators ERK1/ERK2 (p44/p42),
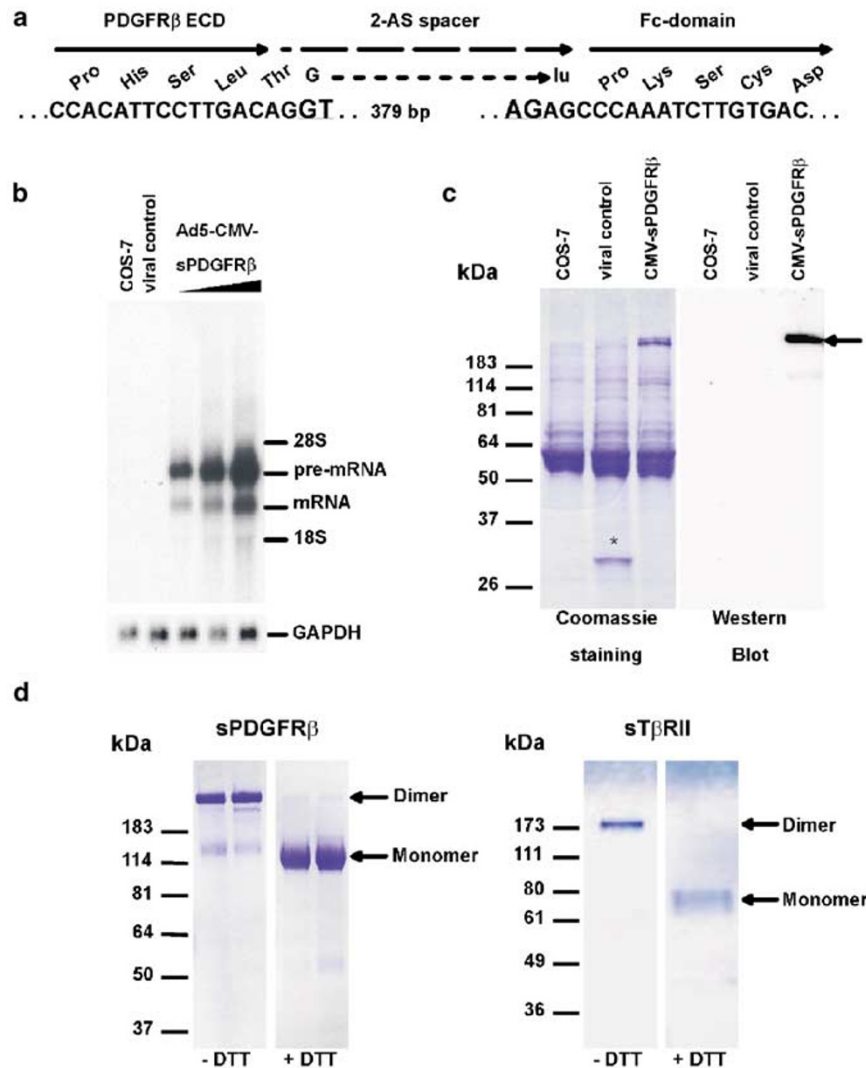
Figure 1 Construction and expression of sPDGFRβ in vitro. (**a**) Schematic diagram of the PDGFRβ containing the ECD of PDGF receptor type β and human IgG₁ Fc-domain separated by a 379-bp intron. In the diagram, splice donor and acceptor sites are underlined. (**b**) Northern blot analysis of Ad5-CMV-sPDGFRβ-infected COS-7 cells. Note that the expression of respective mRNAs is dependent on viral doses. (**c**) SDS-PAGE and Western blot analysis of supernatants taken from COS-7 cells. Supernatants from cells infected with Ad5-CMV-sPDGFRβ or Ad5-CMV-EGFP (viral control) were separated by SDS-PAGE and subjected to Western blot analysis under nonreducing conditions. The infection resulted in strong expression of sPDGFR. Also the expression of EGFP was detectable in Coomassie blue staining (*). (**d**) SDS-PAGE of purified soluble receptors. Both sPDGFRβ and sTβRII show redox-dependency and migrate as dimers under nonreducing (−DTT) conditions and as monomers under reducing conditions (+DTT).

previously shown to be an absolute requirement for triggering a proliferative response,[9] was virtually abolished to background levels after pretreatment with sPDGFRβ (Figure 2c), thus again demonstrating that sPDGFRβ is able to block PDGF signaling in culture-activated HSC. In contrast, incubation with PDGF was associated with the expected appearance of two bands corresponding to the phosphorylated isoforms of ERK1 (pp44) and ERK2 (pp42).

Adenoviral Expression of sPDGFRβ Prevents the Progression of Hepatic Fibrosis

Hepatic fibrosis in rats can be induced by ligature of the common BD for several weeks or months resulting in morphological changes in liver tissue comparable to those seen in human biliary fibrosis.[36–38] We used this experimental model of prolonged BD obstruction for testing the influence of sPDGFRβ on the early steps of ongoing liver fibrogenesis. We applied the adenoviral vehicle 2 days prior to setting of BDL, taken into account recent reports describing that the transduction efficiencies by adenoviral-mediated gene transfer are strongly reduced after liver injury.[27,39,40] The injection with Ad5-CMV-sPDGFRβ resulted in strong hepatic expression of the transgene at the mRNA and protein levels (Figure 3). High amounts of sPDGFRβ were detectable ($>1\,\mu g/ml$) in the serum at day 4 after adenoviral injection (2 days
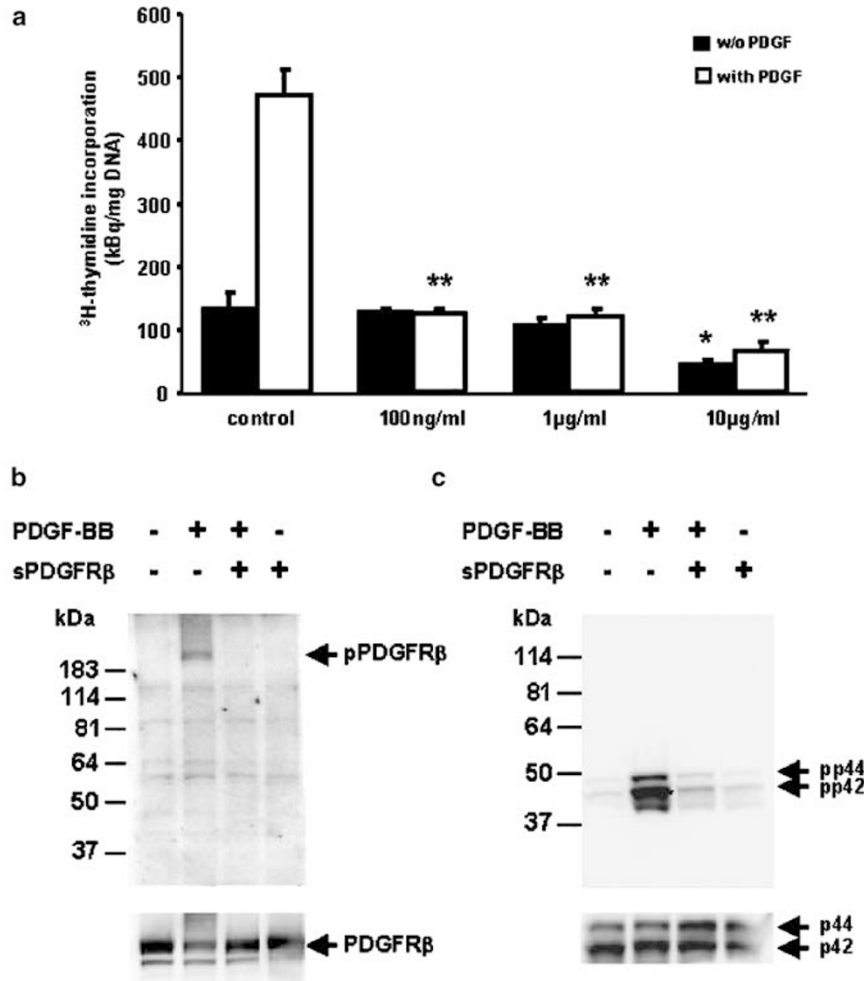
Figure 2 Functionality of sPDGFRβ *in vitro*. (**a**) Proliferation assay of culture-activated HSC. Cells were pretreated with indicated amounts of sPDGFRβ and stimulated with 20 ng/ml PDGF-BB (black bars) or left unstimulated (white bars). The experimental design was performed in triplicate and significant differences compared to control values are indicated with $P \leq 0.05$ (*) or $P \leq 0.01$ (**). (**b, c**) Western blot analysis for detection of total (PDGFRβ, p42, p44) and phosphorylated forms (pPDGFRβ, pp42, pp44) of proteins involved in the PDGF signaling pathway. In the experiments, HSC were treated with indicated combinations of sPDGFRβ and PDGF-BB and protein extracts were analyzed for expression of pPDGFRβ and PDGFRβ (**b**), or pp44/pp42 and p44/p42 (**c**), respectively.

after BDL surgery) and at lower levels (~ 100 ng/ml) at day 9 (7 days after BDL surgery) as assessed by Western blot analysis (Figure 3b, upper part) and ELISA (Figure 3b, lower part). To determine the impact of sPDGFRβ on liver fibrogenesis, the expression and deposition of collagen type I were assessed by Sirius red staining and Northern blot analysis (Figure 4). The deposition and the number of collagen fibrils was significantly decreased in rats receiving Ad5-CMV-sPDGFRβ, while no reduction in the degree of fibrosis was observed in animals receiving a control adenovirus (Ad5-CMV-EGFP), indicating that the prevention was specific for Ad5-CMV-sPDGFRβ (Figure 4a). The suppression of collagen type I expression was also confirmed at the transcriptional level (Figure 4b). However, we found that in this analysis the suppression of collagen type I mRNA was less pronounced but still significant.

We next examined the influence of sPDGFRβ on expression of α-smooth muscle actin (α-SMA),

another marker of ongoing fibrogenesis and cellular activation. In animals receiving Ad5-CMV-sPDGFRβ, the signals obtained in α-SMA immuno-stainings were markedly reduced (Figure 5), again confirming that sPDGFRβ is effective in preventing progression of hepatic fibrosis.

Efficacy of Purified Proteins (sPDGFRβ and sTβRII) in Preventing Liver Fibrosis

In hepatology research, adenoviral delivery approaches are attractive because they mainly target the expression of transgenes into the liver. However, the expressed soluble receptors are secreted and are detectable in circulating fluids (see above). Therefore, the application as purified proteins should have the same beneficial impact on hepatic fibrogenesis as the adenoviral approach. We purified large quantities of sPDGFRβ and sTβRII from the supernatant of COS-7 cells infected by respective
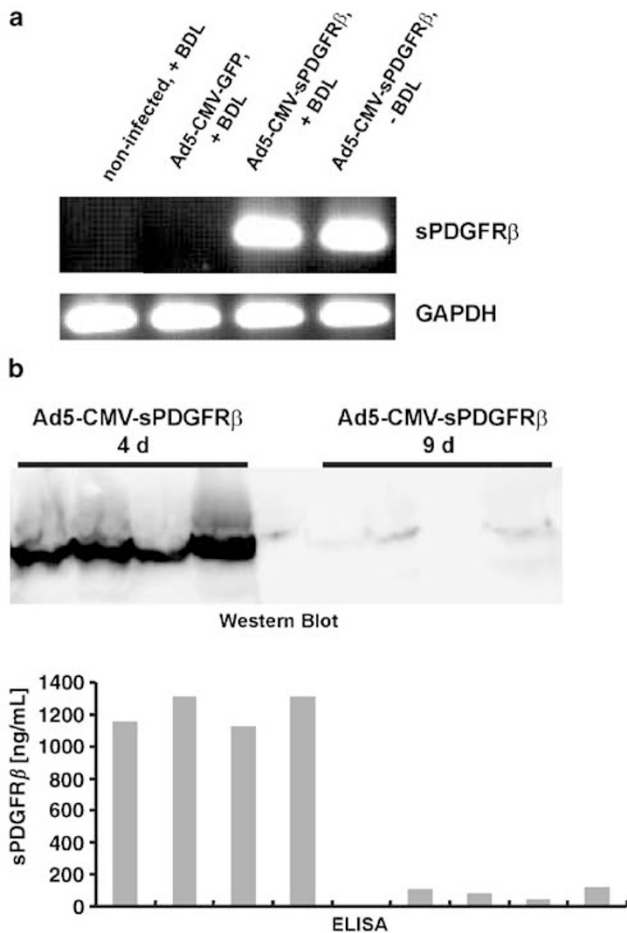
Figure 3 Expression of sPDGFRβ in rats. (**a**) In order to detect sPDGFRβ mRNA expression in BD ligated (+BDL) and non-ligated rats (−BDL), total RNA was isolated 9 days after adenoviral infection (7 days after BDL surgery) from rat livers and subjected to RT–PCR. In this analysis, Ad5-CMV-EGFP-infected rats served as negative controls. To verify the integrity of individual cDNA, a *GAPDH* specific product was amplified from each sample. (**b**) Western blot analysis (upper panel) and quantitative ELISA (lower panel) of serum isolated from bile duct-ligated and Ad5-CMV-sPDGFRβ-infected animals. Blood samples were taken at days 4 and 9 after viral infection.

adenoviruses and repeated our experiments using these purified protein fractions. As expected, the efficacy (eg collagen deposition and expression) of both soluble receptors was also seen when sPDGFRβ or sTβRII were administered as purified proteins (Figure 6), indicating that the observed antifibrotic effects were not the result of adenoviral infection *per se.*

Discussion

HSC are essentially involved in the development and progression of hepatic fibrosis. Profibrogenic cytokines, such as TGF-β, trigger HSC activation that involves proliferation, enhanced production of ECM components, and a phenotypic transition towards MFB, including a loss of the characteristic retinoid-

containing fat droplets.[1] Consequently, local expression of a dominant-negative type II TGF-β receptor (sTβRII) in rat livers treated with dimethylnitrosamine resulted in attenuation of liver fibrosis in a model of persistent liver fibrosis.[16] Further, the beneficial impact of sTβRII was recently demonstrated to have an inhibitory effect on the activation of rat HSC in primary culture resulting from interruption of the TGF-β autocrine signaling-loop.[18] Among other polypeptide growth factors potentially involved in chronic liver inflammation, PDGF has shown to be the most potent mitogen for cultured HSC isolated from rat, mouse, or human liver[3] reflecting the most striking molecular response of HSC to injury. Of the different possible dimeric forms of PDGF, PDGF-BB functions as the strongest stimulator of HSC growth and intracellular signaling with concurrent predominant expression of PDGF-receptor β (or type β) subunits in activated HSC.[41] Importantly, codistribution of PDGF with cells expressing the relative receptor subunits has been clearly demonstrated after both acute and chronic liver tissue damage, thus confirming a functional role of PDGF in the liver tissue repair process and in the development of hepatic fibrosis.[12,5] For these reasons, targeting the PDGF signaling is attractive for therapeutic intervention in hepatic fibrosis. We therefore constructed a transgene, encoding the ECD of the PDGF receptor type β fused to the IgG-Fc domain of human immunoglobulin and tested the impact of this soluble PDGF receptor β (sPDGFRβ) on liver fibrogenesis *in vitro* and *in vivo*. The binding of PDGF to the sPDGFRβ was confirmed in proliferation assays with PDGF-BB using culture-activated HSC. In these studies, the preincubation of HSC with sPDGFRβ was able to reduce PDGF-BB-induced mitogenic response of HSC. The phosphorylation of endogenous PDGFRβ was abrogated in response to sPDGFRβ. We also found that sPDGFRβ selectively inhibited the PDGF-BB-induced postreceptor phosphorylation of p42/p44, consistent with previous findings indicating that autophosphorylation of PDGFRβ is a prerequisite for PDGF downstream signaling *via* the Ras/ERK-regulated kinase pathway, which is likely the signal involved in the mitogenic response to PDGF.[42] These findings demonstrate that the soluble receptor is an effective means to block PDGF signaling in culture-activated HSC. In order to examine the effects of the soluble receptor on hepatic fibrosis, we used the BDL model. Previous studies have demonstrated that expression of the PDGF receptor β-subunit is increased during this experimental injury induced by biliary obstruction.[4] Using this model, we showed that the adenoviral expression or direct application of sPDGFRβ as purified protein significantly attenuates ongoing fibrosis as assessed by reduced expression of α-SMA and collagen. This fits well with previous studies demonstrating that inhibitors of PDGF decrease α-SMA immunoreactivity.[43] However,
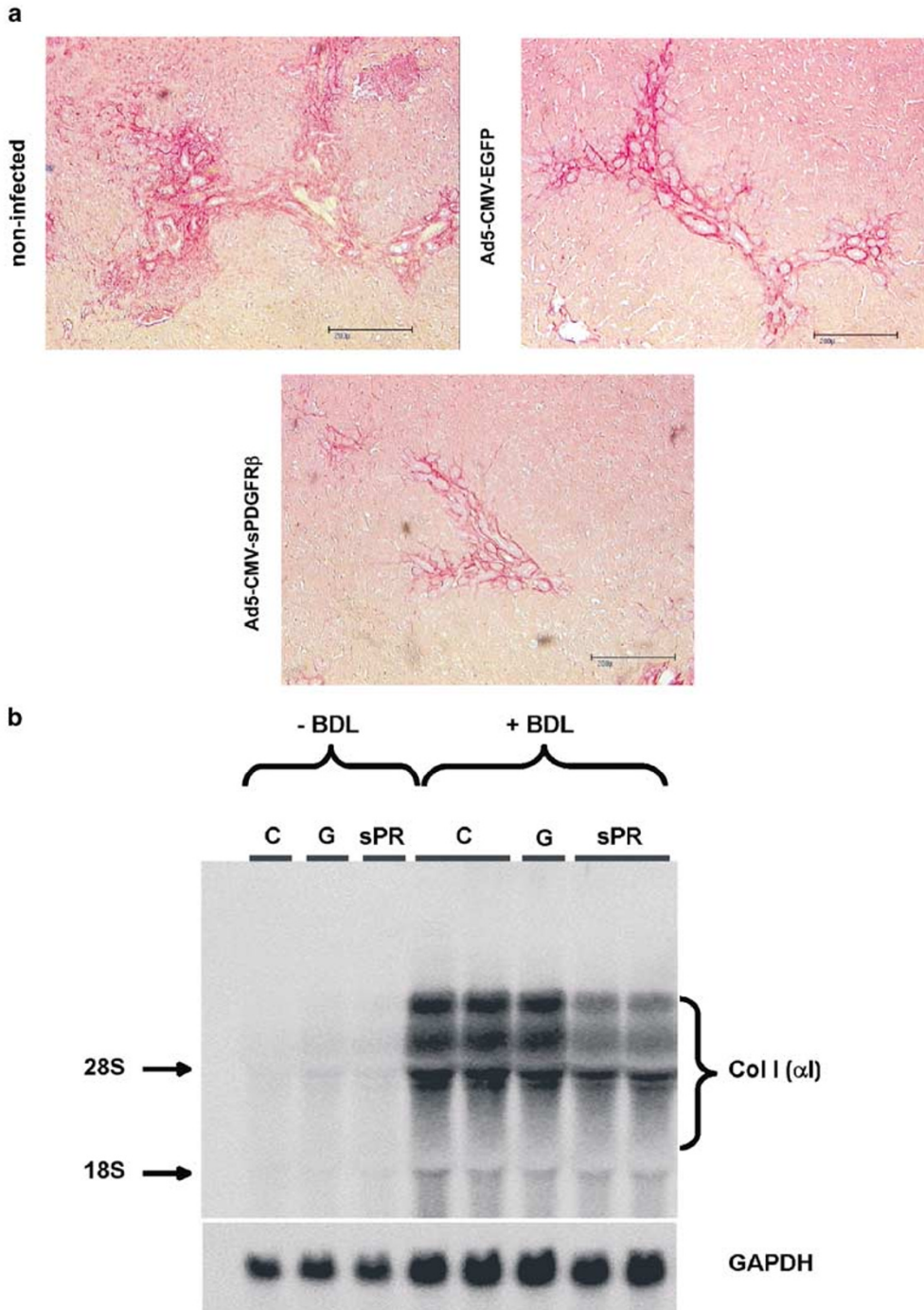
Figure 4 sPDGFRβ decreased collagen expression in BDL model of liver fibrosis. (**a**) Content and deposition of collagen in livers from animals subjected to BDL and injected with saline (non-infected), Ad5-CMV-EGFP (viral control), or Ad5-CMV-sPDGFRβ were analyzed by Sirius red staining. Sirius red staining results in dark red staining of collagen fibers (space bar = 200 μm). (**b**) Northern blot analysis of representative liver samples taken from both non-BD ligated-(−BDL) and BD ligated (+BDL) rats after saline (**c**), Ad5-CMV-EGFP (G), and Ad5-CMV-sPDGFRβ (sPR) application. The sizes of 18S and 28S rRNAs are indicated in the left margin.

compared to sTβRII, the PDGF antagonist exerts an overall weaker antifibrotic effect in experimental-induced liver injury, supporting the notion that TGF-β plays a more pivotal role in the fibro-proliferative changes that occur from tissue damage. Further, the comparison of absolute expression of

type I collagen mRNA and respective protein amount as assessed by Sirius red staining suggests a relatively greater reduction of collagen at the protein level after administration of sPDGFRβ. This discrepancy was previously described in experiments investigating the inhibition of hepatic
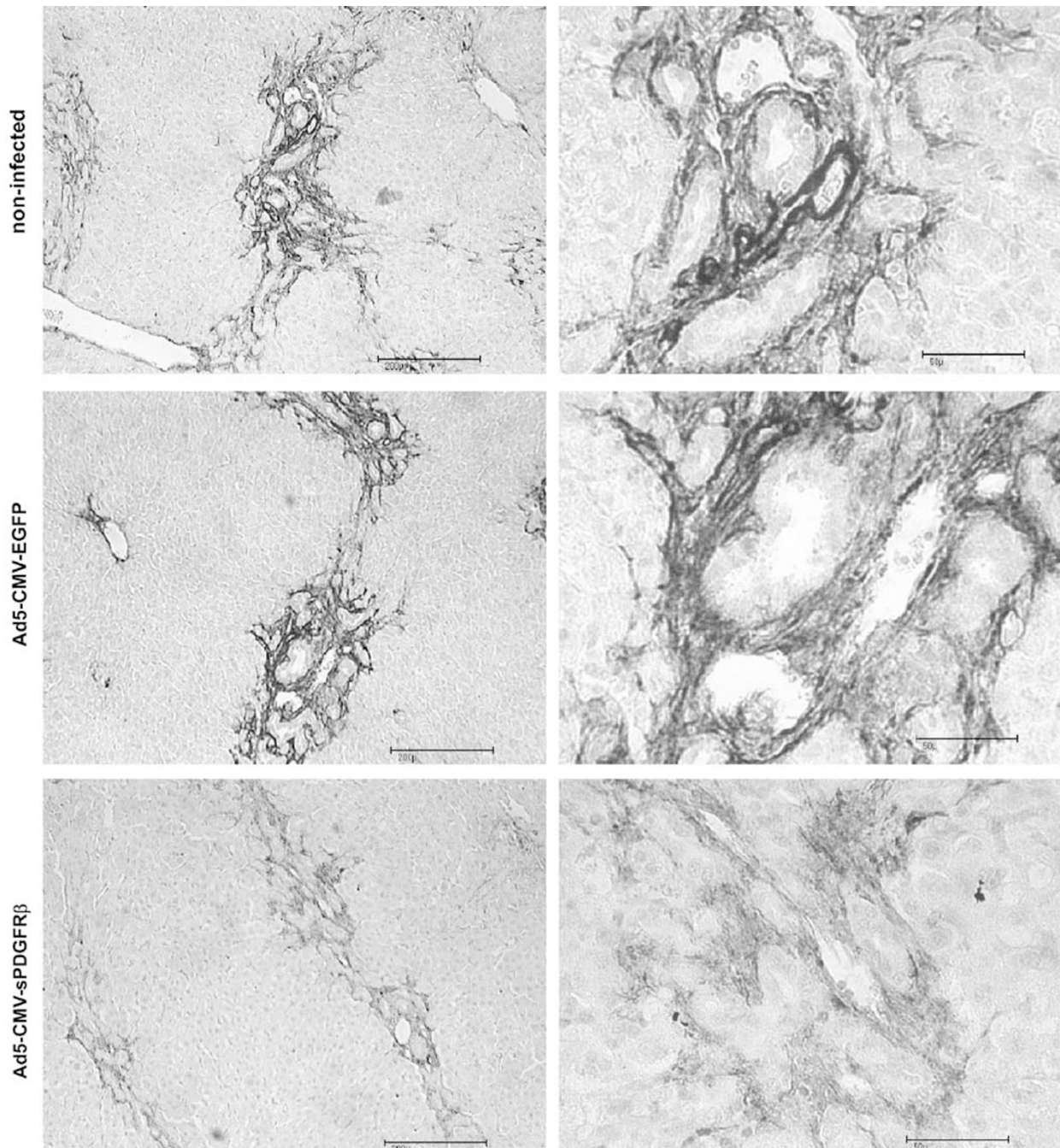
Figure 5 Reduction of smooth muscle α-actin immunoreactivity in rat livers treated with Ad5-CMV-sPDGFRβ. Immunohistological distribution of α-SMA in representative liver sections of BD-ligated rats injected with saline (non-infected), Ad5-CMV-EGFP, or Ad5-CMV-sPDGFRβ. Each liver section is shown at lower (left panel; space bar = 200 μm) and higher (right panel; space bar = 50 μm) magnification.

fibrosis by sTβRII[30] and might be due to the post-transcriptional regulation of collagen expression in cultured HSC.[44]

We have not yet analyzed the impact of sPDGFRβ with regard to chemotaxis of HSC towards BD structures that were shown to contribute to the development of periductular fibrosis in cholestatic disorders.[45] Probably, the reduced fibrosis observed after treatment with sPDGFRβ is not a direct consequence of interfering with the processes involved in controlling the synthesis of fibrogenic markers (α-SMA, collagen) *per se,* but may result from blockade of HSC proliferation and inhibition of chemotaxis, thereby decreasing the number of cells able to form these destructive components. This would suggest that the combined elimination of TGF-β and PDGF could further contribute to a more effective inhibition of hepatic fibrosis. The testing of this hypothesis is a major focus of future investigations in our laboratory.
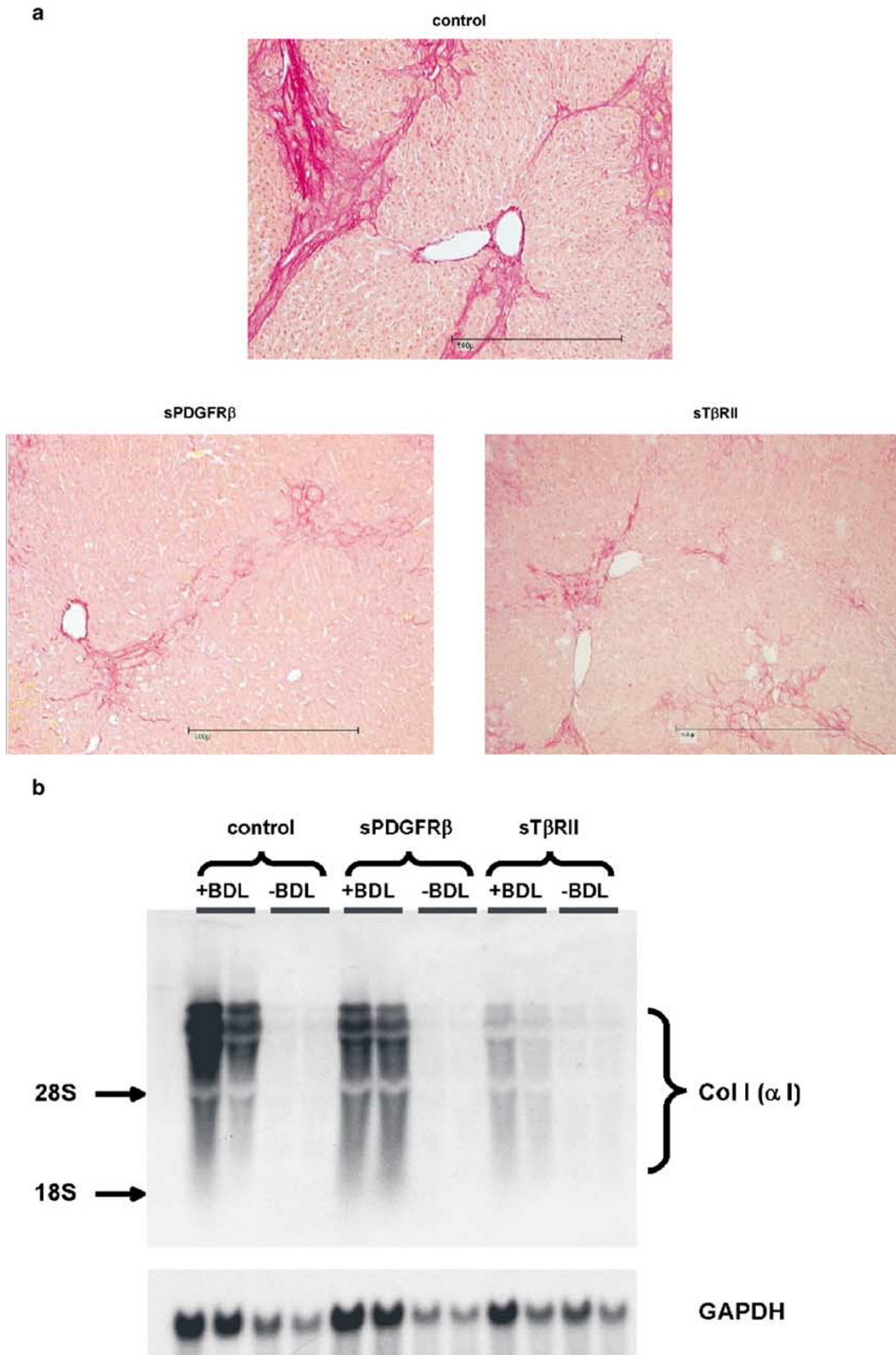
Figure 6 Administration of purified sPDGFRβ protein leads to decreased collagen expression in fibrotic rat liver. (**a**) Sirius red staining of liver sections from BD ligated and rats injected with saline (control), PDGFRβ, or sTβRII (space bar = 500 μm). (**b**) Northern blot analysis of collagen expression in livers obtained from rats with (+BDL) and without (−BDL) receiving injection of saline (control), sPDGFRβ, or sTβRII. The sizes of 18S and 28S rRNAs are indicated in the left margin.

In conclusion, the data presented herein provide evidence that sPDGFRβ is active as an antifibrogenic protein drug able to reduce the biological effects of PDGF in ongoing fibrogenesis. Therefore, the beneficial effects of this compound make it an attractive therapeutic agent for the treatment of fibrotic liver diseases and possibly other fibrotic organ lesions as well.

Acknowledgements

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