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Hepatocyte Growth Factor Receptor Signaling Mediates the Anti-Fibrotic Action of 9-*cis*-Retinoic Acid in Glomerular Mesangial Cells

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Retinoic acid (RA), an active metabolite of vitamin A, plays a critical role in the regulation of cell proliferation, survival, and differentiation. RA action is primarily mediated through its receptors, ligand-dependent transcription factors of the steroid/thyroid/vitamin D nuclear receptor superfamily. Recent studies indicate that administration of RA mitigates progressive kidney disease, underscoring its renoprotective potential. In this study, we investigated the effects of 9-*cis*-RA on glomerular mesangial cell activation induced by transforming growth factor (TGF)- β 1 using an *in vitro* cell culture system. In human mesangial cells 9-*cis*-RA suppressed TGF- β 1-induced α -smooth muscle actin, fibronectin, and plasminogen activator inhibitor-1 expression, but it did not significantly affect cell proliferation and survival. Interestingly, 9-*cis*-RA induced hepatocyte growth factor (HGF) mRNA expression and protein secretion, stimulated HGF promoter activity, and activated c-met receptor phosphorylation. Similar to HGF, 9-*cis*-RA induced expression of the Smad transcriptional co-repressor TGIF in mesangial cells. Overexpression of exogenous TGIF by transfection or 9-*cis*-RA treatment suppressed *trans*-activation of the TGF- β -responsive promoter. Moreover, conditional ablation of the c-met receptor completely abolished the anti-fibrotic effect of 9-*cis*-RA and abrogated TGIF induction. Collectively, these results indicate that 9-*cis*-RA possesses anti-fibrotic ability by antagonizing TGF- β 1 in mesangial cells and that 9-*cis*-RA activity is likely mediated through a mechanism dependent on HGF/c-met receptor signaling. (*Am J Pathol* 2005, 167:947-957)

Retinoic acid (RA) is an active metabolite of vitamin A (retinol) that plays an imperative role in regulating a wide

range of biological processes such as cell proliferation and survival, embryonic development, immune modulation, and tissue homeostasis.¹⁻³ The natural RA exists in different forms and represents a group of active metabolic derivatives of vitamin A, which include all-trans RA, 9-*cis*-RA, 13-*cis*-RA, and others. The action of RA is mediated through binding to its receptors, the ligand-dependent transcription factors that belong to the steroid/thyroid/vitamin D nuclear receptor superfamily. Two subfamilies of RA receptors with different ligand specificities are found, namely retinoic acid receptor (RAR) and retinoid X receptor (RXR), each of them has three isotypes ($-\alpha$, $-\beta$, and $-\gamma$), respectively. Although RARs are stimulated by the all-trans RA and 9-*cis*-RA, the RXRs can be activated exclusively by 9-*cis*-RA. On ligand binding, the retinoid receptors undergo nuclear translocation, and subsequently bind to specific *cis*-acting RA- and RX-responsive elements in the promoter regions of their target genes, thereby directly modulating gene transcription. Retinoid receptors may also heterodimerize with the other members of the nuclear receptor superfamily, including the vitamin D receptor and peroxisome proliferator-activated receptors.² This will result in significant cross-talks among the different hormonal systems. In addition, retinoid receptors may modulate gene expression by interacting with other general transcription factors, such as activator protein-1 (AP-1) and nuclear factor (NF)- κ B.^{4,5} In such way, retinoids may integrate the signaling cascade of multiple regulatory pathways and control the distinctive cellular responses in a cell- and context-specific manner.

Kidney cells express all major isotypes of retinoid receptors and respond to their stimulation.^{6,7} In the embry-

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onic stage, retinoid is a major modulator of renal tubulogenesis and plays an essential role in determining the total number of nephrons per kidney.^{8–10} Double knock-out of the RARs and RXRs result in several malformations, including kidney agenesis, hypoplasia, or aplasia of the ureteral bud.^{11,12} In adult kidneys, the retinoid receptor system is activated in the early phase of the experimental nephropathies, suggesting a potential role of this system in tissue repairs after injuries.⁷ Consistent with this notion, administration of either natural or synthetic retinoids has been shown to be renoprotective in numerous animal models of renal diseases.^{13–20} In anti-Thy1.1 glomerulonephritis, treatment with all-trans RA or 13-*cis*-RA effectively ameliorates renal damage and mesangial cell proliferation, attenuates capillary occlusion and glomerular inflammation, and reduces albuminuria.^{13,21–24} The beneficial effects of retinoids are also clearly evident in many other models of renal diseases, such as anti-glomerular basement membrane glomerulonephritis,¹⁷ lupus nephritis,^{14,20} obstructive nephropathy,¹⁶ puromycin aminonucleoside-induced nephrosis,^{15,18} and acute renal allograft nephropathy.¹⁹

Despite increasing evidence demonstrating that RA is a potent renoprotective agent that may have therapeutic potential for human kidney diseases, relatively little is known regarding the mechanisms of its action. In light of the interactions between retinoid receptors and the AP-1 or NF- κ B, previous studies are primarily emphasized on its anti-proliferative and anti-inflammatory capacity.^{2,25} It remains ambiguous whether RA can directly block the fibrogenic processes of the kidney cells. Likewise, it is also to be determined whether RA counteracts the action of transforming growth factor (TGF)- β 1, the principal profibrotic cytokine that has been implicated in the pathogenesis of virtually all kinds of fibrotic disorders.^{26–28}

In this study, we have demonstrated that 9-*cis*-RA effectively suppresses TGF- β 1-mediated glomerular mesangial cell activation. Further examination reveals that 9-*cis*-RA also induces anti-fibrotic hepatocyte growth factor (HGF) expression. Interestingly, conditional ablation of HGF receptor, c-met, completely abolishes the action of 9-*cis*-RA in mesangial cells. These studies establish that HGF/c-met signaling is essential for mediating the anti-fibrotic activity of 9-*cis*-RA.

Materials and Methods

Antibodies, Expression Vectors, and Reagents

The mouse monoclonal anti- α -smooth muscle actin (α -SMA, clone 1A4) was obtained from Sigma (St. Louis, MO). Mouse monoclonal anti-fibronectin (clone 10) was purchased from BD Pharmingen (San Jose, CA). The anti-TGIF (sc-17800), anti-plasminogen activator inhibitor-1 (PAI-1) (sc-5297), and anti-actin (sc-1616) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phospho-Met (Tyr1234/1235) antibody that detects Met only when phosphorylated at tyrosine 1234/1235 and monoclonal anti-Met antibody (25H2) were obtained from Cell Signaling Technology, Inc. (Beverly,

MA). Antibody against Cre recombinase was purchased from EMD Biosciences, Inc. (San Diego, CA). Monoclonal antibody against human HGF (clone H-14) was prepared by our laboratory and described elsewhere.²⁹ This antibody specifically recognizes human HGF protein. Affinity-purified secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The TGF- β -responsive reporter vector p3TP-Lux, Smad2, Smad3, and TGIF expression vectors were kindly provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY).³⁰ Adenoviral vectors containing Cre recombinase (Ad.Cre) or green fluorescent protein (Ad.GFP) were provided by Dr. A. Gambotto at the Vector Core Facility of the University of Pittsburgh. Recombinant human TGF- β 1 was purchased from R&D Systems Inc. (Minneapolis, MN). Recombinant human HGF was provided by Genentech Inc. (South San Francisco, CA). 9-*cis*-Retinoic acid (9cRA) was obtained from Sigma. Cell culture media, fetal bovine serum, and supplements were obtained from Invitrogen (Carlsbad, CA). All other chemicals were of analytic grade and were obtained from Sigma or Fisher (Pittsburgh, PA) unless otherwise indicated.

Cell Culture and Treatment

Human glomerular mesangial cells (HMCs) and media were purchased from ScienCell Research Laboratories (San Diego, CA). These cells are characterized by the manufacturer using morphological appearances and immunofluorescent method with various antibodies, including anti-Thy-1 and fibronectin. Rat mesangial cells were provided by Dr. C. Wu at the University of Pittsburgh and described previously.³¹ HMCs were cultured in HMC medium and rat glomerular mesangial cells were seeded in Dulbecco's modified Eagle's medium-F12 medium supplemented with 5% fetal bovine serum. Twenty-four hours later, the cells were changed to serum-free medium and then incubated for 16 hours. Cells were then treated with various agents at the concentrations specified. The supernatants or whole cell lysates were collected at different time points for various analyses.

MTT Assay

Cell proliferation was assessed by using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]-based cell growth determination kit (Sigma). Briefly, HMCs were seeded in 96-well plates at the same concentration (2×10^3 /well). After incubation in complete medium containing 5% fetal bovine serum overnight, cells were changed to serum-free medium for 24 hours. HMCs were then treated with 9-*cis*-RA in the absence or presence of TGF- β 1 for 48 hours. MTT (5 mg/ml) was added to the culture at 10 μ l per well, followed by incubation at 37°C for 2 hours. Medium was aspirated, and cells were lysed with dimethyl sulfoxide. Absorbance of each well was recorded in a microplate reader at 540 nm. Data are presented as means $\pm$ SEM. Three wells per treatment were used.

Lactate Dehydrogenase Assay

The cytotoxicity of various agents to mesangial cells was assessed by measuring lactate dehydrogenase release using a cytotoxicity detection kit (Roche Diagnostics GmbH, Penzberg, Germany). HMCs were treated with various agents for 48 hours as indicated. The supernatants of the cultures were harvested and transferred to an optically clear 96-well plate. After incubation with 100 μ l of the reaction mixture for 30 minutes at room temperature, absorbance of each well was read at 490 nm. Data are presented as means $\pm$ SEM. Three wells per treatment were used.

Terminal dUTP Nick-End Labeling (TUNEL) Staining

Apoptotic cell death was detected by using TUNEL staining with an apoptosis detection system (Promega, Madison, WI), as described previously.³² Briefly, HMCs after various treatments for 48 hours were fixed and permeabilized with methanol at -20°C for 15 minutes. After washing with phosphate-buffered saline (PBS), cells were incubated with 100 μ l of equilibration buffer at room temperature for 10 minutes, followed by incubation with terminal deoxynucleotidyl transferase at 37°C for 60 minutes. The reaction was terminated by immersing the slides in $2\times$ standard saline citrate for 15 minutes at room temperature. The slides were mounted in Vectashield mounting medium with propidium iodide (Vector Laboratories Inc., Burlingame, CA) and observed on a Nikon Eclipse E600 epifluorescence microscope equipped with a digital camera (Nikon Inc., Melville, NY). Apoptotic cells were counted in high-power ($\times 400$) fields and expressed as apoptotic cells per field.

Western Blot Analysis

Whole cell lysates were prepared as described previously.³³ Cells were lysed with sodium dodecyl sulfate sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mmol/L dithiothreitol, and 0.1% bromophenol blue). Samples were heated at 100°C for ~ 5 to 10 minutes before loading and separated onto 10% sodium dodecyl sulfate-polyacrylamide gels. After the proteins were electrotransferred to Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ), nonspecific binding to the membrane was blocked for 1 hour at room temperature with 5% Carnation nonfat milk (Nestlé, Wilkes-Barre, PA) in TBST buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20). The membranes were then incubated for 16 hours at 4°C with various primary antibodies in blocking buffer containing 5% milk at the dilutions specified by the manufacturers. After extensive washing in TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature in 5% nonfat milk dissolved in TBST. Membranes were then washed with TBST buffer and the signals were visualized using the SuperSignal West Pico

chemiluminescent substrate kit (Pierce Biotechnology, Rockford, IL).

Immunofluorescence Staining

Indirect immunofluorescence staining was performed using an established procedure.³⁴ Briefly, cells cultured on coverslips were washed twice with cold PBS and fixed with cold methanol/acetone (1:1) for 10 minutes at -20°C . After three extensive washings with PBS containing 0.5% bovine serum albumin, the cells were blocked with 20% normal donkey serum in PBS buffer for 30 minutes at room temperature and then incubated with the specific primary antibody described above. Slides were then incubated with fluorescein-conjugated secondary antibody for 1 hour before being extensively washed with PBS. As a negative control, the primary antibody was replaced with nonimmune IgG, and no staining occurred. For some samples, cells were double stained with 4',6-diamidino-2-phenylindole to visualize the nuclei. Stained cells were mounted and viewed with a Nikon Eclipse E600 epifluorescence microscope.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time quantitative RT-PCR was performed to determine the steady-state levels of HGF c-met mRNA. Briefly, total RNA was extracted from HMCs using TRIzol RNA isolation system according to the manufacturer's instructions (Invitrogen). RNA samples were quantified by determination of ultraviolet absorbance at 260 nm. The first strand cDNA synthesis was performed by using a reverse transcription system kit according to the instructions of the manufacturer (Promega, Madison, WI). Real-time PCR amplification was performed on ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The PCR reaction mixture at a 25- μ l volume contained 12.5 μ l of $2\times$ SYBR Green PCR Master Mix (Applied Biosystems), 5 μ l of diluted RT product (1:20), and 0.5 μ mol/L sense and anti-sense primer sets. The primer sequences were as follows: HGF, 5'-AAGGT-GACTTTGAATGAGTC (sense), and 5'-GGCACATCC-ACGACCAGGAACAATG (anti-sense); β -actin, 5'-AG-GCATCCTCACCCTGAAGTA (sense), and 5'-CACACG-CAGCTCATTGTAGA (anti-sense). Each sample was added in duplicate. PCR reaction was run by using standard conditions. After sequential incubations at 50°C for 2 minutes and 95°C for 10 minutes, respectively, the amplification protocol consisted of 50 cycles of denaturing at 95°C for 15 seconds, annealing and extension at 60°C for 60 seconds. The standard curve was made from a series of dilutions of template cDNA. Expression levels of HGF mRNA were calculated after normalizing with β -actin.

DNA Transfection and Luciferase Assay

The reporter construct 0.9HGF-Luc, which contains 0.9 kb of the 5'-flanking region of mouse HGF gene and the

coding sequence for firefly luciferase, was described previously.³⁵ At 24 hours before transfection, rat mesangial cells were seeded onto six-well plates at 4×10^5 cells per well. The cells were then transfected with 0.9HGF-Luc (0.9 μ g). A fixed amount (0.1 μ g) of internal control reporter *Renilla reniformis* luciferase driven under thymidine kinase (TK) promoter (pRL-TK; Promega) was also co-transfected for normalizing the transfection efficiency. After transfection with Lipofectamine 2000 reagent (Invitrogen), the cells were incubated for an additional 48 hours in the absence or presence of 10^{-6} mol/L 9cRA. For investigating the effect of 9cRA on the transcription of TGF- β 1-responsive genes, reporter plasmid p3TP-Lux (1.0 μ g) together with or without Smad2 (0.5 μ g)/Smad3 (0.5 μ g), and/or TGIF (1.0 μ g) expression vectors were co-transfected into mesangial cells. A fixed amount (0.1 μ g) of control reporter pRL-TK was also co-transfected. The transfected cells were pretreated with 9cRA (10^{-6} mol/L) for 16 hours, followed by incubation with or without TGF- β 1 (1 ng/ml) for another 36 hours. After being washed in PBS, the cells were pelleted, resuspended in 250 μ l of lysis buffer, and then disrupted by three freeze-thaw cycles. The protein suspension was clarified by centrifugation at $15,000 \times g$ for 5 minutes at 4°C, and the supernatant was collected and analyzed. Luciferase assay was performed using the dual luciferase assay system kit essentially according to the manufacturer's protocols (Promega). Relative luciferase activity of each construct (arbitrary unit) was reported as fold induction after normalizing for transfection efficiency.

Mouse Mesangial Cell Culture and Adenovirus Infection

The c-met-floxed mice in which the exon 16 of the c-met gene was flanked by loxP sites were kindly provided by Dr. S. Thorgeirsson of the National Cancer Institute, National Institutes of Health (Bethesda, MD).³⁶ Mouse primary cultured mesangial cells from outgrowths of isolated whole glomeruli were prepared essentially as described previously.³⁷ Briefly, glomeruli were isolated from the c-met-floxed mice under sterile conditions by differential sieving of cortical tissue. After the final wash, the isolated glomeruli were resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum and 5 μ g/ml of insulin, and plated on culture dishes for outgrowth of the mesangial cells. When the cells reached confluency, they were subcultured at 1:4 dilution. The cells showed the typical smooth muscle cell-like morphology and positive staining for Thy1.1 and α -SMA, while negative staining for cytokeratin. Passages 4 through 10 of subcultured mesangial cells were infected with adenoviral vectors (Ad.GFP or Ad.Cre) in serum-free medium ($\sim 10^7$ particles/ml). Infected cells were incubated for 4 hours, and then restored to complete medium. After 24 hours, infected cells were treated either with or without TGF- β 1 (1 ng/ml) and/or 9cRA (10^{-6} mol/L), respectively, for 48 hours. Whole cell lysates were then collected for analyses.

Statistical Analysis

Statistical analysis was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparisons between groups were made using one-way analysis of variance, followed by the Student's *t*-test. A *P* value of less than 0.05 was considered significant.

Results

9-cis-RA Suppresses Mesangial Activation Induced by TGF- β 1

Mesangial cell activation, characterized by cell proliferation, α -SMA induction, and matrix overproduction, is a predominant pathological feature of many primary glomerular diseases. We first examined the effects of 9-cis-RA on cell proliferation and survival in cultured HMCs. As shown in Figure 1, 9-cis-RA at the concentrations ranging from 10^{-8} to 10^{-6} mol/L did not significantly affect mesangial cell proliferation and survival, as detected by MTT assay, lactate dehydrogenase release, and TUNEL staining, respectively. Co-incubation of 9-cis-RA with TGF- β 1 also failed to modulate HMC growth and survival (Figure 1; B, D, and F).

We next investigated the effects of 9-cis-RA on mesangial cell activation by examining α -SMA expression. As demonstrated in Figure 2, 9-cis-RA at different concentrations did not affect α -SMA expression in HMCs at basal conditions. TGF- β 1 induced *de novo* expression of α -SMA in HMCs, as reported previously.³⁸ However, simultaneous incubation of HMCs with 9-cis-RA markedly suppressed TGF- β 1-initiated α -SMA expression (Figure 2A). Figure 2B shows that 9-cis-RA was able to block the TGF- β 1-mediated α -SMA induction at different time points. Immunofluorescence staining also revealed that TGF- β 1 induced α -SMA expression and assembly in HMCs, as illustrated by the presence of cytoplasmic α -SMA-positive microfilaments (Figure 2D). Concurrent treatment of 9-cis-RA primarily abolished the α -SMA expression induced by TGF- β 1 (Figure 2F). These results suggest that 9-cis-RA is able to suppress α -SMA induction, a hallmark for mesangial cell activation.

9-cis-RA Inhibits Fibronectin Expression and Deposition at Both Resting and Stimulated Conditions

Mesangial cell activation is accompanied by extracellular matrix overproduction, which ultimately leads to mesangial expansion and glomerulosclerosis. To study the effects of 9-cis-RA on matrix production and deposition in mesangial cells, we examined the expression of fibronectin in HMCs after incubation with 9-cis-RA and/or TGF- β 1. Figure 3 shows that cultured HMCs expressed considerable amounts of fibronectin protein at resting state. Incubation with 9-cis-RA resulted in a reduced fibronectin expression in a time- and dose-dependent manner (Figure 3, A and B). As expected, TGF- β 1 substantially increased fibronectin expression in HMCs. Simultaneous

Figure 1. The effects of 9-*cis*-RA on mesangial cell proliferation and survival. HMCs were treated with increasing amounts of 9-*cis*-RA as indicated (**A**, **C**, and **E**) or with 9-*cis*-RA (10⁻⁶ mol/L) or/and TGF-β1 (1 ng/ml) (**B**, **D**, and **F**) for 48 hours. Cell proliferation was assessed by MTT assay (**A** and **B**). Cell cytotoxicity was estimated by lactate dehydrogenase release (**C** and **D**), whereas apoptosis was detected by TUNEL staining (**E** and **F**). No statistical difference was found between the treatment groups and controls ($n = 3$).

incubation of HMCs with 9-*cis*-RA also suppressed TGF-β1-stimulated fibronectin expression.

We further examined the fibronectin deposition by HMCs after various treatments. Consistent with the Western blot results, immunofluorescence staining also exhibited an inhibitory effect of 9-*cis*-RA on fibronectin deposition at both resting and TGF-β1-stimulated conditions. HMCs at basal conditions displayed moderate fibronectin-positive microfilaments in the cytoplasm (Figure 3C). TGF-β1 induced a marked increase in fibronectin deposition, in which the majority of the fibronectin-positive filaments were deposited in the extracellular compartments (Figure 3D). Treatment with 9-*cis*-RA essentially abolished the fibronectin staining in the HMCs in both basal and TGF-β1-stimulated states (Figure 3, E and F).

9-*cis*-RA Abolishes TGF-β1-Mediated Type 1 Plasminogen Activator Inhibitor Induction

TGF-β1 not only activates mesangial cells to produce excess amounts of matrix components, but also inhibits matrix degradation through up-regulating plasminogen activator inhibitor-1 (PAI-1) expression. We found that 9-*cis*-RA also inhibited TGF-β1-mediated PAI-1 induction in mesangial cells (Figure 4). Although 9-*cis*-RA did not exhibit any effect on PAI-1 production at basal conditions, it drastically inhibited TGF-β1-stimulated PAI-1 expression in a dose-dependent manner (Figure 4). The inhibitory effect of 9-*cis*-RA on TGF-β1-mediated PAI-1 expression was also confirmed at different time points in the HMCs after various treatments (data not shown). Therefore, 9-*cis*-RA elicits potent anti-fibrotic activity by specifically antagonizing TGF-β1's action in mesangial cells.

9-*cis*-RA Induces HGF mRNA Expression and Protein Secretion, Stimulates Its Promoter Activity, and Activates c-Met Receptor Phosphorylation

The anti-fibrotic activity of 9-*cis*-RA in mesangial cells in many ways resembles the action of HGF,³⁸ a cytokine that has emerged as an important anti-fibrotic factor *in vitro* and *in vivo*.³⁹ This prompted us to explore the potential interconnection between 9-*cis*-RA and HGF. We found that incubation with 9-*cis*-RA induced HGF protein secretion by mesangial cells. As shown in Figure 5, A and B, Western blot analysis demonstrated that 9-*cis*-RA up-regulated HGF protein abundance in the supernatants of mesangial cells in a dose-dependent manner. To examine whether this increase in HGF protein secretion is driven by an enhanced gene expression, we measured the steady-state level of HGF mRNA in mesangial cells after 9-*cis*-RA treatment. As shown in Figure 5C, 9-*cis*-RA significantly induced HGF mRNA expression in the HMCs, as demonstrated by real-time quantitative RT-PCR. Furthermore, 9-*cis*-RA was able to induce HGF mRNA expression in the presence of TGF-β1 (Figure 5D).

Sequence analysis revealed the presence of a putative retinoid X response element (RXR) in the promoter region of the HGF gene.³⁵ To test whether 9-*cis*-RA activates HGF promoter, we transfected luciferase reporter construct (0.9HGF-Luc) that contains RXR into mesangial cells. As demonstrated in Figure 5E, 9-*cis*-RA could significantly stimulate the HGF promoter activity. Incubation of HMCs with 9-*cis*-RA appeared to induce the tyrosine phosphorylation of HGF receptor, c-met. As shown in

Figure 2. 9-*cis*-RA inhibits TGF- β 1-mediated α -SMA expression in mesangial cells. **A:** HMCs were treated with or without TGF- β 1 (1 ng/ml) and different amounts of 9-*cis*-RA as indicated for 72 hours. Whole cell lysates were immunoblotted with specific antibodies against α -SMA and actin, respectively. **B:** HMCs were incubated without or with TGF- β 1 (1 ng/ml) and/or 9-*cis*-RA (10^{-6} mol/L) for different periods of time as indicated. 9-*cis*-RA inhibited α -SMA expression induced by TGF- β 1 at 48 and 72 hours, respectively. **C to F:** Immunofluorescence staining for α -SMA in HMCs after various treatments. HMCs were treated without (**C**) or with TGF- β 1 (1 ng/ml) (**D**), 9-*cis*-RA (10^{-6} mol/L) (**E**), or both (**F**) for 72 hours.

Figure 5F, 9-*cis*-RA stimulated c-met phosphorylation in HMCs, as detected by immunoblotting with specific antibody that detects c-met only when phosphorylated at tyrosine 1234/1235.

9-*cis*-RA Induces Smad Transcriptional Co-Repressor TGIF and Blocks TGF- β /Smad-Mediated Gene Transcription

Previous studies show that HGF antagonizes TGF- β 1 action in mesangial cells by stabilizing Smad transcrip-

tional co-repressor TGIF, thereby sequestering TGF- β /Smad-mediated gene transcription.³⁸ To elucidate the potential mechanism by which 9-*cis*-RA counteracts the fibrogenic action of TGF- β 1, we investigated the effect of 9-*cis*-RA on TGF- β 1-mediated Smad signaling. As shown in Figure 6A, similar to HGF, 9-*cis*-RA markedly induced TGIF expression in mesangial cells in a delayed manner. Because HGF rapidly increases TGIF abundance that takes place as early as 1 hour after treatment,³⁸ it is likely that the TGIF induction by 9-*cis*-RA is mediated by HGF.

Figure 3. 9-*cis*-RA suppresses TGF- β 1-induced fibronectin expression and deposition. **A:** 9-*cis*-RA suppresses fibronectin expression at both resting and TGF- β 1-stimulated conditions in a time-dependent manner. HMCs were treated with or without TGF- β 1 (1 ng/ml) in the absence or presence of 9-*cis*-RA (10^{-6} mol/L) for 48 and 72 hours, respectively. Whole cell lysates were immunoblotted with specific antibodies against fibronectin and actin, respectively. **B:** 9-*cis*-RA suppresses fibronectin expression in HMCs in a dose-dependent manner. **C to F:** Indirect immunofluorescence staining shows fibronectin deposition after various treatments. HMCs were treated without or with TGF- β 1 (1 ng/ml) and/or 9-*cis*-RA (10^{-6} mol/L) for 72 hours. **C:** Control; **D:** TGF- β 1; **E:** 9-*cis*-RA; **F:** TGF- β 1 plus 9-*cis*-RA.

Figure 4. 9-*cis*-RA abolishes TGF- β 1-mediated plasminogen activator inhibitor-1 (PAI-1) induction in HMCs. HMCs were treated with or without TGF- β 1 (1 ng/ml) and different concentrations of 9-*cis*-RA as indicated for 72 hours. Whole cell lysates were immunoblotted with specific antibodies against PAI-1 and actin, respectively.

To evaluate the effect of 9-*cis*-RA on TGF- β /Smad-mediated gene transcription, we transiently transfected rat mesangial cells with p3TP-Lux, a TGF- β -responsive luciferase reporter plasmid that has been widely used in examining TGF- β responsiveness.⁴⁰ As shown in Figure 5B, TGF- β 1 activated the luciferase reporter activity of p3TP-Lux. Co-transfection with Smad2/3 expression vectors increased both basal and TGF- β 1-stimulated luciferase activity. However, ectopic expression of TGIF by transfection significantly suppressed Smad-mediated promoter activity in either basal or TGF- β 1-stimulated conditions. Likewise, direct incubation with 9-*cis*-RA also mimicked TGIF action and repressed Smad-me-

diated *trans*-activation of the TGF- β -responsive gene (Figure 6B).

Conditional Ablation of c-Met Receptor in Mesangial Cells Abolishes the Anti-Fibrotic Effect of 9-*cis*-RA

To provide direct evidence for a definite role of HGF signaling in mediating the anti-fibrotic action of 9-*cis*-RA, we examined the impact of c-met receptor ablation on 9-*cis*-RA action in mesangial cells. To this end, mesangial cells were prepared from the mice in which the c-met gene was flanked by LoxP sites. To achieve mesangial cell-specific ablation of c-met receptor, these cells were infected with adenovirus containing Cre recombinase. As shown in Figure 7A, the c-met receptor was almost completely ablated in the mesangial cells infected with Ad.Cre adenovirus, whereas the cells infected with control Ad.GFP adenovirus retained abundant c-met protein. Immunostaining showed that more than 95% of the mesangial cells infected with Ad.Cre displayed nuclear staining for Cre recombinase (data not shown), presumably leading to c-met deletion. Incubation of the mouse mesangial cells infected with Ad.GFP with 9-*cis*-RA dramatically blocked TGF- β 1-mediated fibronectin expression (Figure 7B), a finding similar to that in the HMCs (Figure 3).

Figure 5. 9-*cis*-RA induces HGF protein and mRNA expression, stimulates HGF promoter activity, and activates c-met receptor phosphorylation. **A:** HMCs were incubated with different doses of 9-*cis*-RA as indicated for 72 hours. The supernatants were collected and concentrated, followed by Western blot analysis with anti-HGF antibody. The authenticity of HGF was confirmed by loading the purified human recombinant HGF (10 ng) on the adjacent lane (not shown). Cell lysates were probed with actin to ensure same number of cells used. **B:** Graphical presentation of the relative abundance of HGF protein in the supernatants of the HMCs after treatment with different concentrations of 9-*cis*-RA. Data are presented as mean $\pm$ SEM of three experiments. **P* < 0.05 versus control (*n* = 3). **C:** Real-time RT-PCR shows that 9-*cis*-RA induced HGF mRNA abundance in HMCs. Cells were treated with 9-*cis*-RA (10⁻⁶ mol/L) for different periods of time as indicated. **P* < 0.05 versus control (*n* = 3). **D:** 9-*cis*-RA induced HGF expression in the presence of TGF- β 1. HMCs were treated with TGF- β 1 (1 ng/ml) alone or TGF- β 1 plus 9-*cis*-RA (10⁻⁶ mol/L) for 24 hours. **E:** 9-*cis*-RA stimulated HGF promoter activity. Rat mesangial cells were transfected with HGF promoter-Luc reporter construct and internal control reporter pRL-TK, followed by incubation with 9-*cis*-RA (10⁻⁶ mol/L) for another 48 hours. **P* < 0.01 versus control (*n* = 3). **F:** 9-*cis*-RA activated c-met receptor phosphorylation. HMCs were incubated without or with 10⁻⁶ mol/L 9-*cis*-RA for 24 hours. Cell lysates were immunoblotted with antibodies against phospho-specific and total c-met, respectively. Fold induction after quantitative analysis was also presented in the bottom of the picture.

Figure 6. 9-cis-RA induces Smad transcriptional co-repressor TGIF expression, which in turn blocks TGF- β 1/Smad-mediated gene transcription. **A:** HMCs were incubated with 10^{-6} mol/L 9-cis-RA for various periods of time as indicated. Whole cell lysates were immunoblotted with antibodies against TGIF and actin, respectively. **B:** 9-cis-RA and TGIF repress TGF- β 1/Smad-mediated gene transcription. Rat mesangial cells were transfected with reporter plasmid p3TP-Lux together with internal control plasmid pRL-TK; with or without Smad2/Smad3 and TGIF expression vectors as indicated. The transfected cells were pretreated with 9-cis-RA (10^{-6} mol/L) for 16 hours, followed by incubation with or without TGF- β 1 (1 ng/ml) for another 36 hours. * $P < 0.01$ versus control groups without Smad2/3 ($n = 9$). † $P < 0.01$ versus pSmad2/3-transfected groups ($n = 9$).

However, 9-cis-RA completely failed to suppress TGF- β 1-induced fibronectin expression (Figure 7B), suggesting that ablation of c-met receptor abolishes the anti-fibrotic effect of 9-cis-RA. Similarly, ablation of c-met receptor also completely abrogated the inhibitory effect of 9-cis-RA on TGF- β 1-mediated α -SMA expression (Figure 7C). Furthermore, 9-cis-RA was unable to induce TGIF expression in the mesangial cells lacking c-met receptor (Figure 7D). These results strongly indicate that HGF/c-met signaling pathway is essential for mediating the anti-fibrotic action of 9-cis-RA in mesangial cells.

Discussion

Although growing evidence indicates that retinoids exert renoprotective activity in assorted models of chronic kidney diseases, it remained elusive as to the signaling pathways and the mechanisms by which RA elicits its anti-fibrotic actions. The results presented in this study provide strong evidence that 9-cis-RA antagonizes the fibrogenic action of TGF- β 1 and prevents mesangial cell activation, one of the central events in the pathogenesis

of many types of primary glomerular diseases. Such anti-fibrotic effect of RA is likely to be mediated by induction of HGF expression, which in turn up-regulates Smad transcriptional co-repressor TGIF, leading to the blockade of the TGF- β /Smad-mediated gene transcription. Therefore, our results suggest a unique cascade of signaling pathway, which may offer a rational explanation for the efficacy of RA in ameliorating renal fibrotic lesions.

The retinoid/HGF/TGIF/Smad pathway characterized in this study is supported by several lines of observation. First, 9-cis-RA is able to suppress TGF- β 1-mediated α -SMA, fibronectin, and PAI-1 expression. These phenotypic markers manifest the major features of mesangial cell activation from a quiescent state after fibrotic stimuli. The fact that 9-cis-RA abrogates α -SMA, fibronectin, and PAI-1 induction underscores that it specifically targets several key events in mesangial cell activation. Second, 9-cis-RA induces HGF mRNA expression and protein secretion, stimulates HGF promoter, and activates c-met receptor phosphorylation (Figure 5). Third, 9-cis-RA induces Smad transcriptional co-repressor TGIF expression in a delayed manner (Figure 6), and ablation of c-met receptor abolishes TGIF induction (Figure 7). Finally, ablation of c-met receptor completely abrogates the anti-fibrotic effect of 9-cis-RA (Figure 7). Collectively, these studies build a compelling case that HGF/c-met signaling is essential for mediating 9-cis-RA action.

The interplay between retinoid and TGF- β *in vivo* has been controversial. Previous studies show that RXR-specific agonist (Ro-257386) suppresses TGF- β 1 mRNA expression in rat model of anti-Thy1.1 mesangioproliferative glomerulonephritis.²⁴ This suppression of TGF- β 1 by retinoid is associated with an alleviation of renal pathology, as evidenced by a complete inhibition of procollagen I and fibronectin mRNA induction.²⁴ However, in lupus nephritis in MRL/lpr mice, all-trans RA is able to reduce proteinuria and inhibits glomerular and interstitial inflammation, but increases renal TGF- β 1 mRNA expression.¹⁴ It has been postulated that increased TGF- β 1 may act as an anti-inflammatory signal in this model.¹⁴ Hence, the response of TGF- β 1 to retinoids *in vivo* may primarily depend on different disease models. The present study suggests a novel mode of interaction between retinoids and TGF- β , in which 9-cis-RA up-regulates Smad transcriptional co-repressor TGIF expression (Figure 6), evidently through a HGF-dependent mechanism. It should be noted that previous studies have showed that TGIF suppresses RA-mediated gene transcription in the cellular retinol-binding protein II promoter and competes with retinoid receptors for DNA binding via the core binding sequence of TGIF (5'-TGTC-3') located adjacent to the RXRE.⁴¹ Because the TGIF core binding sequence is absent in the HGF promoter,³⁵ it suggests that TGIF may not bind to the regulatory region of HGF gene. Instead, TGIF primarily acts as a transcriptional antagonist of activated Smads via direct protein-protein interaction,^{30,38} whereby sequestering TGF- β /Smad-mediated gene transcription (Figure 6B). In accordance with this, induction of Smad antagonist TGIF has been demonstrated to play a crucial role in mediating HGF suppression of mesangial

Figure 7. Conditional knockout of c-met receptor in mesangial cells abolishes the anti-fibrotic effect of 9-*cis*-RA. **A:** Mouse mesangial cells (MMC) were isolated from c-met-floxed mice by differential sieving technique. MMCs were infected with adenoviral vectors containing Cre recombinase or GFP (control vector), respectively. Cre expression and c-met ablation in the MMCs were confirmed by Western blot analysis of whole cell lysates. **B:** Ablation of c-met receptor abolished the suppressive effect of 9-*cis*-RA on fibronectin expression induced by TGF-β1. MMCs infected with Ad.GFP (control) or Ad.Cre (c-met knockout) were treated with or without TGF-β1 (1 ng/ml) and/or 9-*cis*-RA (10⁻⁶ mol/L) for 72 hours. Whole cell lysates were immunoblotted with antibodies against fibronectin and actin, respectively. **C:** Ablation of c-met receptor abolished 9-*cis*-RA inhibition of the α-SMA expression induced by TGF-β1. MMCs were treated as indicated for 72 hours. **D:** Ablation of c-met receptor abrogated TGIF induction by 9-*cis*-RA. MMCs after infection with adenoviral vectors were incubated with 9-*cis*-RA for 24 hours.

cell activation *in vitro* and glomerular fibrotic lesions *in vivo*.^{38,42}

The beneficial effects of retinoid in chronic kidney disease may not be limited to its anti-fibrotic potential through counteracting TGF-β actions, but are also contributable to its anti-inflammatory properties. It is well known that retinoid is an inflammation inhibitory agent that specifically suppresses NF-κB signaling. Along this line, previous studies have also documented potent anti-inflammatory actions of retinoid in kidney diseases.^{2,17} For instance, in anti-glomerular basement membrane glomerulonephritic rats, RA administration not only inhibits TGF-β1 expression and significantly reduces urinary protein excretion, but also suppresses a wide variety of inflammation-related genes such as tumor necrosis factor-α and interleukin-1β.¹⁷ It is interesting to point out that HGF may also play a role in mediating these anti-inflammatory activities of retinoids, in view of the fact that HGF can block inflammatory infiltration of monocytes by inhibiting proinflammatory cytokine expression.⁴³ On the other hand, we cannot exclude the possibility that other mechanisms independent of HGF may also account for the beneficial effects of 9-*cis*-RA. This appears likely because the magnitude of HGF induction by 9-*cis*-RA is moderate in mesangial cells (Figure 5).

Mesangial cells express all major isotypes of the RARs and RXRs,⁶ suggesting retinoids play an important role in

specifying mesangial cell phenotypes. Each subtype of retinoid receptors is believed to regulate the expression of specific genes, because they display distinctive patterns of expression during embryonic development and different distributions in adult tissues.^{1,44} It should be noted that 9-*cis*-RA is a pan-agonist that binds to RAR and RXR, and thus both RAR- and RXR-specific pathways could contribute to the HGF induction and blockade of TGF-β signaling. At this stage, we cannot distinguish or assign specific retinoid receptor-mediated processes to any action of 9-*cis*-RA in mesangial cells. Such studies have to be performed by using specific experimental approaches, such as usage of the mesangial cells deficient in specific retinoid receptors, or synthetic retinoid receptor-specific agonists.

9-*cis*-RA can effectively antagonize the profibrotic action of TGF-β1 at concentrations as low as 10⁻⁸ mol/L in mesangial cells. Earlier studies show that 9-*cis*-RA was toxic only at 50 μmol/L in human mesangial cells,⁶ a concentration 5000 times higher than the effective dose. It is worthwhile to emphasize that 9-*cis*-RA at the concentrations used in the present study induces neither apoptosis nor cytotoxicity (Figure 1), and its dosage is also comparable to the blood levels found in cancer patients receiving this retinoid.⁴⁵ Therefore, the potential use of 9-*cis*-RA as a therapeutic agent for the treatment of chronic renal diseases is feasible in a clinical setting.

In summary, the present study demonstrates that 9-*cis*-RA, a naturally occurring retinoid receptor agonist, exhibits potent anti-fibrotic potential by specifically antagonizing TGF- β 1 action in mesangial cells. This activity of 9-*cis*-RA is primarily mediated by inducing the expression of HGF, an endogenous anti-fibrotic cytokine, which leads to up-regulation of the Smad transcriptional co-repressor TGIF, thereby suppressing TGF- β /Smad-mediated gene transactivation of the TGF- β -responsive genes. This provides an integral connection between retinoid and HGF, suggesting induction of endogenous HGF expression could be an effective strategy for amelioration of chronic renal diseases.

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