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RESEARCH PAPER

An *in vitro* model for the pro-fibrotic effects of retinoids: mechanisms of action

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BACKGROUND AND PURPOSE

Retinoids, including all-*trans* retinoic acid (tRA), have dose-dependent pro-fibrotic effects in experimental kidney diseases. To understand and eventually prevent such adverse effects, it is important to establish relevant *in vitro* models and unravel their mechanisms.

EXPERIMENTAL APPROACH

Fibrogenic effects of retinoids were assessed in NRK-49F renal fibroblasts using picro-Sirius red staining for collagens and quantified by spectrophotometric analysis of the eluted stain. Other methods included RT-qPCR, immunoassays and matrix metalloproteinase (MMP) activity assays.

KEY RESULTS

With or without TGF-β1, tRA was dose-dependently pro-fibrotic, notably increasing collagen accumulation. tRA and TGF-β1 additively suppressed expression of mRNA for MMP2, 3 and 13 and suppressed MMP activity. tRA, in the presence of TGF-β1, induced plasminogen activator inhibitor-1 (PAI-1) mRNA and they additively induced PAI-1 protein expression. A PAI-1 inhibitor, a pan-retinoic acid receptor (RAR) antagonist and a pan-retinoid X receptor (RXR) antagonist each partially prevented the pro-fibrotic effect of tRA. The dose-dependent pro-fibrotic effects of a pan-RXR agonist were similar to those of tRA. A pan-RAR agonist showed weaker, less dose-dependent pro-fibrotic effects and the pro-fibrotic effects of RARα and RARβ-selective agonists were even smaller. An RARγ-selective agonist did not affect fibrogenesis.

CONCLUSIONS AND IMPLICATIONS

An *in vitro* model for the pro-fibrotic effects of retinoids was established in NRK-49F cells. It was associated with reduced MMP activity and increased PAI-1 expression, and was probably mediated by RXR and RAR. To avoid or antagonize the pro-fibrotic activity of tRA, further studies on RAR isotype-selective agonists and PAI-1 inhibitors might be of value.

Abbreviations

CKD, chronic kidney disease; ECM, extracellular matrix; ITS, insulin-transferrin-selenium; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; PSR, picro-Sirius red; RA, retinoic acid; RAR, retinoic acid receptor; RT-qPCR, reverse transcription quantitative polymerase chain reaction; RXR, retinoid X receptor; TIF, tubulointerstitial fibrosis; TIMP, tissue inhibitors of metalloproteinases; tRA, all-*trans* retinoic acid; TTNPB, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid; UUO, unilateral ureteric obstruction

Introduction

Chronic kidney disease (CKD) represents a significant health problem in the United Kingdom and worldwide (El Nahas and Bello, 2005). Among all predictors of CKD progression, tubulointerstitial fibrosis (TIF), characterized by excessive accumulation of collagens and other extracellular matrix (ECM) proteins in the renal interstitial compartment, is regarded the common pathway to end-stage renal disease (Nangaku, 2004).

It is now known that TIF is mediated by fibrogenic factors such as TGF-β1, which lead to increased production and/or reduced degradation of ECM by effector cells, especially fibroblasts (Liu, 2011). ECM degradation occurs via two major pathways, the serine protease plasminogen–plasmin system and the matrix metalloproteinase (MMP) system. Inhibitors of these systems include plasminogen activator inhibitor-1 (PAI-1) for the former and tissue inhibitors of metalloproteinases (TIMPs) for the latter (Catania *et al.*, 2007; Eddy, 2009).

PAI-1 is an inhibitor of plasminogen activators, which convert plasminogen to plasmin. The latter can degrade matrix proteins directly or indirectly through activating MMPs (Eddy, 2009). PAI-1 has been proposed as an important mediator of TIF: (i) it is up-regulated in many CKDs with progressing TIF (Eddy, 2009); (ii) its non-inhibitory mutant inhibits renal collagen deposition in experimental nephritis and diabetic nephropathy (Huang *et al.*, 2003; 2008); (iii) its deficiency reduces TIF in unilateral ureteric obstruction (UUO; Oda *et al.*, 2001) and TGF-β1 transgenic mice (Krag *et al.*, 2005); and (iv) UUO induces more fibrosis in PAI-1 transgenic mice than in wild-type mice (Matsuo *et al.*, 2005).

The MMPs, a family of zinc-dependent ECM-degrading enzymes, have been implicated in the pathogenesis of TIF and are regarded as potential therapeutic targets (Catania *et al.*, 2007). However, roles for MMPs are likely to be more complex. For example, MMP-2 has been proposed to mediate macrophage infiltration (Nishida *et al.*, 2007) and epithelial-to-mesenchymal transition (Cheng and Lovett, 2003), thus promoting inflammation and fibrosis.

Retinoids are vitamin A and its derivatives, including all-*trans* retinoic acid (tRA) and 9-*cis* retinoic acid (9-*cis* RA). While tRA is a pan-agonist of all the three isotypes (α, β and γ) of RA receptors (RARs), 9-*cis* RA is a pan-agonist of not only RARs, but also retinoid X receptors (RXRs; Chambon, 1996; receptor nomenclature follows Alexander *et al.*, 2011). Pharmacologically, retinoids have shown promise in treating CKD models, with a suggested anti-fibrotic potential (Xu *et al.*, 2004). However, more recently, dose-dependent pro-fibrotic effects of retinoids were reported in experimental models of CKD, although the cellular and molecular mediators remained elusive (Morath *et al.*, 2009; Xu *et al.*, 2010). If retinoids are to be considered further for the treatment of CKD, their pro-fibrotic effects must be better defined.

We hypothesized that tRA was pro-fibrotic by activating renal fibroblasts. This hypothesis was supported by our *in vitro* findings of a dose-dependent, net pro-fibrotic effect of tRA in NRK-49F normal rat kidney fibroblasts, which was associated with reduced MMP activity and increased PAI-1 expression. Studies of RXR and RAR agonists and antagonists indicated that tRA was likely to be acting through retinoid receptor-dependent pathways and that isotype-selective RAR agonists may have reduced pro-fibrotic activities.

Methods

Cell culture

NRK-49F normal rat kidney fibroblasts (LGC Standards, Teddington, UK) were maintained in DMEM (PAA Laboratories GmbH, Pasching, Austria) supplemented with 5% fetal calf serum (FCS; Sigma-Aldrich Company Ltd., Gillingham, UK), penicillin 100 IU·mL^{-1}, streptomycin 100 μg·mL^{-1} (PAA Laboratories GmbH) and amphotericin B 2.5 μg·mL^{-1} (Invitrogen, Paisley, UK) under humidified conditions at 37°C and 5% CO_2. A human foreskin fibroblast primary culture (a kind gift from Dr. Carole Yee, National Institutes of Health, Bethesda, MD, USA) was maintained in DMEM supplemented with 10% FCS and antibiotics and anti-fungals as reported before (Xu *et al.*, 2007). Cells between passages 8 and 12 were used for experiments. NRK-49F cells and human foreskin fibroblasts showed typical morphological appearances of fibroblasts and stained positive for the fibroblast marker vimentin, but negative for the epithelial marker cytokeratin.

Experimental design

NRK-49F cells and human foreskin fibroblasts were cultured in DMEM supplemented with 2.5% FCS and 2.5% Nu-Serum serum replacement (BD Biosciences, Oxford, UK) for 3 days followed by DMEM with insulin-transferrin-selenium supplement (ITS; Sigma-Aldrich) for 4 days as previously defined (Grotendorst *et al.*, 2004). Cells, with or without pretreatment with retinoid receptor antagonists and the PAI-1 inhibitor tiplaxtinin, were then treated with retinoids with or without 5 ng·mL^{-1} TGF-β1 in DMEM supplemented with ITS for 48 h. Vehicle-treated cells were included as controls.

In vitro *model of fibrosis*

An *in vitro* model of fibrosis was used to quantify global fibrogenesis (Xu *et al.*, 2007). In brief, 1×10^4 cells per well cultured in collagen type I-coated 96-well plates (BD Biosciences) were treated with TGF-β1 and other compounds as described earlier, fixed in ice-cold methanol overnight at –20°C, washed with Dulbecco's PBS and then incubated with 0.1% picro-Sirius red (PSR; Sigma-Aldrich) for 4 h. Following rinses in 0.1% acetic acid (VWR International Ltd., Lutterworth, UK), plates were air-dried and examined by photomicroscopy. For quantification of PSR staining, 200 μL 0.1 M sodium hydroxide (Sigma-Aldrich) was added and the plate rocked on a rocking platform for 2 h to elute the stain. Optical density was determined using a spectrophotometer (Prior Laboratory Supplies Ltd., East Sussex, UK) at 540 nm.

Cytotoxicity assays

Cell detachment index was recorded to estimate cytotoxicity and to ensure the reliability of subsequent PSR staining results, which required minimal cell (and matrix) detachment and disruption of cell monolayer. Scores of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 represented an area of approximately 0, 5, 10, 20, 30, 40, 60, 80 and 100%, respectively, of the total adherent surface not covered by cells. Furthermore, LDH release was determined by CytoTox 96® Non-Radioactive Cytotoxicity Assay according to manufacturer's instructions (Promega, Southampton, UK).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Crawley, UK) according to manufacturer's instructions. The RT^2 First Strand Kit (Tebu-bio, Peterborough, UK) and the Omniscript Reverse Transcription Kit (Qiagen) were

used in reverse transcription for qPCR array and standard qPCR studies, respectively. In a pilot screening, a PCR array kit for rat ECM and adhesion molecules (Qiagen) was used according to the manufacturer's instructions on an ABI7900HT qPCR system (Applied Biosystems, Warrington, UK). For standard RT-qPCR, cDNA was mixed with 20 × TaqMan primer and probe mixtures, TaqMan Universal Master Mix (Applied Biosystems) and water, and qPCR was run on an ABI7900HT system. Where TaqMan gene expression assays were unavailable, SABiosciences RT^2 qPCR primer assays were used with RT^2 SYBER Green/ROX 2 × qPCR Master Mix (Qiagen). Supporting Information Table S1 lists the gene expression assays used in this study. Results were presented as fold change of gene expression relative to the untreated control sample and normalized to GAPDH ($2^{-\Delta\Delta CT}$; Livak and Schmittgen, 2001).

Immunocytochemistry and photomicroscopy

NRK-49F cells cultured in 35 mm dishes were fixed with 3.7% paraformaldehyde (Sigma-Aldrich) on ice for 10 min, washed and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 5 min. Cells were washed again and then blocked with 1% BSA (PAA Laboratories GmbH) for 1 h, before incubation with primary antibody (Supporting Information Table S2) in PBS for 1 h. After three washes, cells were incubated with 1:1000 diluted secondary antibody labelled with Alexa Fluor 488 (Invitrogen) overnight at 4°C. Cells were again washed and counterstained with 1 μg·mL^{-1} DAPI in PBS, followed by further washing and examination under a Nikon Eclipse TE2000-S epifluorescence microscope equipped with a standard RGB filter wheel (Nikon Instruments Europe B.V., Amstelveen, The Netherlands). Images were captured with a DXM1200F Nikon digital camera (Nikon UK Limited, Surrey, UK), processed and merged with Adobe Photoshop (Adobe Systems Europe Ltd., Uxbridge, UK).

Protein extraction

Conditioned media of NRK-49F cells cultured in 10 cm dishes were stored at –80°C until use. 500 μL PBS-TDS cell lysis buffer without (for MMP activity assays) or with (for all other applications) protease inhibitors (leupeptin, pepstatin, PMSF and EDTA, all Sigma-Aldrich) was added to the cell monolayer and the dishes incubated on ice for 1 h. Total cell lysate was transferred to a microcentrifuge tube on ice and sheared through a 25 G needle using a syringe. Samples were then centrifuged and supernatants collected and stored at –80°C until use. Protein concentration of samples was determined using the bicinchoninic acid method (Pierce Protein Research Products, Thermo Scientific, Rockford, IL, USA).

Western blotting

Total cell lysates, biotinylated protein ladder (Cell Signalling Technology, Danvers, MA, USA) and Amersham's full range rainbow molecular weight marker (GE Healthcare, Little Chalfont, UK) were separated by routine SDS-PAGE, and protein was transferred onto Amersham's Hybond C-super nitrocellulose membrane (GE Healthcare). Membrane was blocked in 5% skimmed milk blocking solution for 2 h and then incubated with primary antibody (Supporting Information Table S2) overnight at 4°C on a rocking platform. Following washing with Tris-buffered saline with 0.1% Tween 20 the membrane was incubated with secondary antibody (Supporting Information Table S2) conjugated to HRP for 1 h and then washed. The membrane was developed using Amersham's ECL Plus Western Blotting detection Reagents (GE Healthcare) and exposed to Amersham's Hyperfilm ECL chemiluminescence film (GE Healthcare). Band densitometry was analysed using Adobe Photoshop. Results were normalized to a loading control.

MMP activity assay

The Molecular Probes EnzChek Gelatinase/Collagenase Assay Kit (Invitrogen) was used to determine MMP activity in total cell lysates according to the manufacturer's instructions. In brief, 100 μL total cell lysates were added to each assay well in a 96-well assay plate and mixed with 20 μL 1 mg·mL^{-1} DQ™ gelatin and 80 μL 1× reaction buffer. Reaction buffer alone was used as a negative control and *Clostridium* collagenase as a positive control. Reactions were performed in triplicate and the plate was incubated at room temperature protected from light for 2 h. Fluorescence intensity was measured using a BioTek FLx800 fluorescence microplate reader (BioTek UK, Potton, UK) at an absorption of 485 nm and fluorescence emission detection of 530 nm. Results were corrected for background fluorescence by subtracting the value derived from the negative control.

Data analysis

PCR array data were analysed using SABiosciences qPCR Array Data Analysis Web portal (Qiagen). All other data were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Parametric data were analysed using a paired *t*-test when comparing two groups. A repeated measures ANOVA with Tukey's post-test was used for analysis of multiple groups. Results presented as fold change were logarithmically transformed before statistical analysis. $P < 0.05$ was defined as statistically significant.

Materials

Human platelet TGF-β1 (R&D Systems, Abingdon, UK) was reconstituted in sterile 4 mM HCl and 0.1% BSA to make a stock solution of 10 ng·μL^{-1}. tRA (Sigma-Aldrich, Gillingham, UK) and (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB; a kind gift from R.A.S. Chandraratna, Irvine, California, USA) were dissolved in 100% ethanol. AGN194204, AGN193109 (kind gifts from R.A.S. Chandraratna), HX531 (a kind gift from H. Kagechika, Tokyo, Japan), CD437, CD2019, AGN195183 (synthesized in-house by the J Corcoran group) and tiplaxtinin (Axon Medchem, Groningen, The Netherlands) were dissolved in DMSO. All aliquots were stored at –80°C until use.

The dissociation constants (K_d) and EC_{50} of pan-RAR agonist TTNPB are 2.5, 2.7 and 1.8 nM and 10, 3.5 and 2.5 nM for mouse RARα, RARβ and RARγ, respectively (Pignatello *et al.*, 1997; 1999). RARα-selective agonist AGN195183 binds to RARα with a K_d of 3 nM and has an EC_{50} of 200 nM with negligible binding to RARβ and RARγ (Beard *et al.*, 2002). RARβ-selective agonist CD2019 binds to RARβ with a K_d of 26 nM (compared with 920 nM and 160 nM for RARα and RARγ) and has an EC_{50} of 3.80 nM for RARβ

(compared with 19.80 nM and 46.90 nM for RARα and RARγ; Bernard *et al.*, 1992). RARγ-selective agonist CD437 binds to RARγ with a K_d of 77 nM (compared with 6500 nM and 2480 nM for RARα and RARβ) and has an EC_{50} of 7.3 nM for RARγ (compared with 140 nM and 28.40 nM for RARα and RARβ; Bernard *et al.*, 1992). Pan-RXR agonist AGN194204 has K_d values of 0.4, 3.6 and 3.8 nM and EC_{50} of 0.2, 0.8 and 0.08 nM for RXRα, RXRβ and RXRγ, respectively. It does not bind to RARs (Vuligonda *et al.*, 2001). Pan-RAR antagonist AGN193109 binds to RARα, RARβ and RARγ with K_d values of 2, 2 and 3 nM, respectively (Johnson *et al.*, 1995). Pan-RXR antagonist HX531 inhibits both RXR homodimers and RXR heterodimers and has an IC_{50} of 0.018 nM (Ebisawa *et al.*, 1999). The K_d of the PAI-1 inhibitor tiplaxtinin is 480 nM and its IC_{50} is 2.7 μM (Elokdah *et al.*, 2004).

Results

Net pro-fibrotic effect of tRA in NRK-49F cells and human foreskin fibroblasts

In NRK-49F cells, tRA dose-dependently increased PSR staining for total collagen, with or without 5 ng·mL^{-1} TGF-β1 (Figure 1A, B). Up to 5 μM tRA alone did not increase LDH release, but with TGF-β1, 5 μM tRA did (Figure 1C). 0.02–5 μM tRA did not increase cell detachment with and without TGF-β1 (Figure 1D), but higher concentrations of tRA (e.g. 10 μM) induced cell detachment and LDH release, indicating cytotoxicity (data not shown). Immunocytochemistry showed that both with and without 5 ng·mL^{-1} TGF-β1, 2 μM tRA induced collagen types I and III (Figure 1E), while no collagen type IV was detected in any groups (data not shown). Of note, the pro-fibrotic effect of tRA was not unique to NRK-49F cells. A similar dose-dependent pro-fibrotic effect of tRA was also observed in human foreskin fibroblasts (Supporting Information Figure S1A, B).

tRA and TGF-β1 down-regulated MMP expression and activity in NRK-49F cells

RT-qPCR array analysis of mRNA expression in a single biological study showed that, both with and without TGF-β1, tRA tended to suppress many and induce a few MMPs, while it tended to also suppress TIMPs (Supporting Information Figure S2). Since these data were from a single biological study, standard RT-qPCR was also performed for MMPs-2, -3 and -13. tRA and TGF-β1 reduced MMP-3 and MMP-13 mRNA expression at 24 and 48 h; although MMP-2 mRNA was reduced at 24 h, complex changes were observed at 48 h (Figure 2B, C and D). To determine net MMP activities, we examined total cell lysates of NRK-49F cells subjected to different treatments. Although tRA and TGF-β1 both significantly reduced MMP activity, no additive effect was observed in the combined treatment group (Figure 2A).

tRA and TGF-β1 additively induced PAI-1, which might play a role in tRA/TGF-β1 dual treatment induced fibrogenesis in NRK-49F cells

In view of the important role for PAI-1 in renal fibrosis its mRNA expression was examined by RT-qPCR. TGF-β1 caused significant increases in PAI-1 mRNA expression at 6 h and 24 h, which showed a trend of further increases in the combined treatment group (Figure 3A). In total cell lysates, both tRA and TGF-β1 induced a trend towards an increase in PAI-1 protein levels and combined treatment significantly increased PAI-1 protein expression (Figure 3Bi, 3Bii). In conditioned media, tRA, TGF-β1 and combined treatment all significantly increased PAI-1 protein levels (Figure 3Bi, 3Bii).

To examine if tRA-induced fibrogenesis could be prevented by PAI-1 inhibition, cells were pretreated for 2 h with the PAI-1 inhibitor tiplaxtinin before addition of tRA and/or TGF-β1 for 48 h. As expected, a significant increase in fibrogenesis was induced by tRA with and without TGF-β1 (Figure 3Ci). Low concentrations (1 and 2.5 μM) of tiplaxtinin alone had no effect on fibrogenesis in vehicle and TGF–β1-treated cells; however, 2.5 μM tiplaxtinin significantly reduced the increase in PSR staining caused by tRA and combined treatment (Figure 3Ci). As 2.5 μM tiplaxtinin also increased LDH release (Figure 3Cii), the relationship between suppression of PAI-1, suppression of fibrogenesis and induction of cytotoxicity needs further examination.

tRA-induced fibrogenesis was partially inhibited by pan-RAR and pan-RXR antagonists

As shown in Supporting Information Figure S3, NRK-49F cells expressed both RAR and RXR, including all three RAR isotypes. To test the possible roles of RAR and RXR in tRA-induced fibrogenesis, we preincubated cells with a pan-RAR antagonist AGN193109 or a pan-RXR antagonist HX531 before tRA and/or TGF-β1 treatment. To abolish RAR and RXR activity of tRA or 9-*cis* RA, a 5–10 fold excess of AGN193109 and HX531 has been recommended (Johnson *et al.*, 1995; Ebisawa *et al.*, 1999; Xu *et al.*, 2002). We found that neither 1 μM AGN193109 nor 1 μM HX531 had any effects on TGF-β1-induced fibrogenesis, however, they both significantly suppressed fibrogenesis induced by combined treatment with tRA (0.2 μM) and TGF-β1 (Figure 4Ai, Bi). Neither antagonist caused significant cell detachment (Figure 4Aii, Bii).

Effects of pan-RAR and pan-RXR agonists on fibrogenesis in NRK-49F cells

We next examined the effects of a pan-RAR agonist TTNPB and a pan-RXR agonist AGN194204 on fibrogenesis with and without TGF-β1. We found that without TGF-β1, TTNPB caused a small but significant increase in fibrogenesis at the highest dose tested (2 μM), in contrast to tRA, which was pro-fibrotic at ten-fold lower concentrations (Figure 5Ai). In the presence of TGF-β1, 0.02–2 μM TTNPB caused a significant, non-dose-dependent increase in PSR staining, similar to the effects of 0.02 μM tRA (Figure 5Ai), without causing significant cell detachment (Figure 5Aii). 0.02–2 μM AGN194204 induced a dose-dependent increase in PSR staining both without and with TGF-β1, similar to that observed with tRA (Figure 5Bi). In the presence but not the absence of TGF-β1, 2 μM AGN194204 significantly increased cell detachment, suggestive of cytotoxicity (Figure 5Bii).

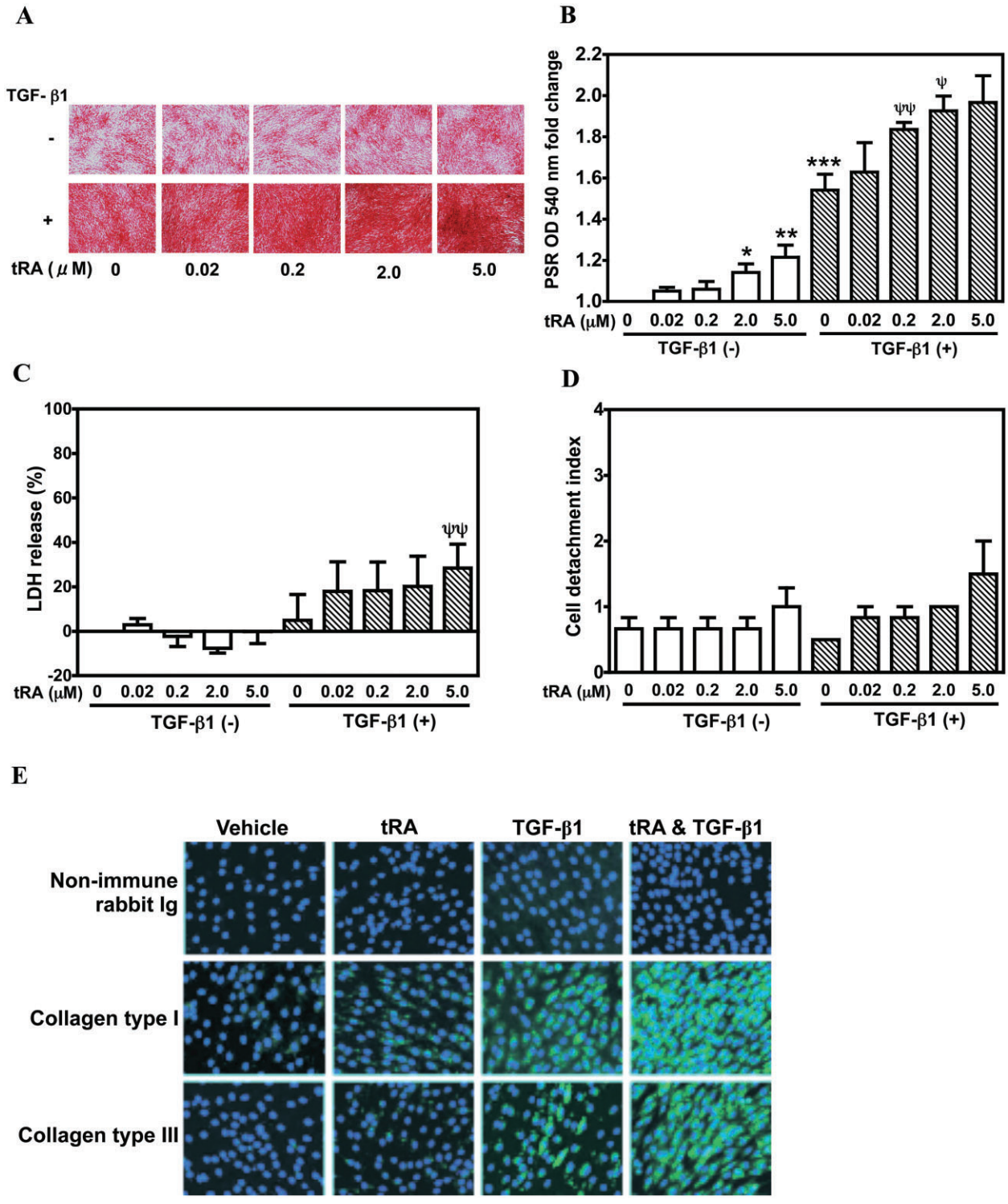

Figure 1

tRA promoted fibrogenesis in NRK-49F cells in the absence and presence of TGF-β1, as indicated by collagen deposition. NRK-49F cells were treated with or without 0.02–5 μM tRA and 5 ng·mL^{-1} TGF-β1 for 48 h. Effects on fibrogenesis were illustrated by representative PSR staining images, which visualized total collagen deposition in red colour (×100 magnification) (A); eluted PSR was quantified by spectrophotometric analysis (B); cytotoxicity was assessed by LDH release (C) and cell detachment (D). Immunocytochemical analysis was used to determine protein expression of collagen types I and III in cells treated without and with 2 μM tRA and 5 ng·mL^{-1} TGF-β1 (fluorescence microscopy ×100 magnification) (E). Data in panels A and E are representative of three independent experiments; data in panels B–D are mean ± SEM of three or more independent cell culture studies. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle (0.1% ethanol) group; $^{\psi}P < 0.05$, $^{\psi\psi}P < 0.01$ versus TGF-β1-treated group.

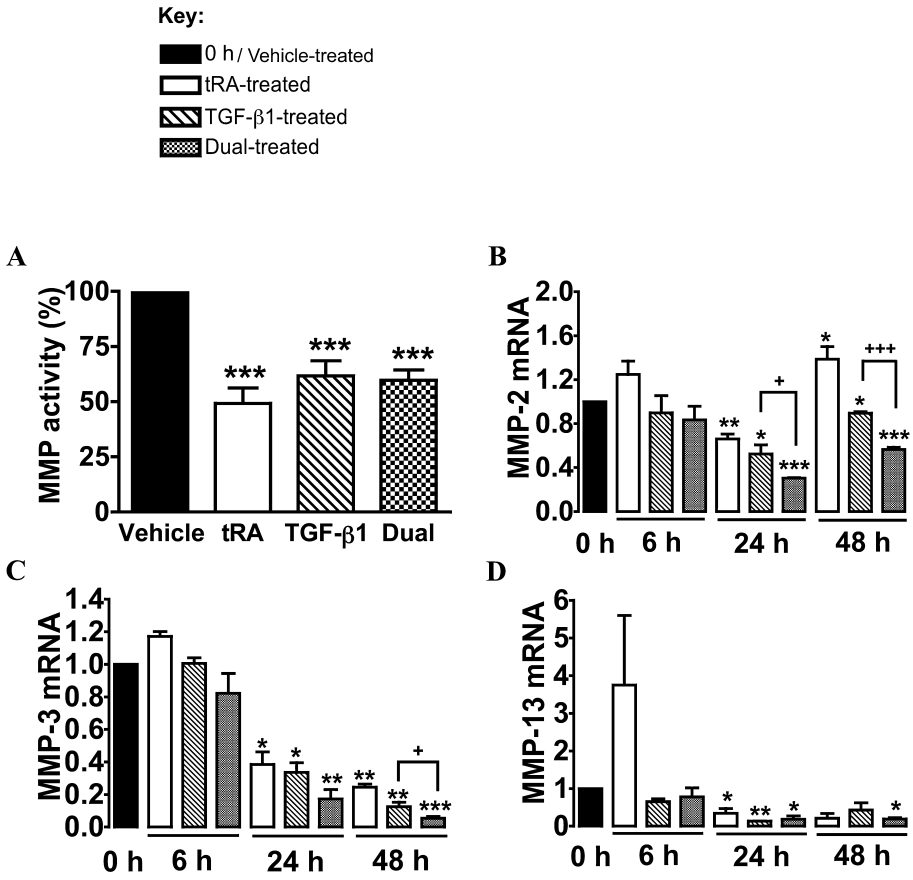

Figure 2

tRA affected MMP activity and MMP-2, MMP-3 and MMP-13 mRNA expression in NRK-49F cells. A. NRK-49F cells were treated with and without 2 μM tRA and 5 ng·mL^{-1} TGF-β1 for 48 h and total cell lysates were subjected to MMP activity assay. Data were normalized to vehicle-treated group (Vehicle) and are shown as means ± SEM from three independent experiments. ***P < 0.001 versus vehicle-treated group. B–D. NRK-49F cells were treated with and without 2 μM tRA and 5 ng·mL^{-1} TGF-β1 for the last 6, 24 and 48 h. RNA was extracted and subjected to RT-qPCR assays for the mRNAs of MMP-2 (B), MMP-3 (C) and MMP-13 (D). Results were normalized to GAPDH and are presented as fold change compared with 0 h. Data represent mean ± SEM of three independent cell culture studies. *P < 0.05, **P < 0.01, ***P < 0.001 in comparison with 0 h; ^{+}P < 0.05, ^{++}P < 0.01, ^{+++}P < 0.001 between groups indicated.

Effects of RAR isotype-selective agonists on fibrogenesis in NRK-49F cells

As a pan-RAR agonist showed smaller pro-fibrotic effects, we further explored the effects of RAR isotype-selective agonists on fibrogenesis. As shown in Figure 5Ci, with or without TGF-β1, 0.2 μM RARα-selective agonist AGN195183 induced significant fibrogenesis. 0.2 and 2 μM RARβ-selective agonist CD2019 did not cause any changes in fibrogenesis alone, however in the presence of TGF-β1 both concentrations mildly, but significantly, increased fibrogenesis. Finally, both with or without TGF-β1, 0.2 and 2 μM RARγ-selective agonist CD437 did not affect fibrogenesis. These isotype-selective RAR agonists did not induce any significant cell detachment (Figure 5Cii).

Discussion

Previous work has identified dose-dependent pro-fibrotic effects of tRA and other retinoids in animal models of CKD, but the cellular and molecular mediators of such an unwanted effect remained elusive. In the present study, a novel *in vitro* model for the dose-dependent pro-fibrotic effects of tRA and other retinoids has been established in a kidney fibroblast cell line, supporting our hypothesis that the pro-fibrotic effects of retinoids might be, at least in part, mediated by their effects on renal fibroblasts. It has also allowed us to identify potential mechanisms behind the pro-fibrotic effect of tRA, including modulation of the expression and activities of MMP and PAI-1, as well as activation of RXR and RAR.

To establish an *in vitro* model of the pro-fibrotic effect of retinoids, one must first try to understand the seemingly opposite effects of retinoids on fibrogenesis documented in the literature. Some retinoids have been suggested to be anti-fibrotic according to their effects on molecular markers of fibrogenesis. For example, in human lung fibroblasts, tRA (but not 9-*cis* RA) decreased the steady-state level of α1(I) collagen mRNA (Krupsky *et al.*, 1994), and suppressed collagen type I and III basal synthesis, while tRA, 13-*cis* RA, 9-*cis* RA and a collection of other retinoids inhibited TGF-β1-

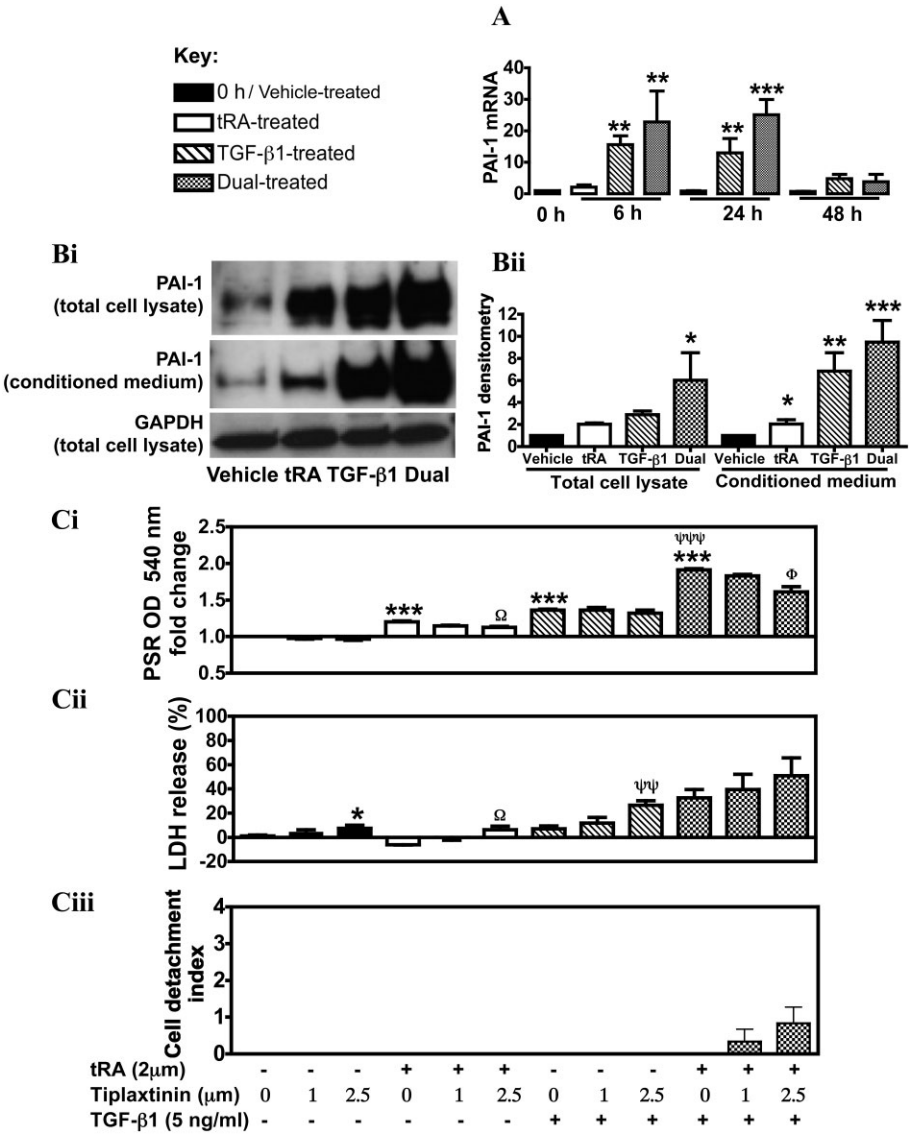

Figure 3

tRA- and TGF-β1- induced PAI-1 expression and the effect of the PAI-1 inhibitor tiplaxtinin on tRA-induced fibrogenesis in NRK-49F cells. A. NRK-49F cells were treated in the last 0, 6, 24, 48 h with 2 μM tRA, 5 ng·mL^{-1} TGF-β1 or both. Total RNA was extracted and subjected to RT-qPCR for PAI-1 mRNA, with GAPDH used as a loading control. B. NRK-49F cells were treated with or without 2 μM tRA and 5 ng·mL^{-1} TGF-β1 for 48 h. Total cell lysates and conditioned media were collected for Western blot analysis, results of which are shown representatively in Bi; the PAI-1 bands of three independent experiments were densitometrically analysed, normalized to the GAPDH band and results were presented as fold change compared with vehicle-treated group (Bii). C. NRK-49F cells were treated with 0–2.5 μM tiplaxtinin, with and without 2 μM tRA and 5 ng·mL^{-1} TGF-β1 for 48 h. The effect on total collagen deposition was determined by PSR staining and quantified by spectrophotometric analysis of eluted PSR (Ci). Cytotoxicity was assessed by LDH release (Cii) and cell detachment index (Ciii). Data represent mean ± SEM of 3 independent cell culture studies. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in comparison with 0 h/vehicle-treated group; $^{\Psi}P < 0.01$, $^{\Psi\Psi\Psi}P < 0.001$ versus TGF-β1-treated group; $^{\Omega}P < 0.05$ versus tRA-only treated group; $^{\Phi}P < 0.05$ versus combined treatment (Dual) group only.

stimulated type I collagen production (Redlich *et al.*, 1995). In human keratinocytes, retinol, retinal, tRA and 13-*cis* RA all suppressed collagen type VII mRNA and protein expression (Chen *et al.*, 1997). tRA, 9-*cis* RA, synthetic RAR agonist TTNPB and RXR agonist AGN194204 have also been reported to reduce synthesis of collagen type I and III and fibronectin in rat hepatic stellate cells (Hellemans *et al.*, 2004). However, in activated hepatic stellate cells, although tRA inhibited synthesis of procollagens type I, III and IV, as well as fibronectin and laminin, 9-*cis* RA increased procollagen I mRNA without affecting the expression of other matrix proteins (Hellemans *et al.*, 1999). The likely ligand type- and cell condition-dependent effects of retinoids have also been reported in skin fibroblasts (Shigematsu and Tajima, 1995; Meisler *et al.*, 1997). In scleroderma and normal human skin fibroblasts, tRA and 13-*cis* RA dose-dependently reduced procollagen production and type I and type III procollagen mRNA expression (Ohta and Uitto, 1987), but in

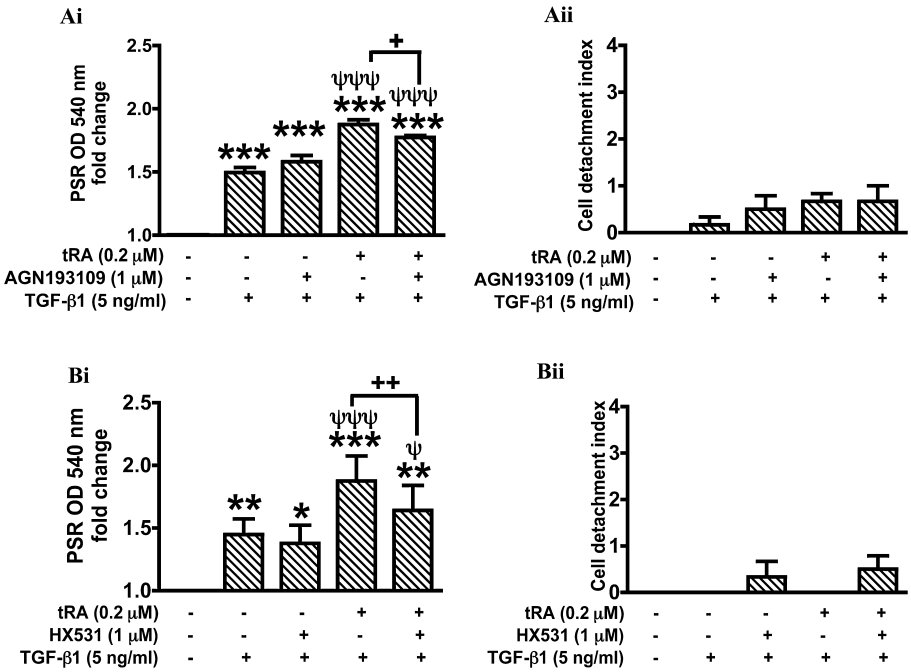

Figure 4

Effects of pan-RAR and pan-RXR antagonists on tRA-induced fibrogenesis in the presence of TGF-β1 in NRK-49F cells. NRK-49F cells were pretreated with 1 μM pan-RAR antagonist AGN193109, 1 μM pan-RXR antagonist HX531 or vehicle for 2 h before treatment with 5 ng·mL^{-1} TGF-β1 with or without 0.2 μM tRA for 48 h. Fibrogenesis was indicated by changes of total collagen deposition as quantified by spectrophotometric analysis of eluted PSR staining (Ai, Bi). Cytotoxicity was assessed by cell detachment index (Aii, Bii). Data represent mean ± SEM of three independent cell culture studies. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle-treated group; $^{\Psi}P < 0.05$, $^{\Psi\Psi\Psi}P < 0.001$ versus TGF-β1-treated group; $^{+}P < 0.05$, $^{++}P < 0.01$ between groups indicated.

growth-inhibited human skin fibroblasts, tRA induced production of fibronectin, thrombospondin, laminin and collagen type I (Varani *et al.*, 1990). These *in vitro* findings are of *in vivo* relevance because, in photodamaged skin, tRA induced collagen types I and III synthesis (Schwartz *et al.*, 1991; Griffiths *et al.*, 1993). Thus, selectivity of retinoids, cell type specificity, choice of pro-fibrotic marker, as well as dose- and condition-dependent factors could all be important in explaining these seemingly opposing effects of retinoids.

To establish an *in vitro* model for the role of retinoids in TIF, we chose NRK-49F renal fibroblasts for the following reasons: (i) this cell type is highly related to TIF and has been widely used in fibrogenesis studies, especially those induced by TGF-β (Roberts *et al.*, 1986; Grotendorst *et al.*, 2004) and (ii) the culture conditions to minimize cell proliferation and maximize the fibrogenic response to TGF-β have already been established (Grotendorst *et al.*, 2004) and used in our efforts to establish TGF-β-induced *in vitro* models of fibrosis in NRK-49F cells (Hu *et al.*, 2009; Wong *et al.*, 2012). In addition, we also screened a number of other cells, which failed to produce a useful fibrotic model due to insignificant increase in PSR staining in response to TGF-β1, with either low or high PSR basal staining (data not shown). The exception was the human foreskin fibroblasts, which showed responses to both tRA and TGF-β1, similar to those of NRK-49F cells (Supporting Information Figure S1). Given the aforementioned condition-dependent effect of retinoids in skin fibroblasts, the pro-fibrotic effect of tRA in human foreskin fibroblasts in our particular conditions could be a useful model for investigating the pro-fibrotic effects of retinoids in skin disorders, if any *in vivo* relevance is established. Likewise, retinoids have been reported to either inhibit or promote liver fibrosis in different animal models (Okuno *et al.*, 1997; Wang *et al.*, 2007) and in hepatic stellate cells, natural and synthetic retinoids are reported to regulate TGF-βl activation, proliferation and synthesis of different ECM proteins (Okuno *et al.*, 1997; Hellemans *et al.*, 2004). However, the net effects of these retinoids on global fibrogenesis and how retinoids could both suppress and potentiate fibrogenesis in hepatic stellate cells are unknown. Thus, it is possible that some of our observations could shed light not only on the dual potential of retinoids in CKD, but also in liver fibrosis.

As demonstrated in Supporting Information Figure S2, tRA and/or TGF-β1 can differentially regulate different markers of fibrosis at the mRNA level. Thus, by selecting only a few molecular markers for measurements, quite different conclusions could be drawn. To avoid this, we visualized total collagen deposition through clinically relevant PSR staining and further quantified it by spectrophotometric analysis, allowing us to define changes of net fibrogenesis (Figure 1A, B), as earlier (Xu *et al.*, 2007; Hu *et al.*, 2009). This was further supported by immunostaining of collagen types I and III (Figure 1E).

Using NRK-49F cells cultured in defined conditions and PSR staining-based fibrogenesis assays, we established dose-dependent, net pro-fibrotic effects of tRA both in the absence

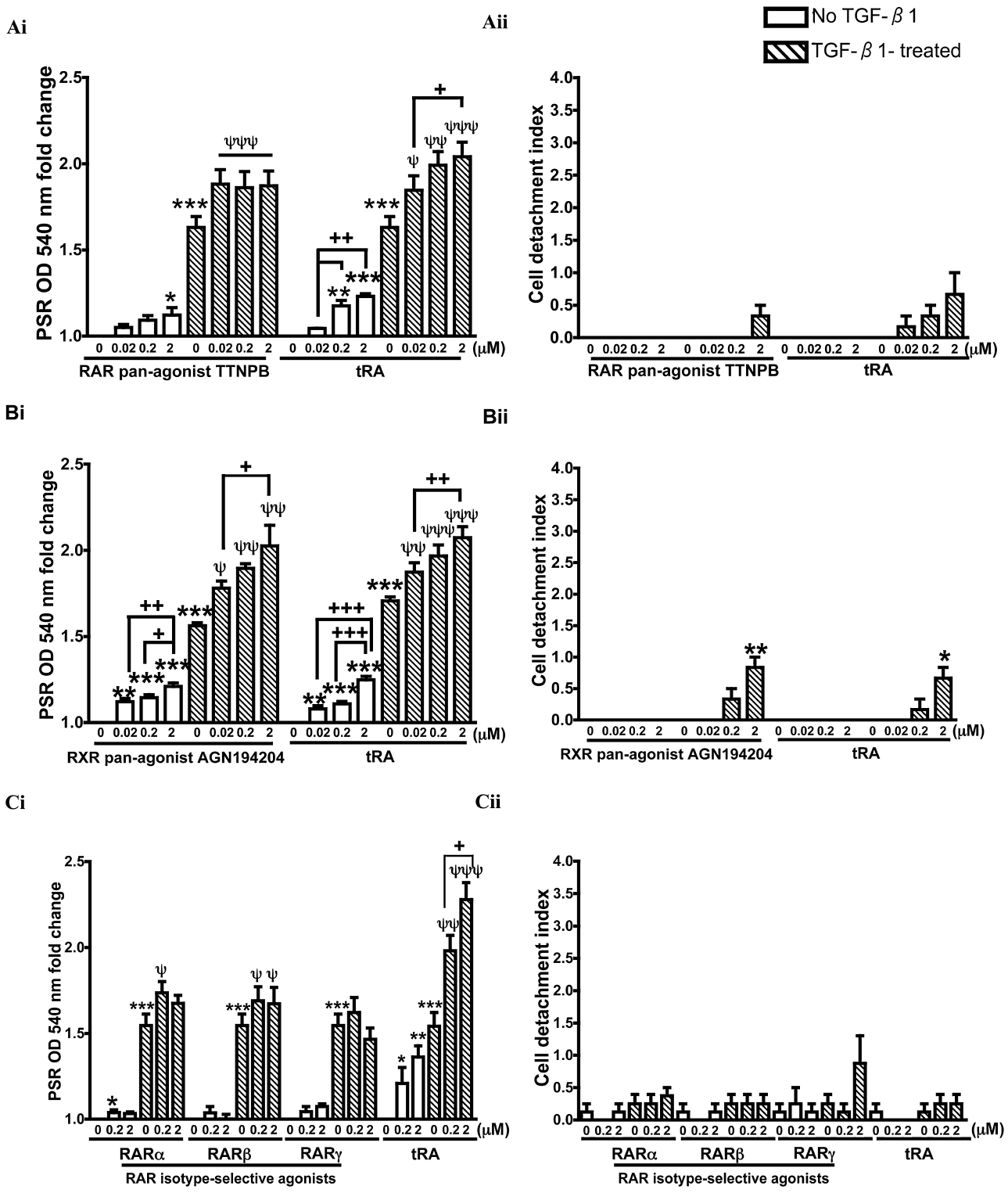

Figure 5

Effects of synthetic RAR and RXR agonists on fibrogenesis in the absence and presence of TGF-β1 in NRK-49F cells. NRK-49F cells were treated for 48 h with vehicle or different concentrations of synthetic RAR and RXR agonists, in the absence and presence of 5 ng·mL^{-1} TGF-β1 and then subjected to quantification of fibrogenesis by spectrophotometric analysis of eluted PSR staining (Ai, Bi and Ci). Cytotoxicity and the integrity of the cell monolayer were assessed by cell detachment index (Aii, Bii and Cii). A. Pan-RAR agonist TTNPB; B. Pan-RXR agonist AGN194204; C. Isotype-selective RAR agonists, including RARα-selective agonist AGN195183, RARβ-selective agonist CD2019 and RARγ-selective agonist CD437. Data represent mean ± SEM of at least three independent cell culture studies. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle-treated group; $^{\psi}P < 0.05$, $^{\psi\psi}P < 0.01$, $^{\psi\psi\psi}P < 0.001$ versus TGF-β1-treated group; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ between groups indicated.

and presence of TGF-β1. In view of the dose-dependent pro-fibrotic effects of tRA in TGF-β1 transgenic mice (Xu *et al.*, 2010) and the synergistic and/or additive effects of tRA and TGF-β1 in inducing net collagen accumulation (Figure 1B), suppressing MMPs (Figure 2) and inducing PAI-1 expression (Figure 3B) in this study, it seems appropriate to call for special caution when tRA is prescribed for CKD patients. This patient group often have increased renal TGF-β1 activity and are thus more prone to fibrosis, therefore they may be more at risk if prescribed tRA either in clinical trials for the treatment of CKD or in other prescriptions for well established indications for tRA. In these *in vivo* and *in vitro* experiments, the pro-fibrotic effect of tRA increased with dose. Thus avoiding over-dose is the first step to consider if pro-fibrotic adverse effects and other dose-related toxicities are to be prevented in CKD.

The pro-fibrotic effects of retinoids might, at least in part, be explained by tRA-induced suppression of ECM-degrading enzymes, including both MMP expression and activation. The latter might be the consequence of tRA-induced PAI-1, which suppresses conversion of plasminogen into plasmin, an activator of MMPs. In fact, both tRA- and TGF-β- induced MMP suppression through inhibition of the AP-1 binding sites in MMP genes has been well documented long before (Benbow and Brinckerhoff, 1997). For example, tRA has been reported to suppress ultraviolet irradiation-induced MMPs in human skin (Fisher and Voorhees, 1998) and to suppress plasma MMP activity and MMP-9/TIMP-1 ratio in cultured macrophages in patients with emphysema (Mao *et al.*, 2003). However, the effect of tRA on PAI-1 expression was somehow surprising. Both tRA and 9-*cis* RA were previously reported to suppress PAI-1 expression in TGF-β-stimulated mesangial cells (Wen *et al.*, 2005; Liu *et al.*, 2008) and in experimental glomerulosclerosis induced by partial nephrectomy (Liu *et al.*, 2011). Furthermore, RAR agonists and antagonists have been previously reported to suppress and potentiate TGF-β-induced PAI-1 promoter activity, respectively (Pendaries *et al.*, 2003). Whether this represents a true cell type-specificity in tRA effect on PAI-1 expression between renal fibroblasts and mesangial cells, two main effectors in TIF and glomerulosclerosis, respectively, deserves further examination. Nevertheless, tRA induction of PAI-1 in NRK-49F cells is somewhat similar to that observed in cultured smooth muscle cells, in which both tRA and 9-*cis* RA induced PAI-1 mRNA expression in a tyrosine kinase-dependent manner (Watanabe *et al.*, 2002). Furthermore, in our study, the PAI-1 inhibitor tiplaxtinin reduced tRA-induced collagen accumulation supporting the hypothesis that PAI-1 was contributing to the pro-fibrotic effect of tRA (Figure 3C). Because chemical inhibitors can have off-target effects as well as their predicted actions, in future work, the results generated from the use of tiplaxtinin will require confirmation via additional interventions such as another PAI-1 inhibitor and/or gene-silencing.

Our data indicated that RXR and RAR were likely to be the mediators of the pro-fibrotic effects of tRA because: (i) NRK-49F cells expressed these receptors (Supporting Information Figure S3); (ii) pan-RAR and pan-RXR agonists, some RAR isotype-selective agonists (Figure 5), but not RAR and RXR antagonists (Figure 4) showed pro-fibrotic effects; and (iii) RAR and RXR antagonists partially blocked tRA-induced total collagen accumulation (Figure 4).

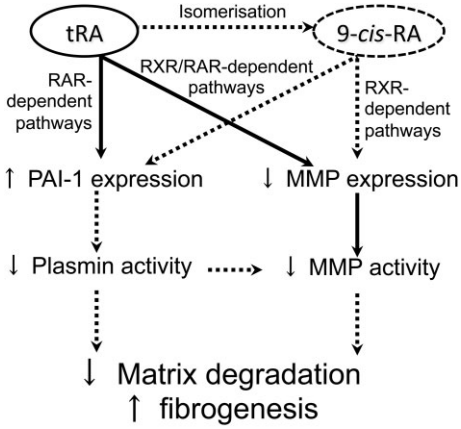

Figure 6

Possible mechanisms of tRA-induced fibrogenesis in this experimental system. tRA-induced pro-fibrotic effect is associated with increased PAI-1 expression and reduced MMP expression. tRA-induced PAI-1 could lead to reduced plasminogen activation into plasmin, which can degrade ECM proteins directly or through activation of MMPs. Thus, both PAI-1 induction and MMP reduction could contribute to decreased degradation and increased accumulation of ECM. Both RXR and RAR pathways are likely to be involved in the pro-fibrotic effects. tRA activation of RXR signalling might be mediated by 9-*cis* RA converted from tRA by isomerases. In terms of the pro-fibrotic effects, RAR agonists were weak and the RXR agonist AGN194204 was as potent as tRA, suggesting that the pro-fibrotic effects of tRA were mainly through RXR-activating metabolites. Solid lines represent pathways directly supported by our existing data; dashed lines are hypothesized pathways.

In this study, RAR and RXR antagonists AGN193109 and HX531 both partially blocked PSR staining induced by tRA and TGF-β1 combined treatment probably by preventing the activation of RARs and RXRs by tRA and its metabolites. Of note, although tRA *per se* is a pan-RAR agonist, in the presence of isomerases, it is believed to produce metabolites such as 9-*cis* RA that can activate RXR as well (Urbach and Rando, 1994). We also found that there were differences between retinoids in terms of their pro-fibrotic potency (Figure 5), ranging from no activity (~0.2–2 μM RARγ-selective agonist CD437) through low (RARα-selective agonist AGN195183, RARβ-selective agonist CD2019) and moderate (pan-RAR agonist TTNPB) to high pro-fibrotic activity (tRA and pan-RXR agonist AGN194204). None of these RAR and RXR agonists showed net anti-fibrotic effects in our system. Nevertheless, given that isotype-selective retinoids have shown promising efficacy in CKD models (Lehrke *et al.*, 2002; Schaier *et al.*, 2004; Zhong *et al.*, 2011), the use of RAR isotype-selective retinoids could be a plausible strategy to avoid unwanted pro-fibrotic effects of tRA.

In summary, we have characterized an *in vitro* model of the pro-fibrotic effect of retinoids in NRK-49F cells, which is associated with reduced MMPs, increased PAI-1 and activation of the RXRs and RARs (Figure 6). In view of the emerging clinical use of retinoids in patients with CKD (Yamane *et al.*, 2009; Kinoshita and Funauchi, 2012), the fibrogenic effects of retinoids need to be better defined *in vitro* and *in vivo* so that the long-term safety profile of retinoids can be improved and

eventually, a viable retinoid-based therapy for CKD, devoid of the unwanted pro-fibrotic effects can be developed.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12348

Figure S1 Effect of tRA and TGF-β1 on fibrogenesis in human foreskin fibroblasts. Fibrogenesis was indicated by total collagen deposition, which was visualized and quantified by PSR staining and spectrophotometric analysis of eluted PSR. Human foreskin fibroblasts cultured in collagen type-I-coated 96-well plates for 3 days in DMEM supplemented with 2.5% FCS and 2.5% Nu then for 4 days in DMEM supplemented with ITS were treated with increasing doses of tRA in the absence and presence of TGF-β1 for 48 h. The effect on total collagen deposition was determined by photomicroscopy of PSR staining (×100 magnification) (A) and quantified by spectrophotometric analysis of eluted PSR (B). Data represent mean ± SEM of three independent cell culture studies. *** $P < 0.001$ versus vehicle; ψ $P < 0.05$, ψψψ $P < 0.001$ versus TGF-β1-treated group.

Figure S2 RT-qPCR array analysis of a pilot study on the effects of tRA and/or TGF-β1 on mRNA expression of selected molecular markers of fibrosis in NRK-49F cells. NRK-49F cells cultured for 3 days in DMEM supplemented with 2.5% FCS and 2.5% Nu then for 4 days in DMEM supplemented with ITS were treated with 2 μM tRA in the absence and presence of 5 ng·mL^{-1} TGF-β1 for 48 h. RNA was extracted and subjected to RT-qPCR using an RT-qPCR array. Fold change in mRNA expression of selected collagens, connective tissue growth factor and fibronectin in TGF–β1- and tRA-treated cells versus vehicle-treated cells (Ai, Bi) and (TGF-β1 + tRA) dual-treated cells versus TGF–β1-treated cells (Ci) are shown in the left panels. The right panels are fold-changes in mRNA expression of the MMPs and TIMPs in TGF–β1-treated cells and tRA-treated cells versus vehicle-treated cells (Aii, Bii) and (TGF-β1 + tRA) dual-treated cells versus TGF–β1-treated cells (Cii). Data represent triplicates from a single cell culture study, therefore statistical analysis was not performed.

Figure S3 Expression of RARs and RXRs in NRK-49F cells without and with tRA and TGF-β1 treatment. (A) Total RNA was extracted from NRK-49F cells cultured for 3 days in DMEM supplemented with 2.5% FCS and 2.5% Nu then for 4 days in DMEM supplemented with ITS and was subjected to RT-qPCR analysis of RAR and RXR mRNAs. The mRNA expression of each receptor was relative to the lowest expressed receptor, RXRγ. Data represent mean ± SEM of nine independent experiments. (B) NRK-49F total cell lysate was extracted from cells cultured as before. Western blotting was performed for RARα (Bi), RARβ (Bii) and RARγ (Biii). A431 cell nuclear extract supplied with the RARγ antibody was used as a positive control for RARγ. (C) NRK-49F cells were cultured with 2 μM tRA and/or 5 ng·mL^{-1} TGF-β1 for 0–48 h. Total RNA was extracted and subjected to RT-qPCR analysis of the mRNA expression of RARα (Ci), RARβ (Cii), RARγ (Ciii), RXRα (Civ), RXRβ (Cv) and RXRγ (Cvi). Data represent mean ± SEM of four independent cell culture studies. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus 0 h group; + $P < 0.05$, ++ $P < 0.01$ between groups indicated.

Table S1 Gene expression assays used in this study.

Table S2 Antibodies used in this study.