Selective delivery of IFN-γ to renal interstitial myofibroblasts: a novel strategy for the treatment of renal fibrosis

Fariba Poosti,*1 Ruchi Bansal,† Saleh Yazdani,‡ Jai Prakash,‖ Eduard Post,§ Pieter Klok,* Jacob van den Born,‡ Martin H. de Borst,‡ Harry van Goor,* Klaas Poelstra,§ and Jan-Luuk Hillebrands*1

*Department of Pathology and Medical Biology, Division of Pathology, †Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen, and §Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, Groningen, The Netherlands; and ‡MIRA Institute, University of Twente, Enschede, The Netherlands

ABSTRACT Renal fibrosis leads to end-stage renal disease demanding renal replacement therapy because no adequate treatment exists. IFN-γ is an antifibrotic cytokine that may attenuate renal fibrosis. Systemically administered IFN-γ causes side effects that may be prevented by specific drug targeting. Interstitial myofibroblasts are the effector cells in renal fibrogenesis. Here, we tested the hypothesis that cell-specific delivery of IFN-γ to platelet-derived growth factor receptor β (PDGFRβ)-expressing myofibroblasts attenuates fibrosis in an obstructive nephropathy (unilateral ureteral obstruction (UUO)) mouse model. PEGylated IFN-γ conjugated to PDGFRβ-recognizing peptide [(PPB)-polyethylene glycol (PEG)-IFN-γ] was tested in vitro and in vivo for antifibrotic properties and compared with free IFN-γ. PDGFRβ expression was 3-fold increased (P < 0.05) in mouse fibrotic UUO kidneys and colocalized with α-smooth muscle actin-positive (SMA+) myofibroblasts. In vitro, PPB-PEG-IFN-γ significantly inhibited collagen I, fibronectin, and SMA mRNA expression in TGF-β-activated NIH3T3 fibroblasts (P < 0.05). In vivo, PPB-PEG-IFN-γ specifically accumulated in PDGFRβ-positive myofibroblasts. PPB-PEG-IFN-γ treatment significantly reduced renal collagen I, fibronectin, and α-SMA mRNA and protein expression. Compared with vehicle treatment, PPB-PEG-IFN-γ preserved tubular morphology, reduced interstitial T-cell infiltration, and attenuated lymphangiogenesis (all P < 0.05) without affecting peritubular capillary density. PPB-PEG-IFN-γ reduced IFN-γ-related side effects as manifested by reduced major histocompatibility complex class II expression in brain tissue (P < 0.05 vs. free IFN-γ). Our findings demonstrate that specific targeting of IFN-γ to PDGFRβ-expressing myofibroblasts attenuates renal fibrosis and reduces systemic adverse effects.—Poosti, F., Bansal, R., Yazdani, S., Prakash, J., Post, E., Klok, P., van den Born, J., de Borst, M. H., van Goor, H., Poelstra, K., Hillebrands, J.-L. Selective delivery of IFN-γ to renal interstitial myofibroblasts: a novel strategy for the treatment of renal fibrosis. FASEBJ. 29, 1029–1042 (2015). www.fasebj.org

Key Words: drug targeting · PDGFRβ · kidney · unilateral ureteral obstruction

Development of renal fibrosis is the final common pathway of chronic kidney disease, which ultimately leads to end-stage renal disease demanding renal replacement therapy. As such, renal fibrosis is an increasing global health problem (1–3). Renal fibrosis is characterized by activation and proliferation of interstitial fibroblasts and excessive matrix deposition (4–6). Despite powerful renoprotective drugs, e.g., renin-angiotensin-aldosterone system blockers that lower blood pressure and proteinuria, many patients still progress toward end-stage renal disease (1).

After kidney injury, several fibrogenic factors activate fibroblasts by ligating their respective receptors resulting in myofibroblast differentiation and activation. Myofibroblasts are the primary source of extracellular matrix (ECM), which is deposited in the renal interstitium during fibrogenesis. The anatomic origin of interstitial myofibroblasts responsible for excessive matrix production is still an area of controversy, and various origins have been proposed including adult kidney nephrogenic progenitors, stroma, bone marrow-derived cells, damaged epithelium, and endothelium (3, 7–15). Most likely, interstitial myofibroblasts in renal fibrosis originate from multiple sources but to various extents; the majority, however, appears to originate from the FOXD1 lineage, which gives rise to pericytes.

Abbreviations: ATA, acetylthioacetate; DMF, dimethylformamide; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GMBS, γ-maleimidobutyryl-α, α-succinimide ester; HRP, horseradish peroxidase; HSA, human serum albumin; IF, interstitial fibrosis; IFN-γR1, IFN-γ receptor 1; JAK/STAT, Janus kinase/signal transducers and activators of transcription signaling pathway; LTA, lotus tetragonolobus; LTL, lotus tetragonolobus lectin; (continued on next page)
residual fibroblasts, mesangial cells, and vascular smooth muscle cells (SMCs) during kidney development (3).

Recent studies suggested that IFN-γ could be a useful therapeutic target to halt and prevent the development of fibrosis (16–18). IFN-γ is a pleiotropic cytokine produced by various activated immune cells including NK cells and T cells. In addition to its proinflammatory effects, IFN-γ has prominent antiproliferative and antifibrotic effects (19, 20). IFN-γ inhibits fibroblast activation and proliferation and also reduces collagen synthesis (18, 20, 21), thereby making it an interesting molecule for therapeutic use to target fibrosis. However, the short half-life and undesirable systemic side effects clearly limit clinical use of IFN-γ (22, 23). Therefore, novel cell-specific delivery strategies are required to improve the therapeutic efficacy of IFN-γ while reducing its systemic side effects. Mesenchymal-derived cells including fibroblasts and pericytes constitutively express platelet-derived growth factor receptor β (PDGFRβ) at a low level at physiologic conditions, whereas tissue injury is accompanied by increased expression of this receptor (24–28). An attractive approach for myofibroblast-specific delivery of IFN-γ could therefore be based on the recognition of PDGFRβ, which can be achieved by the use of a PDGFRβ-recognizing cyclic peptide (PPB) (29–31). This carrier increases the half-life of drugs and also imparts receptor specificity by binding specifically to PDGFRβ and, therefore, can be used for drug targeting to activated fibroblasts. Recently, we employed this carrier to target PDGFRβ-expressing stellate cells in fibrotic liver disease (29, 30, 32). The efficacy of this novel targeting strategy to prevent renal fibrosis is yet unknown. In the present study, we therefore investigated whether cell-specific targeting of IFN-γ to PDGFRβ-expressing myofibroblasts attenuates renal fibrosis. For that purpose, chemically engineered PEGylated IFN-γ was conjugated to the PPB carrier [PPB-polylethylene glycol (PEG)-IFN-γ] and tested in in vitro culture systems as well as in vivo in the unilateral ureteral obstruction (UUO) mouse model for renal fibrosis.

MATERIALS AND METHODS

Human renal tissue

To analyze PDGFRβ expression in human kidneys, nephrectomy specimens from renal transplant recipients with chronic transplant dysfunction (n = 6) were used in this study. The unaffected part of kidneys from patients with renal cell carcinoma was used as control (n = 3). Tissues were fixed in 4% formaldehyde and processed for paraffin embedding. Use of anonymous human renal tissue was performed according to national guidelines.

(continued from previous page)

Mal-PEG-SCM, maleimide-polylethylene glycol-succinimidyl carboxy methyl ester; MHC, major histocompatibility complex; NLS, nuclear signaling sequence; PAS, periodic acid Schiff; PDGFRβ, platelet-derive growth factor receptor β; PEG, polylethylene glycol; PPB, platelet-derived growth factor receptor β-recognition peptide; PTC, peritubular capillary; qRT-PCR, quantitative RT-PCR; SMA, smooth muscle actin; SMIC, smooth muscle cell; TA, tubular atrophy; TBST, 20 mM Tris/ HCl (pH 7.6), 154 mM NaCl, and 0.1% Tween 20; UUO, unilateral ureteral obstruction; Ywhaz, tyrosine 3 mono-oxygenase/tryptophan 5 mono-oxygenase activation protein γ

Male C57Bl/6 mice (9–12 wk old; weight 28−30 g) were obtained from Harlan Laboratories (Zeist, The Netherlands). Animals were housed in cages with free access to food and water. During the operation, the abdomen was opened using a midline incision under general anesthesia (isoflurane/O2). All mice were subjected to UUO by a double ligation of the left ureter proximal to the kidney using 6-0 silk sutures. Right kidneys were used as sham controls and were manipulated but not ligated. The different treatment groups were as follows: 1) UUO, vehicle (saline); 2) UUO, PPB-human serum albumin (HSA) [PDGFRβ-recognizing peptide (PPB)-HSA]; 3) UUO, PPB-PEG-IFN-γ, and 4) UUO, free IFN-γ (5 μg per mouse per injection). Synthesis of the various conjugates is described below. Follow-up time was 3 and 7 d during which mice received 2 (on d 1 and 2 post-UUO) or 3 (on d 2, 4, and 6 post-UUO) i.v. injections of the respective compounds via the penile vein. Group size was 6 per group per time point. The experimental protocol adhered to the national guidelines for the Care and Use of Laboratory Animals and was approved by the local Animal Ethics Committee of the University of Groningen.

Cell lines

Mouse NIH3T3 fibroblasts and RAW macrophages (ATCC, Manassas, VA, USA) were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-Glu, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). Mouse NIH3T3 fibroblasts were used from passage 160 to 170, and RAW macrophages were used between passage 13 and passage 19.

Synthesis of PPB-PEG-IFN-γ and PPB-HSA conjugates

Synthesis of PPB-PEG-IFN-γ and PPB-HSA conjugates has been described in detail elsewhere (30). Briefly, for PPB-PEG-IFN-γ, recombinant murine IFN-γ (0.256 nmol; PeproTech, London, United Kingdom) was mixed with 12.8 nmol maleimide-PEG-succinimidyl carboxy methyl ester (Mal-PEG-SCM) (2 kDa; Creative PEGworks, Winston-Salem, NC) for 2 h and dialyzed overnight against PBS using 10 kDa dispropodialyzer (Harvard Apparatus, Holliston, MA, USA). Mal-PEG-SCM is a 2 kDa hetero-functional PEG linker that provides hydrophilicity, stability, and conformational flexibility for the appropriate receptor interaction. The dialyzed sample (IFN-γ-PEG) was then reacted with 25.6 nmol PPB-acetylthioacetate (ATA) in the presence of deacetylating reagent [0.1 M hydroxylamine, 25 mM EDTA in PBS (pH 7.2)]. PPB-ATA, an 8 amino acid PDGFRβ-recognition binding cyclic peptide (PPB) modified with succinimidyl acetylthioacetate, was synthesized by Ansynth Service B.V. (Roosendaal, The Netherlands). Finally, PPB-PEG-IFN-γ was extensively dialyzed against PBS using dispropodialyzer and stored at −80°C until use.

For PPB-HSA, HSA (1.5 μmol dissolved in PBS) was mixed with γ-maleimidobutryloxy succinimidyl ester (GBMS) [30 μmol, dissolved in dimethylformamide (DMF)] for 2 h and extensively dialyzed against PBS using a 10 kDa cutoff dialysis membrane cassette (Thermo Scientific, Rockford, IL, USA). Next, PPB-ATA (34.5 μmol; dissolved in DMF) was added to the GBMS-modified HSA for overnight, dialyzed against PBS. The final product (PPB-HSA) was freeze-dried for storage at −20°C.

Biochemical characterization of PPB-PEG-IFN-γ

PPB-PEG-IFN-γ was characterized by Western blotting using rabbit polyclonal anti–IFN-γ antibodies and custom-made rabbit polyclonal anti-PPB antibodies. PPB-PEG-IFN-γ was subjected to
10% SDS-PAGE, and the separated proteins were transferred to a PVDF membrane and blocked with TBST [20 mM Tris/HCl (pH 7.6), 154 mM NaCl, and 0.1% Tween 20] containing 5% skimmed milk. The blots were then incubated with anti–IFN-γ (1:1000 dilution) or anti-PPB (1:1000 dilution) antibodies. After washing with TBST, the blots were incubated with horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibodies (1:2000 dilution; DAKO, Glostrup, Denmark) for 2 h. The bands then were visualized using the Western Lighting-ECL reagent (PerkinElmer, Waltham, MA, USA) according to the manufacturer’s instructions, followed by Kodak X-ray film exposure (Kodak, Rochester, NY, USA). In addition, PEG staining using barium iodide was performed following electrophoresis of PBB-PEG-IFN-γ on 10% SDS-PAGEs. The gels were rinsed with water, followed by fixation in perchloric acid (0.1 M) for 15 min. The gels were rinsed again and treated with barium chloride (5%) for 10 min. Subsequently, the color was developed using Tritisol iodine solution (Sigma-Aldrich, St. Louis, MO, USA). The gel was photographed using G-Box (Syngene, Cambridge, United Kingdom).

Biologic activity of free and targeted IFN-γ on mouse RAW macrophages

The bioactivity of free IFN-γ, PBB-PEG-IFN-γ, and PBB-HSA was assessed by measuring the accumulation of nitrite (NO₂−), a stable end product of NO produced by murine RAW macrophages in response to IFN-γ in the presence of LPS. RAW cells (1 × 10⁴ cells/200 μl per well) were seeded in 96-well plates and incubated with different concentrations (5, 10, 20, and 50 ng/ml) of free IFN-γ, PBB-PEG-IFN-γ, and PBB-HSA or with medium alone in the presence of 100 ng/ml LPS (from Escherichia coli 055:B5; Sigma-Aldrich). After 24 h, the secreted nitrite was measured as absorbance at 550 nm using Griess reagent (1% sulfanilamide, 0.1% naphthylethendiamine dihydrochloride, and 3% H₃PO₄). Experiments were performed in triplicate and repeated 3 times.

In vitro binding and antifibrotic effects of free and targeted IFN-γ on mouse NIH3T3 fibroblasts in vitro

For binding assay, cells were seeded on Lab-Tek (Nunc, Roskilde, Denmark) and allowed to adhere overnight. Cells were then incubated with PBB-PEG-IFN-γ (1 μg/ml). In order to block PDGFRβ-mediated binding, anti-PDGFRβ IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added 1 h before adding PBB-PEG-IFN-γ. After 2 h, cells were extensively washed, fixed, and immunofluorescence staining for PPB was performed.

In order to assess the antifibrotic effects of PBB-PEG-IFN-γ, mouse NIH3T3 fibroblasts (75,000 cells/well) were seeded in 12-well Falcon culture plates (Becton Dickinson, Heidelberg, Germany) and allowed to attach overnight. Cells were starved with 0.5% serum containing medium for 24 h and then incubated with free IFN-γ, PBB-HSA, and PBB-PEG-IFN-γ (equivalent 1 μg/ml) or medium (0.5% serum) alone in the presence of 5 ng/ml human recombinant TGF-β1 (Roche, Mannheim, Germany) for 48 h. As a control for the profibrotic effect of TGF-β1, cells were cultured in medium alone (without TGF-β1 and conjugates). Thereafter, cells were lysed with lysis buffer constituted with 2-ME (Stratagene, Agilent Technologies, Santa Clara, CA, USA) to perform real-time PCR analysis for procollagen 1α1, α-SMA, and procollagen 1α2 as described below. Experiments were repeated 3 times independently.

Antibodies

The primary antibodies used for immunohistochemistry and immunofluorescence, and the lectin used to detect proximal epithelial cells, are listed in Supplemental Table 1.

Immunohistochemistry

Periodic acid Schiff (PAS) and immunohistochemistry for α-SMA, fibronectin, PDGFRβ, CD31, podoplanin, lotus tetragonolobus lectin (LTL), and CD3 were performed on 2 μm paraffin sections. Sections were deparaffinized in xylene and rehydrated in graded alcohol and distilled water. Antigen retrieval was achieved by overnight incubation at 60°C in 0.1 M Tris/HCl buffer (pH 9.0) for fibronectin staining, proteinase K for LTL, treatment in the microwave for 15 min at 300 W with 1 mM EDTA (pH 8.0) for PDGFRβ staining, and 10 mM citrate buffer (pH 6.0) for podoplanin and CD31 staining. No antigen retrieval was performed for α-SMA staining. Endogenous peroxidase activity was blocked with 3% H₂O₂ (in PBS) for 30 min. Primary antibody binding was detected by sequential incubations with HRP-labeled appropriate secondary and tertiary antibodies (all obtained from DAKO). Peroxidase activity was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAKO) as chromogen (10 min incubation). Sections were counterstained with hematoxylin for 1 min and mounted with Kaiser’s glycerin gelatin.

Quantification of immunostaining

The sections were scanned using a NanoZoomer HT (Hama-matsu Photonics K.K., Shizuoka Prefecture, Japan). The extent of cortico-interstitial α-SMA, fibronectin, and CD3 expression (number of positive pixels), and number of podoplanin–vesSEL-like structures were measured and counted, respectively, using Aperio ImageScope software (version 9.1.772.1570; Aperio Technologies Incorporated, Vista, CA, USA) at ×200 magnification. For CD31 staining, 10 cortical areas at ×200 magnification have been selected, and the percentage of CD31+ area has been measured using ImageJ 1.46r (NIH, Bethesda, MD, USA). To assess renal tubular atrophy (TA), PAS-stained sections were evaluated in 5 randomly selected fields for each kidney. Tubular dilation was determined by dividing area_int (i.e., surface area tubular lumen) by area_ext (i.e., surface area tubular lumen + epithelium) for all individual tubules present within a fixed surface area using Aperio ImageScope software. For each kidney, the mean ratio area_int:area_ext was calculated; an increased ratio represents tubular dilation.

Immunofluorescence

Specific binding of IFN-γ conjugates to PDGFRβ first was checked in vitro. Mouse NIH3T3 fibroblasts were grown overnight at 37°C/5% CO₂. Then, cells were incubated with PBB-PEG-IFN-γ (1 μg/ml). After 2 h, cells were fixed with acetone/methanol (1:1) and were incubated with the PPB antibody. Subsequently, they were incubated with tetramethylrhodamine-5-(and 6)-isothiocyanate-conjugated goat anti-rabbit secondary antibodies. The binding studies were performed 3 times independently.

To confirm PDGFRβ expression on renal myofibroblasts, immunofluorescent double labeling was performed using the primary antibodies for PDGFRβ and α-SMA described above. Briefly, 4 μm acetone-fixed frozen sections were blocked for endogenous peroxidase activity with 0.07% H₂O₂ (in PBS) followed by incubation with primary antibodies. Binding of PDGFRβ and α-SMA primary antibodies was detected by incubation with goat anti-rabbit HRP-conjugated (DAKO) and goat anti-mouse IgG2a FITC (Southern Biotech, Birmingham, AL, USA), respectively. HRP activity was visualized using the TSA Tetramethylrhodamine System (PerkinElmer). DAPI was used for nuclear counterstaining, and sections were coveredslipped with DAKO Fluorescent Mounting Medium. To examine the binding of PBB-PEG-IFN-γ to target receptor, staining for PDGFRβ and PPB was performed as described above using donkey anti-goat Alexa 647 and donkey
anti-hamster HRP and goat anti-rat FITC were used as secondary antibodies. Images were taken with a Zeiss Axiol Observer Z1 (Carl Zeiss AG, Oberkochen, Germany) and TissueFAXS acquisition software (TissueGnostics, Vienna, Austria).

Quantitative real-time PCR

Total RNA from cells and kidney tissues was extracted using the RNA MicroPrep kit (Stratagene, Agilent Technologies) and RNasey Mini Kit (QIAGEN, Hilden, Germany), respectively, according to the manufacturer’s instructions. The RNA concentrations were measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). For NIH3T3 fibroblasts, total RNA was reverse transcribed using a cDNA synthesis kit (Promega, Madison, WI, USA) in a volume of 50 µL, and 20 ng cDNA was used for quantitative real-time PCR. The reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) in a volume of 50 µL. The sequences of primers used in the study were forward primer 5′-ctgatggcagagctggtgta-3′ and reverse primer 5′-atccatcggtcatgctctct-3′; procollagen 1α1, forward primer 5′-actactgcagcgtgagat-3′ and reverse primer 5′-actactgcagcgtgagat-3′; α-SMA, forward primer 5′-atgttgccagcttcacctct-3′ and reverse primer 5′-actactgcagcgtgagat-3′; α-SMA, forward primer 5′-atgttgccagcttcacctct-3′ and reverse primer 5′-actactgcagcgtgagat-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward primer 5′-acagtccatgccatcactgc-3′ and reverse primer 5′-gatccacgacggacacattg-3′. Single-stranded cDNA for kidney tissues was synthesized using SuperScript II and random hexamer primers (Invitrogen) in a volume of 20 µL. cDNAs were diluted to a concentration of 2 ng/µL, and 2.5 µL/reaction (5 ng) was used for quantitative RT-PCR (qRT-PCR) analysis. PCRs were performed in a 10 µL reaction volume containing 1× qRT-PCR MasterMix (EUrogentec, Liege, Belgium) and 1× TaqMan Gene Expression Assay mix (Applied Biosystems). The primer reference numbers were as follows: tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein ζ (Ywhaz), Mm03950126_s1; Col 1a1, Mm00801666_g1; Col 3a1, Mm01254476_m1; α-SMA/Acta2, Mm01546133_m1; Ftn, Mm01256744_m1; major histocompatibility complex (MHC) II/CD74, Mm00658576_m1; and PDGFRβ, Mm00435546_m1. qRT-PCRs were performed on an ABI7900HT thermal cycler (Applied Biosystems). Relative gene expression (expressed as 2−ΔΔCt) was determined using GAPDH (for mouse NIH3T3 fibroblasts) and Ywhaz (for mouse kidney tissues) as housekeeping genes.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 (La Jolla, CA, USA). Differences between groups were calculated...
using Kruskal-Wallis with \( P < 0.05 \) as the minimal level of significance. Data are expressed as the mean ± SEM.

RESULTS

PDGFRβ expression is increased on interstitial myofibroblasts in fibrotic renal tissue

We first analyzed PDGFRβ protein expression in human normal and fibrotic renal tissue [i.e., renal allografts with interstitial fibrosis (IF)/TA] as well as mouse sham and UUO kidneys. Fibrosis is characterized by increased interstitial PDGFRβ expression in both human and mouse renal tissue, as revealed by immunohistochemistry (Fig. 1A). Increased PDGFRβ expression was confirmed at the mRNA expression level in mouse UUO kidneys, both at d 3 and 7 postobstruction (Fig. 1B). This increase appeared to be confined to the obstructed kidney because other organs from these mice (liver, heart, lung, and spleen) did not express detectable levels of PDGFRβ at the protein level (Fig. 1C). Double-immunofluorescent labeling showed colocalization of α-SMA and PDGFRβ on interstitial myofibroblasts, which was increased in UUO compared with sham (Fig. 1D). Arteriolar α-SMA+ medial SMCs did not express detectable levels of PDGFRβ protein (Fig. 1D).

Synthesis and characterization of PPB-PEG-IFN-γ conjugate

To target IFN-γ to PDGFRβ-expressing myofibroblasts, IFN-γ was conjugated to PPB via a 2 kDa hydrophilic hetero-bifunctional PEG linker resulting in formation of the PPB-PEG-IFN-γ conjugate as illustrated in Fig. 2A. The synthesized conjugate was characterized by Western blot analyses using anti-IFN-γ and anti-PPB antibodies (Fig. 2B, C). Successful coupling and conjugation were confirmed by PEG staining (Fig. 2D). Biologic activity of PPB-PEG-IFN-γ was then examined in a NO release assay in mouse RAW macrophages (Fig. 2E). Collectively, these data indicate that PEG-IFN-γ was successfully conjugated to PPB and retained its biologic activity.

PPB-PEG-IFN-γ is antifibrotic in TGF-β1–stimulated mouse 3T3 fibroblasts

To investigate the binding of PPB-PEG-IFN-γ to PDGFRβ and to test its antifibrotic properties in vitro, mouse 3T3 fibroblasts were stimulated by TGF-β1 (5 ng/ml) in the presence of PPB-PEG-IFN-γ. PPB staining revealed the binding of PPB-PEG-IFN-γ to the PDGFRβ on fibroblasts, which was almost completely blocked after preincubation...
with PDGFRβ-blocking antibody (Fig. 3A). Both free IFN-γ and PPB-PEG-IFN-γ significantly reduced collagen 1α2 mRNA expression. However, a significant reduction of α-SMA and collagen 1α1 mRNA expression was only observed in PPB-PEG-IFN-γ–treated cells (Fig. 3B–D). These data indicate that targeted IFN-γ is at least as effective (or potentially even more) as free IFN-γ with regard to its antifibrotic properties in vitro.

**PPB-PEG-IFN-γ is targeted toward PDGFRβ-expressing interstitial myofibroblasts in vivo**

Binding of PPB-PEG-IFN-γ to its target receptor PDGFRβ was revealed by double labeling for PPB and PDGFRβ. As shown in Fig. 4A, in sham-operated mice treated with PPB-PEG-IFN-γ, colocalization of PPB and PDGFRβ was found in interstitial myofibroblasts, although the number of PDGFRβ-expressing cells was relatively low. UUO resulted in a substantial increase in PDGFRβ expression (confirming our data shown in Fig. 1). Treatment with PPB-PEG-IFN-γ revealed colocalization of PPB and PDGFRβ (Fig. 4B), although not 100%. The discrepancy in colocalization is attributed to the dynamic interaction of PPB with its receptor. In other words, following interaction/binding of the conjugate to PDGFR, the complex is internalized, degraded, and IFN-γ (or its signaling moiety) will be released into the cytoplasm for IFN-γ–mediated effects. Furthermore, binding of PPB hinders the interactions of PDGFRβ antibody to the PDGFR, leading to reduced PDGFR staining. No PPB staining was observed in vehicle-treated sham-operated (Fig. 4A) and UUO (Fig. 4B) mice and demonstrates the specificity of the PPB staining.

**PPB-PEG-IFN-γ reduces α-SMA and fibronectin expression**

To investigate whether PPB-PEG-IFN-γ treatment ameliorates IF, qRT-PCR analysis was performed on UUO and sham kidneys. UUO significantly increased α-SMA mRNA expression 7 d after surgery, which was prevented by PPB-PEG-IFN-γ (Fig. 5A). Similar results were obtained for fibronectin mRNA expression, which was significantly increased after UUO (Fig. 6A). PPB-PEG-IFN-γ treatment significantly attenuated the UUO-induced enhancement of fibronectin mRNA expression 7 d after surgery (Fig. 6A).

We next analyzed whether reduced α-SMA and fibronectin mRNA expression also translated into reduced protein expression levels. UUO induced a significant increase in interstitial α-SMA (Fig. 5B) and fibronectin (Fig. 6B) protein expression at both d 3 and 7 post-UUO when compared with sham-operated mice. At d 3, both PPB-HSA and PPB-PEG-IFN-γ significantly reduced UUO-induced α-SMA expression. At d 7, however, only PPB-PEG-IFN-γ

---

**Figure 3.** In vitro binding and antifibrotic effect of PPB-PEG-IFN-γ. A) In vitro binding of PPB-PEG-IFN-γ to PDGFRβ in mouse NIH3T3 fibroblasts incubated for 48 h in the presence of TGF-β1 (5 ng/ml) and anti-PDGFRβ IgG. Representative images stained for PPB demonstrate binding of IFN-γ conjugate to the targeted receptor, which was almost completely blocked in the presence of PDGFRβ-blocking antibody. Relative mRNA expression is shown of the fibrotic markers (B) procollagen 1α1, (C) α-SMA, and (D) procollagen 1α2 (n = 3) in NIH3T3 cells incubated with medium only (control), TGF-β1 (5 ng/ml) in combination with PPB-HSA, unmodified IFN-γ, and PPB-PEG-IFN-γ (1 μg/ml). *P < 0.05 vs. medium; †P < 0.05 vs. vehicle; ‡P < 0.05 vs. PPB-HSA.
significantly reduced UUO-induced α-SMA expression (Fig. 5B). Although fibronectin expression was not affected by any of the treatments 3 d after surgery, at 7 d, again PPB-PEG-IFN-γ, but not PPB-HSA, significantly tempered fibronectin expression (Fig. 6B). Representative immunofluorescent photomicrographs of α-SMA and fibronectin staining are shown in Figs. 5C and 6C, respectively. In addition to α-SMA and fibronectin, also collagen I and III mRNA expression levels were determined. Compared with vehicle, PPB-PEG-IFN-γ significantly reduced UUO-induced collagen I mRNA expression at d 7, as well as a trend toward reduced collagen III expression (Supplemental Fig. 1). Collectively, these data indicate that PPB-PEG-IFN-γ is highly effective in reducing IF 7 d after UUO.

**PPB-PEG-IFN-γ preserves tubular morphology**

UUO not only results in IF (as discussed above) but also in tubular dilation. We therefore analyzed whether PPB-PEG-IFN-γ-induced attenuation of renal fibrosis is accompanied by preservation of tubular morphology. To this end, the cortical tubular luminal area in the various UUO groups was determined and compared with sham-operated mice at both d 3 and 7 after surgery. UUO induced tubular dilation as indicated by a significant increase in mean tubular luminal area (Fig. 7B), which was significantly attenuated by PPB-PEG-IFN-γ at d 7. Tubular dilation after UUO was mirrored by reduced LTL staining, a specific marker for proximal tubular epithelial cells (Fig. 7C). Reduced LTL staining in UUO kidneys results from TA as well as IF. Treatment with PPB-PEG-IFN-γ resulted in less reduction of LTL staining at both d 3 and 7. Representative photomicrographs of PAS and LTL staining used for quantification are shown in Fig. 7A.

**PPB-PEG-IFN-γ treatment reduces T-cell infiltration**

Because the development of renal IF is associated with T-cell infiltration (33, 34), we investigated whether increased T-cell influx occurs in UUO and whether this is influenced by any of the treatments. Quantitative analysis following immunohistochemical staining for CD3 revealed that UUO kidneys are indeed characterized by significant infiltration of CD3+ T lymphocytes at both d 3 and 7 after induction of disease (Fig. 7D). T-cell infiltration was significantly reduced by PPB-PEG-IFN-γ treatment 7 d after surgery, whereas free IFN-γ and PPB-HSA did not show this effect. Representative photomicrographs of CD3-stained sections used for quantification are shown in Fig. 7A.

**PPB-PEG-IFN-γ reduces the number of lymphatic vessels but does not affect PTC density**

UUO has been associated with time-dependent angiogenesis and capillary rarefaction (35). We therefore analyzed the effect of PPB-PEG-IFN-γ on PTC density by quantifying the number of CD31+ PTCs expressed as the percent CD31 surface area. Moreover, we and others have shown earlier that renal fibrosis is associated with increased lymph vessel formation in different renal disease models (36–40). This led us to hypothesize that UUO-induced renal

---

**Figure 4.** Fibroblast-specific delivery of PPB-PEG-IFN-γ in mouse UUO kidneys. In vivo localization of PPB-PEG-IFN-γ conjugate and PDGFRβ receptor in sham (A) and UUO (B) mouse kidneys. Representative immunofluorescent photomicrographs depict specific binding of PPB-PEG-IFN-γ to PDGFRβ in conjugate-treated animals in comparison to vehicle-treated mice. Expression of PDGFRβ and accumulation of PPB-PEG-IFN-γ were increased in UUO kidneys compared with sham kidneys. PPB is shown in red, PDGFRβ in green, and nuclei in blue (×630). Arrowheads point to myofibroblasts; asterisk indicates glomerulus.
lymphangiogenesis might be attenuated by PPB-PEG-IFN-γ. By use of antibodies against CD31 and podoplanin, we were able to discriminate between PTCs and lymphatics because virtually no podoplanin+ lymphatic vessels showed CD31 positivity (Fig. 8A). Figure 8B, D, respectively, shows photomicrographs of podoplanin and CD31 immunohistochemistry used for quantitative analyses. In our study, UUO was associated with significantly increased PTC density at d 3 and 7 post-UUO compared with sham. No effect of any of the interventions on PTC density was observed (Fig. 8E).

In contrast to PTCs, the number of podoplanin+ lymphatic capillaries at 7 d post-UUO was significantly increased compared with sham. At 7 d post-UUO, PPB-PEG-IFN-γ treatment significantly prevented the UUO-induced increase in lymphatic capillaries, whereas free IFN-γ and PPB-HSA did not affect lymphatic capillary numbers (Fig. 8C).

**PPB-PEG-IFN-γ attenuates IFN-γ–related brain MHC class II expression**

Systemic adverse effects of free IFN-γ resulting from its proinflammatory activity hamper its clinical use as an antifibrotic agent. We previously reported reduced side effects of PPB-PEG-IFN-γ treatment when compared with

---

**Figure 5.** Effect of PPB-PEG-IFN-γ on α-SMA expression in obstructed mouse kidneys. A) Relative gene expression of α-SMA in fibrotic and sham kidneys both at d 3 and 7. B) Computerized quantitative analysis and (C) representative photomicrographs of α-SMA expression in UUO mice treated with PBS, PPB-HSA, (free) IFN-γ, or PPB-PEG-IFN-γ (both d 3 and 7) (×200). PPB-PEG-IFN-γ significantly attenuated UUO-induced α-SMA expression at both mRNA and protein expression levels. Dotted line in graphs represents sham-operated mice. *P < 0.05 denotes significance vs. vehicle groups; **P < 0.05 vs. sham.
free IFN-γ (30). To follow up on this, we here determined MHC II mRNA expression in brain tissue (as readout for systemic adverse effects) from UUO mice (d 7). As shown in Fig. 9, treatment with free IFN-γ significantly increased brain MHC class II expression, which was markedly reduced in the PPB-PEG-IFN-γ–treated mice.

DISCUSSION

The present study reveals that specific targeting of IFN-γ to myofibroblasts using a PDGFRβ-recognizing carrier leads to attenuation of fibrosis in obstructed kidneys. Specifically, the results demonstrate that PPB-PEG-IFN-γ reduces α-SMA expression and ECM deposition, improves tubular morphology, and decreases T-cell infiltration and lymphangiogenesis, as schematically summarized in Fig. 10.

During tubulointerstitial fibrosis, renal fibroblasts maintain their activated phenotype (myofibroblasts), even after the removal of the initial insult (2, 41). Following recurrent tissue injury, myofibroblasts undergo reactivation (42, 43). Although data on the reversibility of fibrotic processes in the kidney are scarce, few studies indicate that resolution of

Figure 6. PPB-PEG-IFN-γ down-regulates fibronectin expression in obstructed mouse kidneys. A) Relative gene expression of fibronectin in UUO and sham kidneys at both d 3 and 7. B) Computerized quantitative analysis and (C) representative photomicrographs of fibronectin expression in kidney sections of UUO mice treated with PBS, PPB-HSA, (free) IFN-γ, or PPB-PEG-IFN-γ (both d 3 and 7) (×200). PPB-PEG-IFN-γ significantly attenuated fibronectin expression at both mRNA and protein levels. Dotted line in graphs represents sham-operated mice. *P < 0.05 vs. sham; **P < 0.05 vs. vehicle; ¥P < 0.05 vs. (free) IFN-γ; ¥¥P < 0.05 vs. PPB-HSA.
renal fibrosis does exist (44–46). Therefore, the clearance of activated fibroblasts is particularly important in order to provide therapeutic strategies to inhibit the progression of tubulointerstitial fibrosis. Selective targeting of antifibrotic compounds to these cells, as shown with IFN-γ in the present study, may therefore pose a novel approach for the development of antifibrosis therapy.

In the field of fibrosis research, IFN-γ has been well recognized as an antifibrotic cytokine due to its direct effect on fibroblasts (18, 47). However, in clinical studies, IFN-γ treatment for idiopathic pulmonary fibrosis and liver fibrosis remained ineffective (17). The main reasons for the lack of beneficial clinical effects are the poor pharmacokinetics of IFN-γ and severe side effects due to ubiquitous expression of the IFN-γ receptor. Therefore, targeted delivery of IFN-γ to specific cells key to the disease process is thought to be a prerequisite to enhance its therapeutic efficacy and reduce side effects. In this study, our strategy was to conjugate the cyclic PDGFRβ-binding peptide (PPB) to mouse IFN-γ via a PEG linker to achieve specific delivery of IFN-γ to PDGFRβ. Although PDGFRβ is known to be expressed on many cell types in several organs, as a matter of fact, its expression is mainly high during early developmental stages but quite low in adult tissues (48, 49). In many pathologic conditions, PDGFRβ expression increases remarkably, especially in fibrotic diseases (24–27). In this study, we also demonstrated increased renal PDGFRβ expression on tubulointerstitial cells in fibrotic mouse kidneys and human kidney biopsies. α-SMA/PDGFRβ double staining revealed selective accumulation of PPB-PEG-IFN-γ in myofibroblasts. To identify the efficacy of our strategy, we examined α-SMA expression both on mRNA and protein levels. We indeed observed a clear inhibitory effect of PPB-PEG-IFN-γ on myofibroblast activation in vitro and in vivo. In addition, we analyzed the expression of ECM components fibronectin and collagen I and III. It has been shown that ECM composition is significantly altered and expanded during fibrosis. This aberrant matrix deposition affects normal resident cell behavior as well as tissue stiffness (2, 4). Compared to free IFN-γ, targeted PPB-PEG-IFN-γ showed enhanced antifibrotic effects (reduced fibronectin and collagen I expression), which stresses the added value of cell-specific targeting.

If coincides with tubular dilation, together referred to as tubulointerstitial injury present in kidneys subjected to UUO (50). Tubular epithelial injury can initiate or sustain progression of fibrosis by different mechanisms such as cytokine production (2, 21, 51). Accumulation of myofibroblasts is also associated with the loss of epithelial integrity and tissue architectural distortion (2). We showed
that targeted IFN-\(\gamma\) improved tubular morphology, which might be explained by direct targeting of myofibroblasts. Another feature of the fibrogenic response in chronic kidney disease is increased inflammation. There is evidence for a direct role of T cells in causing renal IF, which can be explained through 1) direct effects of T cells on fibroblast and pericyte transdifferentiation, and ECM deposition (possibly through the TGF-\(\beta\)1 pathway); 2) induction of a profibrotic phenotype of infiltrating macrophages leading to the secretion of proliferative and profibrotic cytokines and growth factors, such as TGF-\(\beta\)1, connective tissue growth factor, and PDGF, that can induce fibroblast proliferation, migration, and differentiation; and 3) acting directly on tubular epithelial cells to induce cytokine and growth factor secretion, which in turn act on fibroblasts (33, 34). We found a clear difference in the number of infiltrating T cells in PPB-PEG-IFN-\(\gamma\)-treated mice when compared with control groups. The mechanism by which T cells enhance fibrosis still needs to be elucidated (34). Reduced T-cell influx observed in our study can be explained by 2 different mechanisms: myofibroblasts have been shown to be capable of secreting several kinds of proinflammatory cytokines and mediators, such as RANTES, which is a potent chemoattractant to T cells (52–54). Therefore, inactivation of myofibroblasts due to the local delivery of IFN-\(\gamma\) reduces the release of these mediators, thereby resulting in the reduced influx of T cells. Another explanation for T-cell influx reduction could be via reduced lymphangiogenesis (as discussed below). However, further studies are needed to unravel the detailed mechanism(s).

**Figure 8.** Effect of PPB-PEG-IFN-\(\gamma\) on the number of PTCs (angiogenesis) and lymphatic vessel capillaries (lymphangiogenesis). (A) Double staining for CD31 (green) and podoplanin (red) revealed no colocalization, indicating that these markers can be used to discriminate between PTCs (CD31) and lymphatic capillaries (podoplanin). (B) and (D) show, respectively, representative images of podoplanin and CD31 stainings used for quantification (d 7) (\(\times 400\)). (C) and (E) display quantitative analyses of the number of podoplanin\(^+\) lymphatic capillaries and CD31\(^+\) PTCs, respectively, at both d 3 and 7. Arrowheads point to lymph vessel.

**Figure 9.** Brain MHC class II mRNA expression in UUO mice. Free IFN-\(\gamma\) increases MHC class II expression (an adverse effect of systemic delivery), which is attenuated by PPB-PEG-IFN-\(\gamma\). *\(P < 0.05\) vs. vehicle; \#\(P < 0.05\) vs. PPB-HSA; §\(P < 0.05\) vs. (free) IFN-\(\gamma\).
Angiogenesis and lymphangiogenesis are common findings in various renal fibrotic diseases (55–58). Injured tubular epithelium and inflammatory cells secrete several mediators and growth factors that promote angiogenesis and/or lymphangiogenesis. Angiogenesis was shown to occur during the early stage of fibrosis in UUO, which is followed by capillary rarefaction (58, 59). We evaluated angiogenesis in our study and showed increased PTC density in UUO, which was not influenced by any of the IFN-γ interventions. This also justifies that binding of PPB (PPB-HSA) to PDGFβR does not lead to the receptor blocking therefore not influencing angiogenesis. Recently, fibrosis in UUO was shown to be associated with lymphangiogenesis (40), and we previously showed similar results in other experimental kidney disease models in rats (36, 38, 39). Because antilymphangiogenic effects of IFN-γ have been reported (60), we analyzed the potential inhibitory effect of PPB-PEG-IFN-γ on lymphangiogenesis. As reported by others (6, 40), we also observed lymphangiogenesis in vehicle-treated UUO mice, which was, in contrast to free IFN-γ, significantly attenuated by PPB-PEG-IFN-γ. A potential consequence of reduced lymphangiogenesis was reduced T-cell influx (61), which then also contributed to reduced fibrosis.

To determine that the antifibrotic effects were not attributed to PPB-induced blockade of PDGFRβ signaling, we coupled PPB to HSA. PPB-HSA significantly attenuated α-SMA and ECM (e.g., fibronectin) expression on both the mRNA and protein (3) level. The reduction in fibrosis was accompanied by preserved tubular morphology, reduced T-cell influx, and reduced lymphangiogenesis (4).

Figure 10. Schematic representation of the in vivo data obtained in this study on the efficacy of the targeted delivery of IFN-γ to PDGFRβ-expressing myofibroblasts in renal fibrosis. PPB-PEG-IFN-γ was synthesized (1), which was then applied in vivo in the UUO model for renal fibrosis in mice (2). Histologic analyses revealed that PPB-PEG-IFN-γ treatment significantly reduced α-SMA and ECM (e.g., fibronectin) expression on both the mRNA and protein (3) level. The reduction in fibrosis was accompanied by preserved tubular morphology, reduced T-cell influx, and reduced lymphangiogenesis (4).
effects of IFN-γ take place via the nuclear signaling sequence (NLS), which is present in its C terminus region (63, 64). This region is able to modulate IFN-γ-responsive genes through activation of the JAK/STAT (Janus kinase/signal transducers and activators of transcription signaling pathway) pathway (65, 66). We indeed previously showed activation of STAT1 by PEG-PPB-IFN-γ (30). We therefore propose that PEG-PPB-IFN-γ is taken up via PDGFRβ, and the internalized construct next releases IFN-γ or its metabolite intracellularly, which binds to IFN-γ receptor 1 (IFN-γR1). IFN-γR1 has the JAK1 and STAT1-binding site on the intracellular surface. IFN-γ containing the NLS was capable of binding to IFN-γR1 and initiating a cascade of events, which is required for nuclear import of STAT1 and generation of biological activity. However, this premise needs to be further explored.

The main reason for the limited clinical utility of IFN-γ so far is its adverse side effects following systemic administration. IFN-γ is a proinflammatory cytokine, and its receptor is expressed on almost all nucleated cells. Therefore, systemic delivery of IFN-γ results in immune cell and endothelial cell activation, flu-like symptoms, neurotropic effects, hyperlipidemia, elevated TNF-α and IL-6, CNS inflammation, and elevated triglycerides. Conspicuously, these side effects were almost completely absent following PEG-PPB-IFN-γ administration (30), as also evidenced in this study by reduced brain MHC class II expression.

In conclusion, in this study, we demonstrate the benefit of targeted delivery of IFN-γ to attenuate fibrosis in the UUO mouse model. We provide a novel strategy of IFN-γ therapy by directing it to myofibroblasts, which increases its therapeutic potential due to enhanced efficacy and reduced off-target side effects. We believe this strategy is a promising approach to target antifibrotic cytokines to myofibroblasts to effectively halt renal fibrosis.

The authors thank Catharina Reker-Smit, Geert Harms, Marian Bulthuis, and Sippie Huitema for their technical assistance. This work was supported by the J.K. de Cock Foundation (University Medical Center Groningen). Microscopical imaging was performed at the UMCG Imaging Center, which is supported by The Netherlands Organisation for Health Research and Development (ZonMW Grant 40/00506-08/0021). F.P. was supported by the Graduate School of Medical Sciences (University of Groningen, Groningen, The Netherlands). M.H.d.B. was supported by a grant from The Netherlands Organization for Scientific Research (Venin grant).

REFERENCES


IFN-γ DELIVERY TO PDGFRβ REDUCES RENAL FIBROSION

fasebj.org by Bibliothek der Rijksuniversiteit van Groningen (129.125.136.170) on June 17, 2019. The FASEB Journal Vol. 31(article.issue.getVolume()) No. 8(article.issue.getIssueNumber()) pp. 1029-1042.


Received for publication July 20, 2014. Accepted for publication October 30, 2014.