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Transforming growth factor β2 (TGF-β_2)-induced connective tissue growth factor (CTGF) expression requires sphingosine 1-phosphate receptor 5 ($S1P_5$) in human mesangial cells

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ABSTRACT

Transforming growth factor β2 (TGF-β_2) is well known to stimulate the expression of pro-fibrotic connective tissue growth factor (CTGF) in several cell types including human mesangial cells. The present study demonstrates that TGF-β_2 enhances sphingosine 1-phosphate receptor 5 ($S1P_5$) mRNA and protein expression in a time and concentration dependent manner. Pharmacological and siRNA approaches reveal that this upregulation is mediated via activation of classical TGF-β downstream effectors, Smad and mitogen-activated protein kinases. Most notably, inhibition of G_i with pertussis toxin and downregulation of $S1P_5$ by siRNA block TGF-β_2-stimulated upregulation of CTGF, demonstrating that G_i coupled $S1P_5$ is necessary for TGF-β_2-triggered expression of CTGF in human mesangial cells. Overall, these findings indicate that TGF-β_2 dependent upregulation of $S1P_5$ is required for the induction of pro-fibrotic CTGF by TGF-β. Targeting $S1P_5$ might be an attractive novel approach to treat renal fibrotic diseases.

1. Introduction

Renal fibrosis is the final outcome of many forms of chronic kidney diseases. The two major forms that constitute renal scarring comprise tubulointerstitial and glomerular fibrosis. The progression of renal glomerular fibrosis is characterized by enhanced secretion of cytokines and growth factors, activation of mesangial cells, subsequent mesangial cell proliferation, and enhanced formation and deposition of extracellular matrix components, overall leading to glomerulosclerosis. In this context, transforming growth factor β (TGF-β) is known as a key cytokine mediating characteristic pro-fibrotic changes (for review, see [1]). TGF-β binds to the TGF-β receptor (TβR) type II, which interacts with TβR type I, classically followed by activation of TβR type I kinase and subsequent phosphorylation of Smad proteins. Smad proteins then translocate in a dimeric form into the nucleus to act as transcription factors. In addition, it was shown that TGF-β activates classical mitogen-activated protein kinase (MAPK) family members, p38 MAPK, p42/p44 MAPK, and c-Jun N-terminal kinase (JNK), which in turn mediate the expression of pro-fibrotic factors (for review, see [1]). In this context, TGF-β is well known to upregulate connective tissue growth factor (CTGF) expression [2], a member of the CCN family of matricellular proteins (for review, see [3]). CTGF itself is an important factor for the development of renal fibrosis by mediating some of the fibrogenic effects of TGF-β, e.g. enhanced matrix production and increased adhesion and migration of several cell types [4,5].

Besides TGF-β and CTGF, previous studies identified sphingosine 1-phosphate (S1P) as another important factor in the progression of renal fibrosis (for review, see [6,7]). S1P is generated in cells by two isoforms of sphingosine kinase (SK), SK-1 and SK-2. S1P can then act either intracellularly, or via activation of five specific transmembrane receptors, denoted as $S1P_{1-5}$ (for review, see [8]). S1P receptor subtypes couple to different G proteins, including G_i ($S1P_{1-5}$), G_q ($S1P_{2/3}$), $G_{12/13}$ ($S1P_{2-5}$) and small G proteins, and thereby regulate a wide range of cellular functions upon activation (for review, see [9]). We previously demonstrated that TGF-β_2 enhanced the expression and activity of SK-1 and supposedly of intracellular S1P, which in turn attenuated renal fibrosis by impeding CTGF expression in human podocytes [10]. Moreover, we demonstrated that extracellular S1P, via activation of S1P receptors, acts pro-fibrotic by mimicking TGF-β dependent cell response, e.g. the induction of pro-fibrotic CTGF [11].

Abbreviations: ActD, actinomycin D; CTGF, connective tissue growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; SK, sphingosine kinase; TGF-β2, transforming growth factor β2; TβR, TGF-β receptor; αSMA, alpha smooth muscle actin

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However, so far nothing is known about the effect of TGF-β on S1P receptor expression in renal cells. We therefore investigated whether TGF-β_2 alters S1P receptor expression and whether such an alteration influences the pro-fibrotic capacity of TGF-β_2 in human mesangial cells.

2. Material and methods

2.1. Reagents

SB203580, SP600125, U0126, TGF-β receptor I kinase inhibitor II, SKI II (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole) and pertussis toxin (PTX) were purchased from Merck (Darmstadt, Germany). TGF-β_2 and the pan-specific anti-TGF-β antibody were from R & D Systems (Minneapolis, MN, USA).

2.2. Cell culture and stimulation

Human primary mesangial cells were a gift from Prof. H. H. Radeke (Frankfurt, Germany) and cultured exactly as described previously [12]. All cell culture media and supplements were purchased from Life Technologies (Darmstadt, Germany). For experiments in this study, human mesangial cells between passages 14 and 20 were seeded in 6-well plates or 60-mm-diameter dishes and used after reaching subconfluency. The cells were pre-incubated with Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg/ml of fatty acid-free bovine serum albumin for 24 h and then stimulated with the indicated substances. Control cells were treated with vehicle alone. Final solvent concentration did not exceed 0.2% (v/v).

2.3. Two step PCR analysis

Two step PCR analysis was performed as described [13]. Briefly, 1.2 μg of total RNA was isolated with TRIZOL™ reagent (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol and used for reverse transcriptase polymerase chain reaction (RT–PCR; RevertAid™ first strand cDNA synthesis kit, Thermo Fisher Scientific, Waltham, MA, USA) utilizing an oligo (dT) primer for amplification. Real-time PCR (TaqMan®) was performed using the Applied Biosystems 7500 Fast Real-Time PCR System. Probes, primers, and the reporter dyes 6-FAM and VIC were from Life Technologies (Darmstadt, Germany). The cycling conditions were as follows: 95 °C for 15 min (1 cycle), 95 °C for 15 s and 60 °C for 1 min (40 cycles). The threshold cycle (C_t) was calculated by the instrument's software (7500 Fast System SDS Software version 1.4). Analysis of the relative mRNA expression was performed using the $\Delta\Delta C_t$ method. Results in Fig. 1A are analyzed as $2^{-\Delta Ct}$. The housekeeping gene GAPDH was used for every normalization.

2.4. Western blot analysis

Following stimulation the medium was removed and the cells were washed once with phosphate-buffered saline (PBS) solution. Thereafter, cells were scraped into ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 40 mM β-glycerophosphate, 50 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM phenylmethyl sulphonyl fluoride) and homogenized by sonication. The samples were then centrifuged for 10 min at 16,200 *g* and the supernatant was taken for protein determination. For determination of secreted CTGF protein levels, cell supernatants were centrifuged (10 min; 16,200 *g*) and the supernatants were precipitated using 70% trichloroacetic acid. After incubation for 30 min on ice and an additional centrifugation (30 min; 16,200 *g*) pellets were resuspended in Tris buffer (pH 8.5). Cell lysates containing equal amounts of protein (30 μg) and cell supernatant were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane and subjected to Western blot analysis as described previously [14]. CTGF (1:1000) and β-actin (1:3000) antibodies were from Santa Cruz Biotechnologies (Dallas, TX, USA); the Smad4 (1:1000) antibody was obtained from Cell Signaling Technology (Danvers, MA, USA) and the $S1P_5$ (1:200) antibody was from Novus Biologicals (Cambridge, UK). The SK-1 antibody (1:2000) was generated as previously described [15].

2.5. Enzyme-linked immunosorbent assays (ELISA)

CTGF concentrations in human mesangial cell lysates were determined by sandwich ELISA, using two distinct monoclonal antibodies specifically recognizing the N-terminal part of the CTGF protein (FibroGen, San Francisco, CA, USA) as described previously [16]. This assay detects both CTGF N-terminal fragments as well as full-length CTGF. Purified recombinant human CTGF (FibroGen) was used as standard.

2.6. siRNA transfection

siRNA transfection was performed as described [17]. Briefly, human mesangial cells (2×10^6 cells per assay) were washed with PBS and suspended in 100 μl Cell Line Nucleofector™ Solution V (Lonza, Cologne, Germany). Then, 0.5 μM Smad4 siRNA (Santa Cruz Biotechnologies, Dallas, TX, USA) and 0.5 μM $S1P_5$ siRNA (Santa Cruz Biotechnologies, Dallas, TX, USA) were added to the cell suspension and transfection was performed according to the manufacturer's instructions using the protocol A-033. Control cells were transfected with 0.5 μM non-targeting siRNA (Thermo Fisher Scientific, Waltham, MA, USA). Immediately after nucleofection, cell suspensions were transferred to a 6-well culture plate. To reach confluency, all cells were cultured at 37 °C for 2 days as described above. Thereafter, quiescent cells were used for experiments.

2.7. Statistical analysis

All data were statistically analyzed by Student's *t* test for unpaired analysis or one-way analysis of variance in case of multiple comparisons. Differences with $P < 0.05$ were considered to be significant (GraphPad Prism version 5; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. TGF-β_2 enhances $S1P_5$ expression in human mesangial cells

In a first step, we checked the effect of TGF-β_2 on mRNA expression levels of S1P receptor subtypes in human mesangial cells using TaqMan® analysis. As illustrated in Fig. 1A, $S1P_1$, $S1P_2$ and $S1P_5$ are well expressed in human mesangial cells. In contrast, $S1P_3$ was very weakly expressed and $S1P_4$ expression was not detectable in our experimental setting (Fig. 1A). Stimulation with 5 ng/ml TGF-β_2 significantly enhanced $S1P_5$ mRNA expression (Fig. 1A) in a time and concentration dependent manner (Fig. 1B and C). In addition to the induction of $S1P_5$ mRNA, we detected significantly reduced $S1P_1$ mRNA levels after 16 h treatment of human mesangial cells with 5 ng/ml TGF-β_2 (Fig. 1A). The mRNA expression levels of $S1P_2$, $S1P_3$, and $S1P_4$ were not altered by TGF-β_2 treatment (Fig. 1A). Next, we analyzed the effect of TGF-β_2 on $S1P_5$ protein expression. Treatment for 2, 4, 16, and 24 h with 5 and 10 ng/ml TGF-β_2 led to significantly higher protein expression levels (Fig. 1D). In human mesangial cells, we detected a highly regulated $S1P_5$ protein signal at approximately 40 kDa (Fig. 1D), which we confirmed by siRNA treatment (Fig. 7B). Incubation of human mesangial cells with $S1P_5$ siRNA completely abolished the TGF-β_2 dependent induction of the $S1P_5$ protein signal described above (data not shown).