Ceramide inhibits CCN2 expression in fibroblasts

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Abstract Connective tissue growth factor (CTGF, CCN2) is induced in response to TGF β in fibroblasts. In this report, we show that C2 ceramide reduced the ability of TGF β to induce CCN2 protein, mRNA and promoter activity in fibroblasts. C2 ceramide reduced the ability of TGF β to induce the generic Smad responsive promoter/reporter construct SBE-luciferase. These results suggest that C2 ceramide reduces the action of TGF β in fibroblasts via Smad antagonism.

Keywords CCN2 · TGF β

Introduction

Connective tissue growth factor (CTGF/CCN2) is a matricellular protein found in the extracellular matrix (ECM) which acts via integrins and heparin sulfate-containing proteoglycans to modify of adhesive signaling in response to ECM and cytokines (Babic et al. 1999; Chen et al. 2004; Shi-wen et al. 2006; Chen et al. 2007; Kennedy et al. 2007). CCN2 is induced during tissue repair and is overexpressed in fibrotic disease and is believed to play key roles in these processes (Blom et al. 2002; Leask and Abraham 2006). CCN2 expression is controlled primarily at the transcriptional level (Blom et al. 2002). CCN2 promoter is induced in response to the pro-fibrotic protein transforming growth

factor β (TGF β) (Igarashi et al. 1993; Grotendorst et al. 1996; Holmes et al. 2001). In normal fibroblasts and mesangial cells, CCN2 induction in response to TGF β requires Smad 3 and Ets-1 and also requires ras/MEK/ERK (Holmes et al. 2001; Stratton et al. 2002; Chen et al. 2002; Leask et al. 2001, 2003; Van Beek et al. 2006). CCN2 promoter activity is elevated in pathologies such as fibrosis and cancer (Leask et al. 2001; Holmes et al. 2001; Pickles and Leask 2007). Identifying methods to control CCN2 promoter activity is likely therefore to be of benefit in understanding how to control these pathological conditions (Blom et al. 2002; Leask and Abraham 2006). For example, we have found that CCN2 is repressed by the pro-inflammatory cytokine tumor necrosis factor α (TNF α), through elements in the CCN2 promoter (Abraham et al. 2000).

The sphingolipid sphingolipid ceramide has emerged as a lipid messenger of cell functions, including differentiation and apoptosis, in response to diverse kinds of stresses and biological factors including TNF α (Adam-Klages et al. 1998). Although the role of TNF α in suppressing TGF β signaling has been well-established, the role of ceramide in this process is less well researched (Tellier et al. 2007). Moreover, whether ceramide modifies CCN2 expression is unknown. In this report we assess whether ceramide may modify CCN2 expression in primary human fibroblasts.

Materials and methods

Cell cultures and western blotting Human foreskin fibroblasts (ATCC) were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS) and antibiotic/antimycotic solution (Invitrogen,

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Burlington, ON). Alternatively Cells were then treated with or without TGF β (4 ng/ml, R and D Systems) for 24 h. Prior to addition of TGF β , cells were pretreated for 45 min with or without C2-ceramide (10 μ M; Calbiochem). Cells were also treated, when indicated, with an identical concentration of dihydroceramide (Calbiochem). CCN2 was then detected by Western blot analysis of cell layer as previously described (Abcam; Holmes et al. 2001). Anti-GAPDH antibody was used as a control (Abcam).

Transfections and DNA constructs For transfections, fibroblasts were used and cultured in six well plates and transfected with FuGene (Roche, Indianapolis, IN) at a ratio of 3 μ l FuGene:1.75 μ g DNA per well. Cells were transfected with plasmids containing a CCN2 promoter (−805 to + 17, 1.50 μ g/well) fused to a secreted alkaline phosphatase (SEAP) reporter gene (CCN2-SEAP) (Abraham et al. 2000). Alternatively, a construct containing multiple copies of the Smad binding element CAGA upstream of the luciferase reporter gene was used (SBE-lux) (Jonk et al. 1998). To control for differences in transfection efficiency, cells were co-transfected with 0.25 μ g of a cytomegalovirus promoter- β -galactosidase (CMV- β -gal) reporter gene (Clontech, Palo Alto, CA) construct. Cells were allowed to grow in 0.5% serum for 18 h in 37°C. Cells were then treated with or without TGF β 1 (4 ng/ml, R and D Systems) for 24 h. Prior to addition of TGF β , cells were pretreated for 45 min with or without C2-ceramide (10 μ M; Calbiochem). Promoter assays were performed with a Phospha-Light kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol and SEAP reporter expression was adjusted for differences in β -galactosidase expression as determined by a Galacto-star kit (Applied Biosystems) according to manufacturer's protocol. Alternatively, a Dual-Light (Applied Biosystems) kit was used to detect luciferase and β -galactosidase expression. Data was expressed as average values $\pm$ standard deviation of at least three replicates and at least two independent trials. Measurement of SEAP levels were obtained from an LMax II 384 luminometer and SoftMax Pro 4.7.1 (Molecular Devices, Sunnyvale CA). Samples were run in sextuplicate, and levels were measured in relative light units and were standardized to control values from β -gal. Statistical tests were done using one-way ANOVA and Tukey's post-hoc test on GraphPad.

Real time RT-PCR Real time PCR was performed as previously described (Kennedy et al. 2007; Shi-wen et al. 2006). Briefly, cells were then treated with or without TGF β (4 ng/ml, R and D Systems) for 24 h. Prior to addition of TGF β , cells were pretreated for 45 min with or without C2-ceramide (10 μ M; Calbiochem). RNA was harvested using the Qiashredder and RNeasy kit (Qiagen)

and used for Real-Time RT-PCR. Twenty-five nanograms of RNA was reverse transcribed and amplified using TaqMan Assays on Demand (Applied Biosystems) in a 15 μ l reaction containing primers for TaqMan probes (Applied Biosystems) and 6-carboxyfluorescein labeled TaqMan MGB probe. Reverse Transcriptase one-step qPCR Mastermix (Applied Biosystems) was added to samples and the ABI Prism 7900 HT sequence detector (Perkin-Elmer-Cetus, Vaudreuil, QC) was used according to manufacturer's instructions to detect amplified sequences. Samples were run in triplicate, and expression values from CCN2 primers (Hs01026927_g1) were standardized to control values from ribosomal RNA primers (catalog 4308329) using the $\Delta\Delta$ Ct method (Livak and Schmittgen 2001). Statistical analysis was done using one way ANOVA and Tukey's post hoc test on GraphPad.

Cell proliferation assay To detect the effect of C2 ceramide on cell proliferation in fibroblasts, an 3-[4,5-dimethylthiazol-2-yl]=2,5-diphenyltetrazolium bromide (MTT) assay (Roche) was performed as described by the manufacturer. Briefly, cells at 0.4×10^6 cells/ml in DMEM, 0.5% FBS were treated with 1 μ g/ml actinomycin D for 3 h, 37°C. Equal numbers of cells were plated into wells of a 96 well plate (125 μ l/well) equal numbers of cells were placed in wells of a 96 well plate (four wells/data point assessed). Cells were then incubated with or without C2 ceramide (10 μ M) for 45 min prior to addition of TGF β (4 ng/ml) for 24 h. After labeling with MTT reagent, results at A_{575} with a reference wavelength of A_{700} were measured (Safire, Tecan, San Jose, CA, USA).

Results

C2 ceramide blocks the TGF β -mediated induction of CCN2 protein and mRNA in fibroblasts

To determine whether C2 ceramide modified CCN2 expression in fibroblasts, fibroblasts were cultured in 0.5% serum and pretreated for 45 min with or without C2-ceramide prior to addition of TGF β 1 for an additional 24 h. To detect CCN2, Western blot analysis of extracts prepared from cell layers was performed. We found that whereas TGF β induced CCN2 protein production, C2 ceramide blocked this response (Fig. 1a). Under the conditions of the assay, C2 ceramide had no effect on the viability of fibroblasts (Fig. 1b). Based on these data, we sought to examine if C2 ceramide could block the TGF β -mediated induction of CCN2 mRNAs. To address this question, cellular mRNA was harvested 6 h post-TGF β treatment. Real-time PCR analysis revealed that the overall amounts of basal and

Fig. 1 C2 ceramide reduces the ability of TGFβ to induce CCN2 protein in fibroblasts. **a** Fibroblasts were incubated for 24 h in DMEM/0.5% FBS. Cells were then incubated without or with C2 ceramide (*cer*, 10 μM, 45 min) or dihydroC2 ceramide (*dhcer*, 10 μM, 45 min) prior to incubation in the presence or absence of TGFβ1 (4 ng/ml, 24 h), as indicated. To detect CCN2 protein, equal amounts of cell

layer extracts (20 μg) were subjected to Western blot analysis with anti-CCN2 antibody. Parallel blots were performed using an anti-GAPDH antibody. **b** C2 ceramide does not affect cell viability. Cells were treated with or without C2 ceramide as in **a**. An MTT assay was conducted to assess cell viability, as described in the “Materials and methods” section

TGFβ-induced CCN2 mRNA was significantly reduced in the presence of C2 ceramide (Fig. 2). Collectively, these results indicate that C2 ceramide can modify CCN2 expression in response to TGFβ in fibroblasts.

C2 ceramide reduces TGFβ-induced CCN2 and Smad-dependent promoter activity

Smad, and in particular Smad3/4, generally mediate TGFβ action in fibroblasts (Leask and Abraham 2004). As C2 ceramide reduced TGFβ-induced mRNA and protein expression in fibroblasts, we evaluated whether C2 ceramide could block the activity of a Smad3-responsive promoters. Thus, we probed the effect of C2 ceramide on the TGFβ-mediated induction of the CCN2 promoter. Fibroblasts were transfected with a CCN2 promoter-SEAP reporter construct containing the region of the CCN2 promoter spanning between -805 to +17. The -805 to +17 promoter fragment contains the previously-identified TGFβ response element of the CCN2 promoter, including a Smad3/4-responsive element (Holmes et al. 2001; Leask et al. 2001, 2003). C2 ceramide reduced basal and TGFβ-induced CCN2 promoter activity (Fig. 3). These results collectively suggested that C2 ceramide blocked Smad action. To test this interpretation further, a promoter/reporter construct bearing multiple copies of a Smad binding element (SBE) subcloned upstream of the luciferase reporter (SBE-lux) gene was transfected into fibroblasts. Cells were treated with or without TGFβ for 24 h. Addition of C2 ceramide reduced activity of the SBE-lux construct in response to TGFβ (Fig. 3). Note that basal Smad-dependent promoter activity was observed in fibroblasts, and that this was diminished by

C2 ceramide treatment (Fig. 3). These results indicate that the TGFβ/Smad pathway is active in the fibroblasts (human foreskin fibroblasts) used for this study. Collectively, these data suggest that C2 ceramide acted, at least in part, via suppressing Smad action.

Fig. 2 C2 ceramide reduces TGFβ-induced CCN2 mRNA expression. Fibroblasts were incubated for 24 h in DMEM/0.5% FBS. Cells were then incubated with or without C2 ceramide (*cer*, 10 μM, 45 min) prior to incubation in the presence or absence of TGFβ1 (4 ng/ml, 6 h). RNA was harvested and subjected to real time RT-PCR and was normalized with ribosomal RNA. The experiment was performed in triplicate, on three independent occasions. A representative experiment is shown. The standard deviation within each data point was less than 10%. (* $P < 0.001$; significantly reduced mRNA due to inhibitor). Note that expression of both basal and TGFβ-induced CCN2 mRNAs were affected by C2 ceramide treatment

Fig. 3 C2 ceramide reduces TGFβ-induced promoter activity (*left panel*) CCN2 promoter Fibroblasts were co-transfected with plasmids containing the wild-type CCN2 promoter driving the SEAP reporter gene and a CMV-β-gal construct. Cells were incubated for 18 h in DMEM/0.5% FBS, and treated with or without 10 μM C2 ceramide for 45 min followed by treatment with or without TGFβ1 for 24 h (4 ng/ml). SEAP expression values were measured in relative light units. The experiment was repeated performed twice. Values were expressed in relative light units and normalized with β-galactosidase. Values are mean of four replicates, ±standard deviation from three separate experiments. * $P < 0.05$; significantly different from control (DMSO alone). SBE-lux construct (*right panel*). Fibroblasts were co-

transfected with plasmids containing multimers of a Smad binding element driving the luciferase reporter gene and a CMV-β-gal construct. Cells were incubated for 18 h in DMEM/0.5% FBS, and treated with or without C2 ceramide for 45 min (concentrations as indicated) followed by treatment with or without TGFβ1 for 24 h (4 ng/ml). Luciferase expression values were measured in relative light units. The experiment was repeated performed twice. Values were expressed in relative light units and normalized with β-galactosidase. Values are mean of four replicates, ±standard deviation from three separate experiments. * $P < 0.05$; significantly different from control (DMSO alone)

Discussion

Until now, there has been no report examining the effect of ceramide on CCN2 expression or on Smad activity. Moreover, only a very small number of papers have examined the effects of ceramide on fibrogenic genes in fibroblasts. It is interesting to note that in prior studies it has been shown that endogenous sphingolipids are co-regulators of the TGF- signaling pathway, involved with the induction of collagen type I expression (Sato et al. 2003). In this study, however, it was found that low levels of ceramide potential, whereas higher levels suppress, the TGFβ induction of type I collagen (Sato et al. 2003). In addition, exogenously applied ceramide suppresses collagen production (Hernández-Muñoz et al. 1997; Reunanen et al. 2000). The TGF- induction of the collagen type I promoter was also inhibited by overexpression of SPHK1, an enzyme that promotes formation of sphingosine-1-phosphate (Sato et al. 2003). It is likely, therefore, that depending on the cellular levels, ceramide can either enhance or suppress gene expression in fibroblasts. Nonetheless, our current report indicates, for the first time, that ceramide can suppress Smad-dependent reporter activity. The fundamental mechanism of C2 ceramide action in modulating Smad activity is beyond the scope of our current study and requires further experimentation. In principle, C2 ceramide may act by promoting Smad sequestration or by

blocking Smad phosphorylation or nuclear localization. Overexpressing Smad3/4 rescued the ability of C2 ceramide to suppress activation of the CCN2 promoter (not shown), supporting the notion that Smad sequestering may be involved. C2 ceramide can activate the JNK pathway; however, the JNK pathway is not likely to be involved JNK itself is not involved as, whereas JNK inhibition potentiated the TGFβ-induction of CCN2 mRNA consistent with previous observations (Leask et al. 2003), JNK inhibition did not affect the ability of C2 ceramide suppress CCN2 mRNA expression (not shown). Overall, our results contained within this current report are consistent with previous observations that α-galactosylceramide suppresses bleomycin-induced acute pulmonary inflammation and thus attenuates the development of pulmonary fibrosis (Kimura et al. 2004), although in this study the effects of ceramide on fibroblasts were not assessed.

Collectively, our results indicate that modulation of the sphingolipid pathway is likely to have utility in controlling fibrogenic responses in fibroblasts, including the induction of CCN2. As there is currently no sufficient treatment for fibrosis, obtaining a greater understanding of the mechanisms underlying fibrosis, such as the mechanism underlying the induction of CCN2 in response to TGFβ, is crucial in order to identify novel potential drug targets.

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