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Full-length article

All-*trans* retinoic acid inhibits the increases in fibronectin and PAI-1 induced by TGF-β1 and Ang II in rat mesangial cells[1]

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Key words

retinoids; plasminogen activator inhibitor 1; fibronectins; transforming growth factor beta1; angiotensin II; mesangial cells

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Abstract

Aim: To investigate the effect of all-*trans* RA (atRA) on the increases in plasminogen activator inhibitor-1 (PAI-1) and fibronectin that are induced by transforming growth factor-β1 (TGF-β1) and angiotensin II (Ang II) in cultured rat glomerular mesangial cells. **Methods:** Subconfluent glomerular mesangial cells were serum-starved for 48 h and pretreated with atRA with subsequent stimulation of TGF-β1 and Ang II. Protein expressions of cell-associated fibronectin and PAI-1 in glomerular mesangial cells were evaluated by Western blot analysis. mRNA expression of RA receptors in glomerular mesangial cells was examined by RT-PCR. **Results:** Retinoic acid receptor-α, -γ (RAR-α, -γ) and retinoid X receptor-α, -β, -γ (RXR-α, -β, -γ) mRNA were expressed in rat glomerular mesangial cells. atRA pretreatment effectively reduced fibronectin expression in glomerular mesangial cells stimulated with TGF-β1 or Ang II for 48 h. TGF-β1 stimulated PAI-1 expression reached a maximum at 5 h. atRA didn't affect the early (5 h) PAI-1 induction by TGF-β1, but markedly attenuated the sustained (48 h) PAI-1 induction. atRA also decreased the prolonged effect of Ang II on PAI-1 expression. **Conclusion:** These results indicate that atRA inhibits the increases in fibronectin that are induced by TGF-β1 and Ang II in cultured glomerular mesangial cells. The data also suggest that this effect of atRA is associated with a change in PAI-1 levels.

Introduction

Glomerulosclerosis is characterized by excessive deposition of extracellular matrix (ECM) in glomeruli. It represents a final common pathway leading to renal dysfunction in a variety of primary and secondary glomerular diseases such as diabetic nephropathy, lupus nephritis and chronic glomerulonephritis[1]. Glomerular mesangial cells are the major cells to produce mesangial ECM. Apart from increased synthesis of matrix protein, decreased degradation contributes to ECM buildup. ECM is mainly degraded via two distinct pathways: the matrix metalloproteinases (MMPs) degrading pathway and the plasminogen activators (PA)/plasmin proteolytic axis[2]. Plasmin is involved in ECM turnover by a direct promotion on the degradation of matrix proteins and by an indirect action on the activation of latent MMP. Inactive plasminogen is converted to active plasmin by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Therefore, PAI-1, as a major inhibitor of t-PA and u-PA , is thought to account for ECM accumulation by inhibiting plasmin- and MMP-mediated ECM degradation. ECM degradation has been shown to be inhibited by plasmin inhibitors, and increased by PAI-1 monoclonal antibody in cultured mesangial cells[2]. PAI-1 level is very low and even undetectable in normal kidneys, but is dramatically elevated in various forms of kidney diseases[3]. A significant increase in renal fibrosis is found in transgenic mice overexpressing PAI-1 that are subjected to unilateral ureteral obstruction (UUO) [4]. In contrast, PAI-1-deficient mice have substantially less fibrosis in the kidney compared with wild-type mice in

response to renal injury[5–7]. Furthermore, PAI-1 blockage using a nonfunctional PAI-1 competitor reduces pathological ECM accumulation by restoring plasmin generation and increasing plasmin-dependent degradation of matrix components in experimental glomerulonephritis and in cultured mesangial cells[8,9]. These studies strongly suggest an important role of PAI-1 in the pathogenesis of renal fibrosis. Transforming growth factor-β1 (TGF-β1) and angiotensin II (Ang II), identified as the key fibrogenic cytokines, have been shown to be stimulators of ECM protein synthesis and potent inducers of PAI-1[10–15].

Retinoic acid (RA) is a group of derivatives of vitamin A (retinol), including all-*trans* RA (atRA), 9-*cis* RA and 13-*cis* RA. The action of RA is mediated by its receptors, which are 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 (-α, -β, and -γ). RARs can be stimulated by atRA and 9-*cis* RA, RXRs are exclusively activated by 9-*cis* RA. RA and its receptor agonists have been demonstrated to protect the kidney in terms of renal structure and function in numerous animal models of renal diseases[16–20]. This beneficial effect of RA is ascribed to its anti-inflammatory and anti-proliferative properties. Moreover, previous study from our laboratory has shown that atRA effectively decreases cardiac fibrosis in spontaneously hypertensive rats[21]. However, the effect of atRA on the expression of fibronectin and PAI-1 in mesangial cells remains unknown. In the present study, we investigated the effect of atRA on the increases in PAI-1 and fibronectin induced by TGF-β1 and Ang II in cultured rat glomerular mesangial cells.

Materials and methods

Cell culture and treatment protocols Rat mesangial cells were kindly provided by Professor Guo MY (Shanghai Medical Center, Fudan University, Shanghai). Experiments were conducted with cells within passages 10. The cells were grown to 50%-60% confluence in Dulbecco's modified Eagle's medium (DMEM) (*D*-glucose concentration 5.6 mmol/L) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in a humidified atmosphere (5% CO_2 and 95% air) at 37 °C. Before each experiment, cells were rendered quiescent by culturing in medium containing 1% FBS. 48 h later, cells were pretreated with atRA (Sigma, Lot No.043k1341 with purity of 98% by HPLC) at the indicated concentrations, and then stimulated with 2 μg/L TGF-β1 (R&D Systems Inc) or 0.1 μmol/L Ang II (Sigma) for the indicated periods of time. atRA was prepared and added to cells under reduced lighting conditions to limit spontaneous isomerization to 9-*cis* RA and 13-*cis* RA.

RT-PCR Total cellular RNA was extracted from mesangial cells with TRIzol according to the procedure recommended by the manufacturer (Shenergy Biocolor Co, Shanghai, China). 2 μg of total RNA was reversely transcribed to cDNA using oligodT (Shenergy Biocolor Co) and MMLV reverse transcriptase (Takara, Tokyo, Japan). cDNA was subjected to PCR amplification using specific primers and Taq DNA polymerase (Takara). PCR products were run on agarose gels and visualized by ethidium bromide staining. The DNA ladder was used as a standard. The primers, experimentally determined optimal annealing temperature and expected size of PCR products are pre-

Table 1. Primers, Genebank accession number, annealing temperature used for RT-PCR.

Gene	Primer sequence (5′→3′)	Accession No	T_A (°C)	Size (bp)
RARα	S: ATC GAG ACC CGA AGC AGC AG AS: TGT TCT GAG CTG TTG TTC G	XO6614	64	466
RARγ	S: CTT ACT ACG CAG AGC CAC T AS: ATG ATA CAG TTT TTG TCG CGG	XM217064.2	60	297
RXRα	S: GCC CAT CCC TCA GGA AAT ATG AS: CAG AAT CTT CTC TAC AGG CAT	NM012805	60	327
RXRβ	S: CCA GTC ATC AGT TCT TCC ATG AS: ACC TGG AGG GGG TGG ACA GTG	NM206849	64	187
RXRγ	S: TGT GGA GAG CTC GAC AAA TG AS: ATG CCA TCC TGG ACA GAA AC	NM031765	50	204

AS, antisense; S, sense; T_A, annealing temperature.

sented in Table 1.

Western blot analysis The mesangial cell monolayer was washed 3 times with ice-cold phosphate-buffered solution (PBS). Cells and matrix were lysed with 1× reducing Laemmli buffer containing a 1:50 dilution of a protease inhibitor cocktail (Roche) on ice, scraped off the plates. Cell debris was pelleted by centrifugation (10 000×*g*, 10 min, 4 °C). Supernatant was collected and protein concentrations measured using BCA protein assay (Shenergy Biocolor Co). Equal amounts of protein from samples were loaded and separated under reducing conditions on SDS-PAGE (7.5% *w*/*v* for fibronectin and 10% for PAI-1 and β-actin). Prestained molecular-mass standards were used to monitor protein migration. Proteins were transferred onto PVDF membranes (Amersham Bioscience). The membranes were blocked in 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST), then incubated with the primary antibody against fibronectin (Chemicon), PAI-1 (BD Biosciences/Pharmingen) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by corresponding peroxidase-conjugated secondary antibody. Immune complexes were detected using the enhanced chemiluminescence (ECL) reagents (Pierce); immunoreactive bands were quantified using Smart viewer software (Furi Technology Co, Shanghai, China). The results were normalized against the intensity of the internal control (β-actin) band for each sample.

Statistical analysis Data were expressed as the mean±SD. Statistical analysis was carried out using Sigma stat 2.0 software. Differences in mean values between groups were analyzed using ANOVA followed by Student-Newman-Keuls test or by Dunn multiple comparison test as appropriate. A P value of <0.05 was considered statistically significant.

Results

mRNA expression of RA receptors in cultured rat mesangial cells Using RT-PCR analysis, we identified the presence of RAR-α, -γ and RXR-α, -β, -γ mRNA in cultured rat glomerular mesangial cells (Figure 1).

Effect of atRA on fibronectin expression induced by TGF-β1 and Ang II Our preliminary study showed that atRA alone was toxic only at 10 μmol/L, slightly increased the basal fibronectin level at 2 μmol/L, unexpectedly and markedly increased the basal fibronectin level at 0.2 μmol/L or 0.02 μmol/L with lower concentration being more effective (data not shown). Next we used atRA within a range from 0.125 to 8 μmol/L and found that pretreatment with atRA at a concentration of 0.5 to 8 μmol/L attenuated TGF-β1-induced fibronectin expression (P<0.05 *vs* TGF-β1) showing no dose-dependent difference, whereas a lower atRA concentration (0.125 μmol/L) increased TGF-β1-induced fibronectin expression (P<0.05 *vs* TGF-β1) (Figure 2). Here we focused on the inhibitory effect

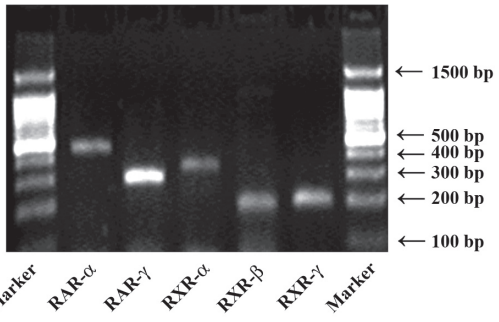

Figure 1. Identification of mRNA expression of five of the six RA receptors in cultured mesangial cells using RT-PCR analysis.

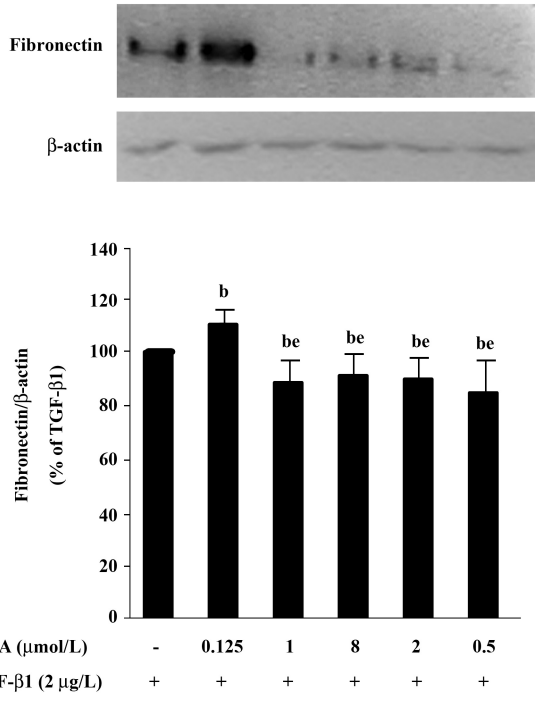

Figure 2. Effect of atRA on TGF-β1-induced fibronectin expression in mesangial cells. Quiescent mesangial cells were pretreated with atRA at the indicated concentrations followed by stimulation with 2 μg/L TGF-β1 for 48 h. Whole cell lysates were analyzed for fibronectin expression by Western blotting. atRA at high concentrations (0.5 to 8 μmol/L) almost equally decreased fibronectin expression induced by TGF-β1 in mesangial cells. In contrast, it exerted an opposing effect at a low concentration of 0.125 μmol/L. Shown are representative blots (top panel) and bar graph of relative fibronectin abundance normalized to β-actin (bottom panel). n=3. Mean±SD. ^bP<0.05 *vs* TGF-β1. ^eP<0.05 *vs* 0.125 μmol/L atRA+ TGF-β1.

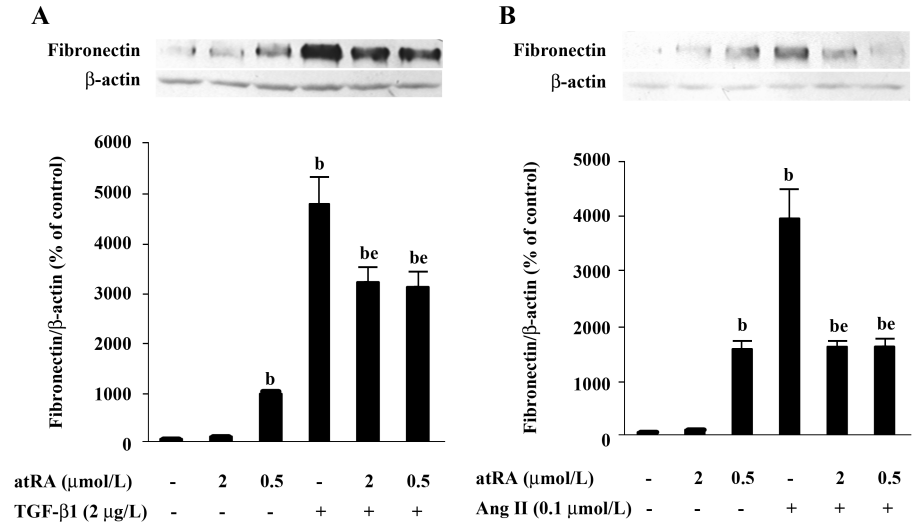

Figure 3. Effect of atRA on the increase in fibronectin induced by TGF-β1 or Ang II in mesangial cells. Quiescent mesangial cells were treated with either TGF-β1(2 μg/L) (A) or Ang II (0.1 μmol/L) (B) in the absence or presence of atRA (2, 0.5 μmol/L) for 48 h. Whole cell lysates were analyzed for fibronectin expression by Western blotting. atRA inhibited the increase in fibronectin in mesangial cells stimulated by TGF-β1 or Ang II. It alone increased the basal fibronectin level at a concentration of 0.5 μmol/L. Shown are representative blots (top panel) and bar graph of relative fibronectin abundance normalized to β-actin (bottom panel). n=3. Mean±SD. ^bP<0.05 *vs* control. ^eP<0.05 *vs* TGF-β1 or Ang II.

of atRA, so the concentrations of 2 and 0.5 μmol/L were chosen in the present study.

As shown in Figure 3A and 3B, 2 μmol/L atRA alone slightly increased, and 0.5 μmol/L atRA alone markedly increased the basal fibronectin level (P<0.05 *vs* control). However, pretreatment with either 2 μmol/L or 0.5 μmol/L atRA effectively inhibited the increase in fibronectin induced by TGF-β1 (2 μg/L) and Ang II (0.1 μmol/L) for 48 h in rat mesangial cells (P<0.05 *vs* TGF-β1 or Ang II) showing no dose-dependent difference.

Effect of atRA on PAI-1 expression induced by TGF-β1 and Ang II PAI-1 protein was increased 2 h after TGF-β1 (2 μg/L) stimulation, peaked at 5 h and decreased at 48 h (Figure 4). As illustrated in Figure 5, the early (5 h) basal and early TGF-β1-induced PAI-1 expressions were not affected by atRA. However, the sustained (48 h) PAI-1 expression under both basal and TGF-β1-stimulated conditions was markedly attenuated by atRA (P<0.05) (Figure 6A). The sustained (48 h) PAI-1 expression under both basal and Ang II-stimulated conditions was also markedly diminished by atRA (P<0.05) (Figure 6B).

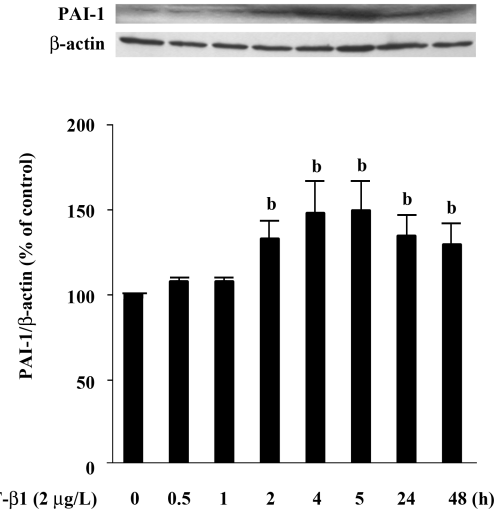

Figure 4. Time course of PAI-1 expression induced by TGF-β1 in mesangial cells. Quiescent mesangial cells were treated with TGF-β1 (2 μg/L) for the indicated periods of time. Whole cell lysates were analyzed for PAI-1 expression by Western blotting. PAI-1 was increased after treatment with TGF-β1, reaching a peak at 5 h. Shown are representative blots (top panel) and bar graph of relative PAI-1 abundance normalized to β-actin (bottom panel). n=3. Mean±SD. ^bP<0.05 *vs* control.

Discussion

The present study provides the first piece of direct evidence about the inhibitory effect of atRA on fibronectin production in mesangial cells. This effect of atRA is associated with a change in PAI-1 levels.

PAI-1 is the major physiological inhibitor of plasmin generation. High levels of PAI-1 are believed to favor the

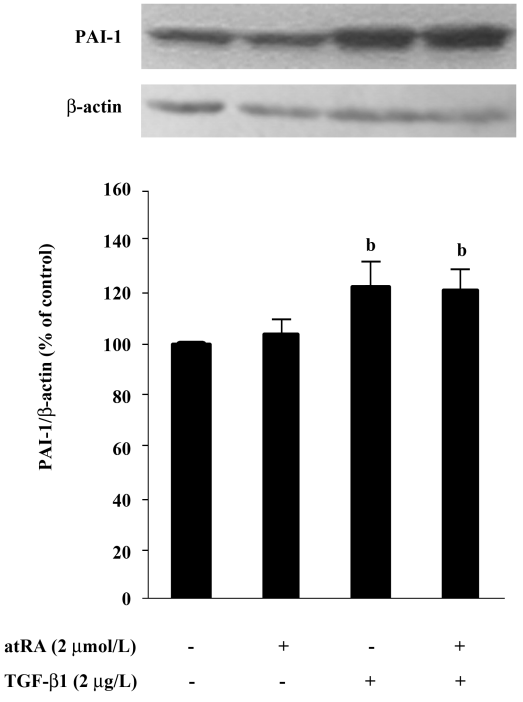

Figure 5. Effect of atRA on the increase in PAI-1 induced by TGF-β1 for 5 h in mesangial cells. Quiescent mesangial cells were treated with or without TGF-β1 (2 μg/L) in the absence or presence of atRA (2 μmol/L) for 5 h. Whole cell lysates were analyzed for PAI-1 expression by Western blotting. Pretreatment with atRA neither affected the increase in PAI-1 induced by TGF-β1 for 5 h, nor altered the basal PAI-1 level at 5 h. Shown are representative blots (top panel) and bar graph of relative PAI-1 abundance normalized to β-actin (bottom panel). n=3. Mean±SD. ^bP<0.05 *vs* control.

development of fibrosis, presumably because plasmin degrades ECM and activates other ECM-degrading MMPs. ECM accumulation is aggravated by PAI-1 overexpression suggesting the involvement of PAI-1 in fibrosis[4]. Moreover, fibrosis is ameliorated by PAI-1 depletion or blockage in both *in vitro* and *in vivo* experiments[2,5–9]. It is noteworthy that plasmin-independent mechanisms may also be implicated in profibrotic effects of PAI-1, because the deadhesive properties of PAI-1 do not require plasminogen or the generation of plasmin, and may play a role in promoting fibrosis by recruiting leukocytes and matrix-producing cells into the damaged tissue[22,23]. TGF-β1 and Ang II have been well recognized as major mediators in progressive glomerulosclerosis[10,13]. TGF-β1 stimulates the expression of ECM proteins such as collagens, laminin and fibronectin. On the other hand it suppresses the degradation of ECM by increasing PAI-1 synthesis [10-12]. Ang II also upregulates genes encoding ECM and PAI-1 proteins[13–15]. Its profibrotic action has been shown to be partially mediated by TGF-β1[14,15]. Therefore, we investigated the *in vitro* effect of atRA on fibronectin and PAI-1 expression induced by TGF-β1 and Ang II to further explore the mechanisms underlying the beneficial action of atRA on renal fibrosis.

In the present study, rat glomerular mesangial cells expressed five of the six RA receptor subtypes mRNA (only RAR-β is absent) using RT-PCR analysis, suggesting that these receptors are present in mesangial cells. The present study showed that TGF-β1 rapidly induced a sustained

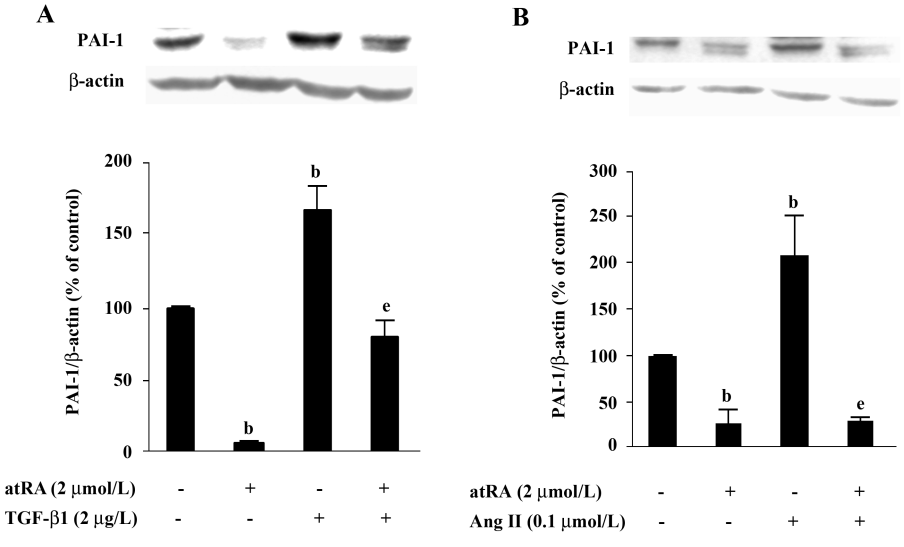

Figure 6. Effect of atRA on the increase in PAI-1 induced by TGF-β1 or Ang II for 48 h in mesangial cells. Quiescent mesangial cells were treated with either TGF-β1 (2 μg/L) (A) or Ang II (0.1 μmol/L) (B) in the absence or presence of atRA (2 μmol/L) for 48 h. Whole cell lysates were analyzed for PAI-1 expression by Western blotting. atRA inhibited the increase in PAI-1 induced by TGF-β1 or Ang II for 48 h. The basal PAI-1 level at 48 h was also decreased by atRA. Shown are representative blots (top panel) and bar graph of relative PAI-1 abundance normalized to β-actin (bottom panel). n=3. Mean±SD. ^bP<0.05 *vs* control. ^eP<0.05 *vs* TGF-β1 or Ang II.

increase in PAI-1 protein in mesangial cells. Interestingly, atRA did not alter the early (5 h) increase in PAI-1 but markedly inhibited the prolonged (48 h) PAI-1 induction. We assume that the mechanisms for PAI-1 induction at early time points are different from those at late time points. atRA may only interfere with the latter mechanisms. Another possible explanation is that atRA generates another mediator, which in turn exerts antifibrotic effects. Thus the inhibitory effect of atRA on PAI-1 expression occurs relatively late. For example, 9-*cis* RA, an isomer of atRA, has been reported to induce mRNA and protein expression of hepatocyte growth factor (HGF) at 24 h and 72 h in cultured mesangial cells[24]. It is possible that atRA inhibition of fibronectin expression stimulated by TGF-β1 is mediated by an inhibition of PAI-1 expression. Nevertheless, we cannot exclude that atRA may interfere with TGF-β1-stimulated fibronectin expression at the transcriptional level. In the present study, atRA also inhibited Ang II-induced increase in fibronectin and PAI-1. Previous studies have shown that atRA downregulates Ang II type I receptor mRNA in vascular smooth muscle cells[25] and that Ang II stimulates ECM and PAI-1 synthesis partially through induction of TGF-β1 expression[14,15], so it is expected that fibronectin and PAI-1 induction by Ang II can be repressed by atRA treatment.

atRA decreased fibronectin expression induced by TGF-β1 and Ang II in mesangial cells at concentrations of 2 and 0.5 μmol/L. However, atRA alone increased the basal fibronectin expression in mesangial cells, suggesting that atRA may increase or decrease fibronectin expression depending on the status of the cells in culture, stimulatory or quiescent. In line with our study, Chen *et al*[26] have reported that atRA alone activates mitogenesis of vascular smooth muscle cells under quiescent conditions. However, atRA inhibits mitogenesis in the presence of endothelin by different signaling pathways. Our data also showed that atRA alone was more effective in increasing the basal fibronectin expression at a very low concentration of 0.02 μmol/L than that at a higher concentration of 0.2 μmol/L. In agreement with our results, atRA alone has been shown to stimulate mitogenesis of vascular smooth muscle cells at low concentrations where the stimulatory effect of atRA at lower concentration is more evident than that at higher concentration[26]. In contrast with the inhibitory effects of atRA at high concentrations (0.5 to 8 μmol/L), it enhanced TGF-β1-induced fibronectin expression at a low concentration of 0.125 μmol/L in mesangial cells. The data suggest that atRA may inhibit or enhance TGF-β1-induced fibronectin expression depending on the concentrations of atRA used. The reasons for this biphasic effect are unclear. However, this is not an unusual biological response. For example, glibenclamide, a stimulator of glucose uptake, exerts opposing effects on high glucose-induced ECM formation at low (0.01 μmol/L) and high (1 μmol/L) concentrations[27]. The mechanisms under this phenomenon remain to be investigated.

At concentrations of 2 and 0.5 μmol/L, atRA was almost equally effective in reducing fibronectin expression stimulated by TGF-β1 and Ang II. A previous study from our laboratory has also shown that the dose-dependence of atRA action on cardiac fibrosis in spontaneously hypertensive rats is not apparent[21]. In addition, we can not show which RA receptor subtype is involved in mediating these effects of atRA. Experimental approaches using mesangial cells deficient in specific RA receptors or synthetic RA receptor ligands may help to address this question.

In summary, the present study shows that atRA at high concentrations inhibits the increase in fibronectin expression induced by TGF-β1 and Ang II in glomerular mesangial cells. This activity of atRA is associated with a change in PAI-1 levels. atRA might be an useful antifibrotic drug in the treatment of kidney diseases.

Author contribution

Xia LIU, Lei LÜ, and Yi-chun ZHU designed research; Xia LIU and Bei-bei TAO performed Western blotting and RT-PCR, respectively; Xia LIU and Lei LÜ analyzed data; Xia LIU and Yi-chun ZHU wrote the paper.

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