Available online at www.sciencedirect.com

SCIENCE DIRECT

Experimental Cell Research 307 (2005) 305 – 314

Experimental Cell Research

www.elsevier.com/locate/yexcr

Modulation of the TGFβ/Smad signaling pathway in mesangial cells by CTGF/CCN2

Nadia Abdel Wahab*[,1], Benjamin S. Weston, Roger M. Mason[1]

Cell and Molecular Biology Section, Division of Biomedical Sciences, Imperial College London, Sir Alexander Fleming Building, South Kensington, London, SW7 2AZ, UK

Received 18 January 2005, revised version received 15 March 2005
Available online 22 April 2005

Abstract

Transforming growth factor-beta (TGFβ) drives fibrosis in diseases such as diabetic nephropathy (DN). Connective tissue growth factor (CTGF; CCN2) has also been implicated in this, but the molecular mechanism is unknown. We show that CTGF enhances the TGFβ/Smad signaling pathway by transcriptional suppression of Smad 7 following rapid and sustained induction of the transcription factor TIEG-1. Smad 7 is a known antagonist of TGFβ signaling and TIEG-1 is a known repressor of Smad 7 transcription. CTGF enhanced TGFβ-induced phosphorylation and nuclear translocation of Smad 2 and Smad 3 in mesangial cells. Antisense oligonucleotides directed against TIEG-1 prevented CTGF-induced downregulation of Smad 7. CTGF enhanced TGFβ-stimulated transcription of the SBE4-Luc reporter gene and this was markedly reduced by TIEG-1 antisense oligonucleotides. Expression of the TGFβ-responsive genes PAI-1 and Col III over 48 h was maximally stimulated by TGFβ + CTGF compared to TGFβ alone, while CTGF alone had no significant effect. TGFβ-stimulated expression of these genes was markedly reduced by both CTGF and TIEG-1 antisense oligonucleotides, consistent with the endogenous induction of CTGF by TGFβ. We propose that under pathological conditions, where CTGF expression is elevated, CTGF blocks the negative feedback loop provided by Smad 7, allowing continued activation of the TGFβ signaling pathway.

Keywords: CTGF; CCN 2; Diabetic nephropathy; TGFβ; Smad 7

Introduction

There is abundant evidence in the literature implicating TGFβ as the major fibrogenic growth factor in the pathogenesis of glomerulosclerosis and interstitial fibrosis. Its role in the development of diabetic nephropathy (DN) has been established [1] and explored both in vitro and in vivo using neutralizing anti-TGFβ antibodies to attenuate its effects [2,3]. CTGF is another prosclerotic growth factor implicated in the pathogenesis of renal fibrosis in DN [4]. Although it is rapidly induced by and appears to mediate at least some of the fibrogenic actions of TGFβ [5], the molecular mechanisms by which CTGF functions are not understood.

The mesangial cell plays a key role in the development of glomerulosclerosis in DN [6]. Recent reports from our laboratory, using CTGF antisense and control oligonucleotides, provide strong evidence that CTGF mediates TGFβ-dependent mesangial cell (MC) dysfunction which is manifest by: (1) induction of cellular hypertrophy due to induction of the cyclin dependent kinase inhibitors (CDKIs) p15, p21, and p27, leading to cell cycle arrest in the G_1 phase [7], (2) increased expression of extracellular matrix proteins (ECM) [4], (3) upregulation of integrins on the cell surface, facilitating the deposition and assembly of ECM proteins [8] (4) reorganization of the actin cytoskeleton (unpublished data). All these changes are prevented by CTGF antisense, but not by control, oligonucleotides. Thus, we investigated the molecular basis of the relationship between TGFβ and CTGF and in particular, the effect of CTGF on the TGFβ/Smad signaling pathway.

* Corresponding author. Fax: +44 20 838 32062.
E-mail address: nadia.wahab@imperial.ac.uk (N. Abdel Wahab).
[1] Present address Renal Section, Division of Medicine, Hammersmith Hospital, Du Cane Road, London, W12 ONN, UK.

0014-4827/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2005.03.022

TGFβ exerts its cellular effects via the Smad signaling pathway which provides the main signal transduction route downstream of TGFβ receptors [9]. On binding TGFβ, the receptors dimerize and autophosphorylate. This in turn phosphorylates Smad 2 and Smad 3 [10], which form a complex with Smad 4 and translocate into the nucleus where they co-operate with other transcription factors to regulate transcription of the many TGFβ-responsive genes containing a Smad binding element (SBE) in their promoters. One such induced gene is Smad 7. Smad 7 protein interacts with the E3-ubiquitin ligases, Smurf1 or Smurf2, in the nucleus. Thus, after TGFβ stimulation, Smad 7–Smurf complex translocates to the plasma membrane where Smurf induces ubiquitination and degradation of the TGFβ receptors [11,12]. This prevents further phosphorylation of Smad 2 and Smad 3. Thus, Smad 7 is a potent endogenous inhibitor that provides a negative feedback response to limit TGFβ effects. Indeed, strategies to overexpress Smad 7 protein have been used successfully to inhibit fibrotic effects of TGFβ on different cell types, including renal cells [13–16]. In contrast, it has been reported that overexpression of a transcription factor, TIEG-1 (TGFβ inducible early gene), enhances TGFβ-induced Smad 2 phosphorylation. It does this by binding to the promoter of Smad 7 and repressing its expression, thus potentiating TGFβ/Smad signaling [17].

In the present work, we provide evidence that CTGF enhances the TGFβ signaling pathway in mesangial cells by stimulating TEIG-1 expression and decreasing the availability of Smad 7.

Materials and methods

Cell cultures, antibodies, and reagents

Primary normal adult human mesangial cells (CC-2259, lot 3F1510) were purchased from Biowhittaker (Wokingham, Berkshire, UK), maintained in culture as described previously [4], and used at passage 9–10. Antibodies against Smad 2, Smad 3, and Smad 7 were from Santa Cruz Biotechnology, Inc. (Autogen Bioclear, Calne, Wilts., UK). TGFβ inducible early gene (TIEG-1) antibodies were a gift from Dr. Steven Johnsen (Mayo Foundation, Minnesota, USA). Recombinant CTGF was expressed in transformed HMC and purified from the medium using Talon metal affinity resin, as reported previously [4]. TGFβ was purchased from R&D Systems (Abingdon, Oxfordshire, UK). Phosphothioate antisense (TGG GCA GAC GAA CG) and control oligonucleotides (ACC GAC CGA CGT GT) directed to CTGF and antisense (TGTGTCTGGACAGTTCAT) and control oligonucleotides (ACTACTACACTAGACTAC) directed to TIEG-1 were designed and manufactured by Biognostik GmbH (Göttingen, Germany), who own the intellectual property rights to the sequences. The SBE4-Luc reporter was a gift from Dr. B. Vogelstein [18].

Western blotting

Cells were lysed in reducing SDS-PAGE loading buffer and immediately scraped off the plate. Cell lysates were sonicated for 10 s to shear DNA. Samples were boiled for 5 min and resolved on 4–12% gradient gels by SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride membrane filter (Immobilin-P, Millipore, Bedford, UK) using a BioRad transfer apparatus. Blots were incubated in blocking buffer containing 1× TBS, 0.1% Tween-20 with 5% (w/v) non-fat dry milk, for 1 h. Immunodetection was performed by incubating the blots in primary antibody at the appropriate dilution in antibody dilution buffer (1× TBS, 0.1% Tween-20 with 5% BSA), overnight at 4°C. Blots were then washed 3 times with washing buffer (1× TBS, 0.1% Tween-20) and incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies for 1 h at room temperature. Bound antibodies were visualized using the enhanced chemiluminescence reagent Luminol (Autogen Bioclear UK Ltd., Wiltshire, UK). Prestained molecular weight standards (Amersham International PLC, Amersham, UK) were used to monitor protein migration.

Nuclear fraction preparation

Cells were scraped in ice-cold PBS, recovered by centrifugation at 500 × *g* for 10 min and resuspended in 500 μl of buffer A [10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 10 mM NaCl, 1× protease inhibitors cocktail (1 mM EDTA, 1 mM EGTA, 0.2 mM TLCK, 1 mM *N*-ethylmaleimide, 0.1 mM TPCK, and 2 mM PMSF, Sigma), 1 mM NaF, 1 mM Na_3VO_4]. After incubation on ice for 20 min, Nonidet P40 was added to a final concentration of 0.6% (v/v) and vigorously vortex-mixed for 10 s. The nuclei were pelleted at 4°C by centrifugation for 5 min at 12,000 × *g*. The nuclear pellet was washed once with buffer A, collected by centrifugation, resuspended in 500 μl of buffer B [10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 450 mM NaCl, 1× protease inhibitors cocktail, 1 mM NaF, 1 mM Na_3VO_4, 20% (v/v) glycerol] and vortex-mixed for 15 min at 4°C. The lysate was centrifuged at 12,000 × *g* for 5 min at 4°C and the supernatant containing the nuclear proteins was transferred to a fresh vial. Protein concentration was measured by Bradford assay. Extracts were stored at −70°C until further use.

Use of antisense oligonucleotides

TIEG-1 and CTGF antisense oligonucleotide (2 μM, as recommended by the manufacturer) or a CG-matched randomized sequence oligonucleotide (negative control) was added directly to cultures 30 min prior to any other treatments [7].

Transient transfection and reporter gene assay

The SBE4-Luc reporter gene construct was transfected into 25 × 10^6 transformed human mesangial cells (THMC),