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Inhibition of Connective Tissue Growth Factor/CCN2 Expression in Human Dermal Fibroblasts by Interleukin-1α and β

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ABSTRACT

Connective tissue growth factor (CTGF/CCN2) is a matricellular protein induced by transforming growth factor (TGF)-β and intimately involved with tissue repair and overexpressed in various fibrotic conditions. We previously showed that keratinocytes in vitro downregulate TGF-β-induced expression of CTGF in fibroblasts by an interleukin (IL)-1 α-dependent mechanism. Here, we investigated further the mechanisms of this downregulation by both IL-1α and β. Human dermal fibroblasts and NIH 3T3 cells were treated with IL-1α or β in presence or absence of TGF-β1. IL-1 suppressed basal and TGF-β-induced CTGF mRNA and protein expression. IL-1α and β inhibited TGF-β-stimulated CTGF promoter activity, and the activity of a synthetic minimal promoter containing Smad 3-binding CAGA elements. Furthermore, IL-1α and β inhibited TGF-β-stimulated Smad 3 phosphorylation, possibly linked to an observed increase in Smad 7 mRNA expression. In addition, RNA interference suggested that TGF-β activated kinase1 (TAK1) is necessary for IL-1 inhibition of TGF-β-stimulated CTGF expression. These results add to the understanding of how the expression of CTGF in human dermal fibroblasts is regulated, which in turn may have implications for the pathogenesis of fibrotic conditions involving the skin. J. Cell. Biochem. 110: 1226–1233, 2010.

KEY WORDS: INTERLEUKIN-1; CONNECTIVE TISSUE GROWTH FACTOR; TRANSFORMING GROWTH FACTOR-β; SMAD 3; TAK1

The process of tissue repair with inflammation and fibrosis is a universal response to various tissue-damaging stimuli. Cutaneous wound healing concludes with the formation of scar tissue that serves to seal and contract the wound. However, fibrosis changes the properties of the connective tissue in a way that often impedes organ function. Indeed, the excessive scar formation often seen after major burns is the main cause of morbidity in these patients. Fibrosis is characterized by an increased synthesis of collagen type I and various profibrotic factors. Connective tissue growth factor (CTGF) belongs to a family of modular proteins with growth factor-like properties collectively denoted CCN (for CTGF, Cyr-61, Nov) [Brigstock, 1999]. CTGF is an immediately-early gene product of approximately 36–38 kDa originally identified by screening for platelet-derived growth factor-like molecules produced by human umbilical vein endothelial cells [Bradham et al., 1991]. Synthesis of extracellular matrix (ECM) by mesenchymal cells, as well as the proliferation and migration of fibroblasts, is promoted by CTGF [Grotendorst, 1997]. Other biologic effects attributed to CTGF are stimulation of endothelial cell proliferation, migration, and adhesion, which implies a role for CTGF as an angiogenetic factor [Babic et al., 1999]. Indeed, CTGF has been demonstrated to promote the formation of granulation tissue and to increase the rate of wound healing [Duncan et al., 1999]. Increased expression of CTGF has been seen in fibrosis of most tissues and organs, and CTGF is considered to be an important factor in the pathogenesis of many different fibrotic conditions [Leask et al., 2002]. Expression of CTGF in fibroblasts is upregulated by transforming growth factor-β (TGF-β), and it has been suggested that the profibrotic effects of TGF-β are in part mediated by CTGF [Grotendorst, 1997]. However, not all effects of CTGF are dependent on TGF-β [Dammeier et al., 1998; Chambers et al., 2000; Holmes et al., 2001] and conversely, not all of the biological effects of TGF-β involve CTGF [Grotendorst, 1997]. It is believed that CTGF is more specific than TGF-β in its profibrotic activity, which could render it a more suitable target for antifibrotic therapy. Activation of the type I TGF-β receptor kinase results in phosphorylation of Smad 3 in the

Abbreviations used: CTGF, connective tissue growth factor/CCN 2; IL-1, interleukin-1; TGF-β, transforming growth factor-β; TAK1, TGF-β activated kinase 1; siRNA, small inhibitory RNA.

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cytosol followed by complex formation with Smad 4. The Smad 3/4 complex translocates to the nucleus where it binds to a Smad-binding element in the CTGF promoter and activates CTGF gene expression [Holmes et al., 2001]. This Smad cascade is suppressed by Smad 7 that inhibits Smad 3 phosphorylation. Smad 7 itself is not phosphorylated but regulated at the level of gene expression, nuclear export and/or other mechanisms such as interaction with the Smurf proteins [Shi and Massague, 2003]. In addition to the Smad cascade activation, mitogen activated protein kinases (MAPK) act in concert with Smad signaling to mediate TGF-β-stimulated CTGF expression in some systems [Leask et al., 2003].

Wound healing with delayed reepithelialization as often seen in burn wounds frequently results in excessive scar formation. Keratinocytes have been demonstrated to downregulate the synthesis of collagen and profibrotic factors by fibroblasts [Lacroix et al., 1995; Le Poole and Boyce, 1999]. We have previously identified interleukin-1α (IL-1α) as the mediator of CTGF mRNA and protein suppression in human dermal fibroblasts by keratinocytes [Nowinski et al., 2002]. Interleukin (IL)-1α and β belong to a family of proinflammatory cytokines with broad biological activity. These interleukins are primarily recognized for their capacity to regulate innate and cognate immune responses, and are produced by a variety of cells participating in host defense against noxious agents [Dinarello, 1994b]. IL-1 has also been shown to regulate connective tissue metabolism, in particular by promoting degradation of ECM [Ito et al., 1988; Qwarnstrom et al., 1989]. Monocytes and macrophages are the principal source of IL-1 but resident cells such as keratinocytes, fibroblasts and endothelial cells can also produce IL-1 [Dinarello, 1994a]. Both IL-1α and β are synthesized as 33 kDa precursor molecules, but only IL-1α is biologically active both as precursor and as mature peptide; IL-1β must be processed by a IL-1β converting enzyme (ICE-1, Caspase-1) in order to be secreted and active [Dinarello, 1996, 1997]. IL-1 activates multiple intracellular signaling pathways [Oda and Kitano, 2006]. The activated transmembrane receptor IL-1R forms a complex with myeloid differentiation factor 88 (MyD88), and phosphorylates IL-1R-associated kinase (IRAK). IRAK dissociates and binds to tumor necrosis factor alpha receptor-associated factor 6 (TRAF6), which activates TGF-β-activated kinase 1 (TAK1/MAP3K7). TAK1 in turn activates either transcription factors NFκB or IκB. TAK1 is a mitogen activated kinase kinase kinase (MAPKKK) originally described as an intermediate in TGF-β and BMP signaling [Yamaguchi et al., 1995; Shibuya et al., 1998]. However, it has also been demonstrated that IL-1 may negatively regulate the Smad pathway through atypical phosphorylation of Smad 3 by TAK1 [Benus et al., 2005]. Hence, TAK1 is an intermediate of both TGF-β and IL-1 intracellular transduction pathways and constitutes a link between the signaling cascades of TGF-β and IL-1 [Lu et al., 2007]. The balance between IL-1 and TGF-β activity is known to be important for the regulation of tissue repair, and antagonistic regulation by IL-1 and TGF-β of target genes important for wound healing has been described [Shephard et al., 2004]. However, mechanistic data on the effects of IL-1 on TGF-β-stimulated CTGF expression is lacking. The purpose of this study was, hence, to further elucidate the intracellular mechanisms by which IL-1 regulates CTGF expression. Possible implications for processes related to tissue repair are discussed.

MATERIALS AND METHODS

REAGENTS

Polyclonal antibodies against Smad 2/3 (FL-425), β-tubulin (H-235) and CTGF (L-20) came from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal phospho-Smad 3 antibody (ab51451) came from Abcam (Cambridge, UK). Reagents used for positive controls were rat thoracic aorta myoblast whole cell lysate (A10) and mink lung epithelial cell whole cell lysate treated with TGF-β (MV1LU + TGF-β), both from Santa Cruz Biotechnology, Inc. CTGF promoter cDNA construct linked to the luciferase gene was a gift from Dr. David Abraham, University College, London. TGF-β responsive element $(CAGA)_{12}$ cDNA linked to a luciferase gene was obtained from Dr. Peter ten Dijke, Uppsala. This construct contains 12 CAGA-repeats which bind Smad proteins [Dennler et al., 1998a]. Recombinant TGF-β1 and IL-1α and β were obtained from Sigma (St. Louis, MO). TAK1 (MAP3K7) siRNA was from Qiagen AB (Solna, Sweden).

CELLS

Human dermal fibroblasts were isolated from samples of skin from young individuals undergoing plastic reconstructive surgery at our department. Approval from the local ethical committee and patient consent was obtained. Immortalized mouse fibroblasts (NIH 3T3) came from American Type Culture Collection. Early passage (3–7) were used for experiments with human dermal fibroblasts.

REAL-TIME PCR

0.3 μg of total RNA per sample was used as template for synthesis of cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A 7500 Fast Real-Time PCR System (Applied Biosystems) and fluorescent probes from the same company (TaqMan™ Gene Expression Assays) were used. Probes used were CTGF, Smad 7 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an internal control. PCR reagents were obtained from Applied Biosystems (TaqMan Gene Expression Master Mix). Crossing threshold (Ct) values were calculated by the 7500 Fast Real-Time PCR System software using the second derivative maximum method. *Calculation of relative changes in gene expression*: Three different cDNA samples for each gene to be analyzed within a run were diluted 10 times prior to amplification in the thermal cycler. Ct values from these samples were subtracted from corresponding undiluted samples (△Ct) to calculate PCR efficiency. PCR efficiency (E) was calculated by the formula $E = 10^{(1/\triangle Ct)}$. Relative level of gene expression (RL) was then calculated using the formula $RL = E^{(Ct\ ref - Ct\ test)}$ where Ct ref is the Ct value of an arbitrary reference sample from which all other samples (Ct test) are subtracted. By this approach the RL of the reference sample always becomes 1.0 and test sample values directly expressed as fold changes. The assayed gene/GAPDH ratio was calculated for each sample. Expression of GAPDH was stable throughout the experiments and negative control (water) samples consistently negative. A two-tailed Student's *t* test (paired) was used to analyze gene expression data. The data were expressed as the means of indicated replicates with values of $P < 0.05$ considered significant.

WESTERN BLOTTING

The culture medium was removed and the cells washed with phosphate-buffered saline. Cells were lysed in phosphate-buffered saline supplemented with 0.1% SDS, 1% Igepal CA-630 (Sigma), 5 mg/ml Na-deoxycholate and protease/phosphatase inhibitors (Thermo Scientific, Rockford, IL). Protein concentration was determined using the Bio Rad ^{D}C protein assay (Bio Rad, Hercules, CA). Equal amounts of proteins were boiled in SDS sample buffer and separated by SDS-PAGE under reducing conditions. Proteins were electro-transferred to Hybond-C nitrocellulose membranes (GE Health Care). Immunoblotted proteins were incubated with primary antibodies as indicated followed by incubation with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence detection (ECL, GE Health Care).

PROMOTER STUDIES

One day before transfection of CTGF promoter or TGF-β-responsive element constructs, fibroblasts were plated in DMEM with 10% FBS in a 24-well plate (5×10^4 cells/well). Transfection of cells was performed by using Lipofectamine Plus kit from Invitrogen. For each well, 0.2 μg DNA construct was transfected into the cells under serum-free conditions according to the kit instructions. After 3 h the medium was replaced with DMEM with 10% FBS. The plates were then incubated for another 24 h to establish signaling from the constructs. The medium was replaced with DMEM containing 0.5% FBS and 100 pg/ml IL-1α or β was added. 100 pM TGF-β was added after 16 h. After 6 h of incubation with TGF-β, cells were harvested and protein lysates analyzed for luciferase activity. NIH 3T3 cell were used for promoter studies as primary fibroblast did not survive transfections.

LUCIFERASE ASSAY

Analysis of luciferase activity was performed using a commercial kit (Luciferase assay kit, Sigma). The cells were washed with ice-cold PBS, cell culture lysis reagent was added and the cells were incubated for 15 min. The cells were scraped of the wells and transferred to microcentrifuge tubes. Cellular debris was separated by centrifugation at 4°C and the samples were stored at −70°C. Luciferase substrate and cell lysates were equilibrated to room temperature, mixed and generation of light was analyzed with a luminometer. Luciferase standard (Sigma) was used to determine the linear range of luminescence.

TAK1 KNOCK-DOWN

TAK1 mRNA knock-down was performed using gene-specific small inhibitory RNA (siRNA) molecules obtained from Qiagen AB. One siRNA duplex (MAP3K7_8 cat no. #S102758749) showing best gene knock-down performance selected from the FlexiTube format of four options was used for electroporation of fibroblasts. siRNA lacking sequence homology to any known mammalian gene was used as negative control (Qiagen, cat no. 1027284). Fibroblasts (100.000) were mixed with 100 pmol of TAK1 or negative control siRNA in 75 μl siPORT™ electroporation buffer (Ambion, Inc., Austin, TX). Electroporation settings were 900 V, 70 μs pulse length and two pulses performed with a 5 s pulse-interval. Transfection was performed in 1 mm cuvettes using BioRad Xcell electroporator. Cuvettes with cells were incubated for 10 min at 37°C and content of each cuvette distributed in duplicate in 24-well Nunclon™ cell culture plate (Nunc A/S, Roskilde, Denmark) containing 1 ml DMEM GlutaMax nutrient medium supplemented with 10% fetal bovine serum and gentamicin (Gibco, Paisley, UK). After 20 h medium was changed to serum-free DMEM. TGF-β was added after an additional 27 h in presence or absence of preincubated IL-1 as indicated. Cells were incubated with TGF-β for 3 h before analysis of CTGF mRNA expression. RNA was extracted from cells using RNeasy Plus Micro Kit from Qiagen. cDNA was generated using High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. TAK 1 knock-down level was 80–85% relative to negative control siRNA in the 24–48 h range post-transfection by real-time PCR using MAP3K7 TaqMan Gene Expression Assay cat no Hs00177373_m1 from Applied Biosystems.

STATISTICAL ANALYSIS

Results are presented as means ± standard deviation. Student's *t* test (paired) was used for statistical analysis. *P*-Values equal or <0.05 were considered statistically significant.

RESULTS

IL-1 INHIBITS CTGF mRNA AND PROTEIN EXPRESSION IN FIBROBLASTS

The effect of IL-1α and β on basal and TGF-β-induced CTGF mRNA in human dermal fibroblasts was measured by real-time PCR. IL-1α, IL-1β, and TGF-β were added as indicated. A suppression of basal and TGF-β-induced CTGF mRNA expression of about 30–50% was seen at IL-1 concentrations in the range of 0.01–0.2 ng/ml (Fig. 1A,B). A similar suppression was also seen in NIH 3T3 cells (not shown), which is in accordance with previous reports [Zhao et al., 2004]. Expression of the 38 kDa CTGF peptide in the cell layer of fibroblasts was measured by Western blotting. There was no detectable CTGF protein when cells were cultured without TGF-β. Incubation with TGF-β resulted in a strong induction of the CTGF protein, and IL-1 inhibited this upregulation at 0.05–0.6 pg/ml (Fig. 1C). Hence, IL-1α and β suppressed basal and TGF-β-stimulated CTGF mRNA levels. Furthermore, IL-1α reversed the induction of CTGF protein expression by TGF-β.

IL-1α AND β INHIBIT TGF-β-STIMULATED Smad 3 PHOSPHORYLATION, TGF-β-INDUCED CTGF PROMOTER ACTIVITY AND THE ACTIVITY OF A TGF-β RESPONSIVE PROMOTER CONSTRUCT

Human primary fibroblasts were incubated with IL-1α or β, TGF-β was added and the level of total and phosphorylated Smad 3 was analyzed by Western blotting. Pre-incubation with both IL-1α and β inhibited TGF-β-induced Smad 3 phosphorylation, without affecting the levels of total Smad 3 (Fig. 2).

The activity of a heterologous CTGF promoter was determined in order to analyze the effect of IL-1α and β on CTGF promoter activity. A 0.8 kb CTGF promoter construct, with the promoter linked to luciferase reporter gene, was transfected into NIH 3T3 mouse fibroblasts as described in the Materials and Methods Section. Promoter activity was analyzed in NIH 3T3 fibroblasts, because

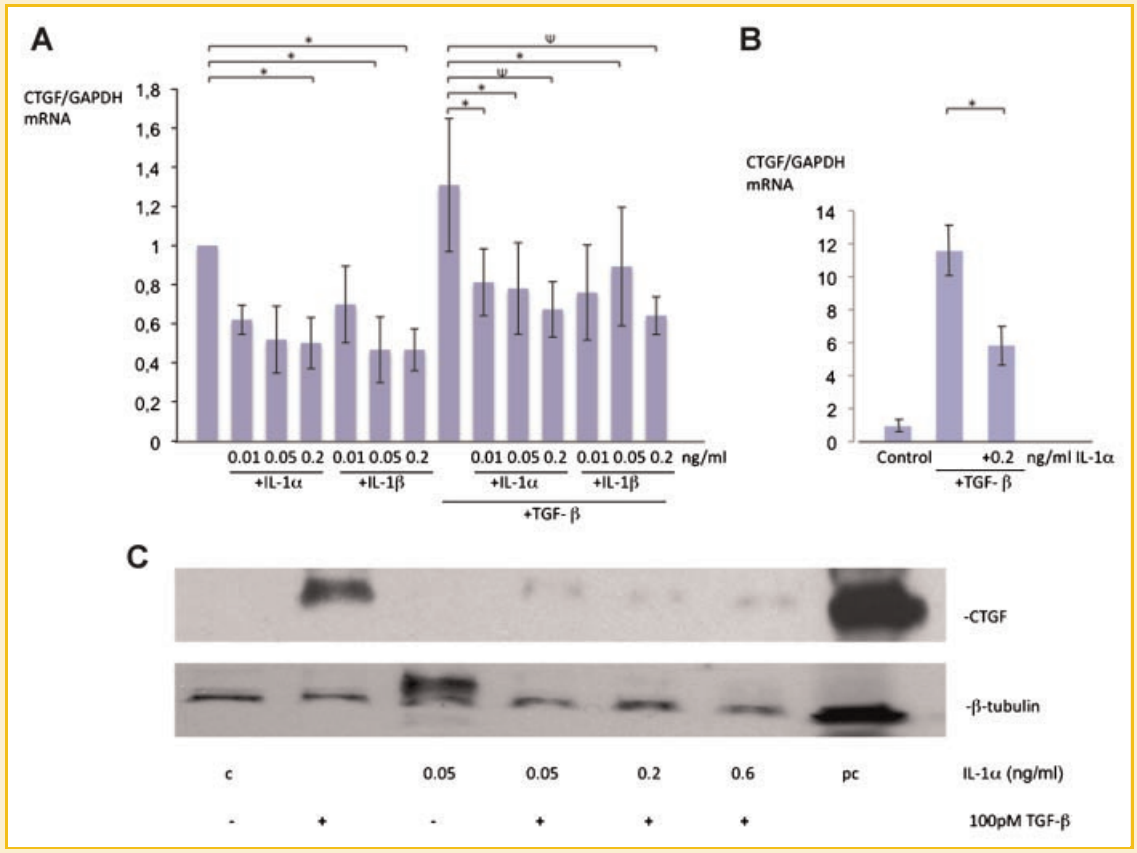

Fig. 1. IL-1 inhibits CTGF mRNA and protein expression in fibroblasts. Fibroblasts were cultured in DMEM supplemented with 0.5% FBS, in the absence or presence of 20 pM TGF-β1 (A) or 100 pM TGF-β1 (B,C). IL-1α or β were added along with TGF-β1 at the concentrations indicated. After 16 h fibroblasts were subjected to real-time PCR ($n = 3$) (A,B) or Western blotting (C) for analysis of CTGF mRNA or protein expression. Levels of GAPDH (A,B) and β-tubulin (C) were used as comparator mRNA and protein, respectively. c, control (no cytokines added); pc, positive control (A-10 whole cell lysate). Western blot was repeated with similar results. *Statistically significant downregulation ($P < 0.05$) ΨStatistically near significant ($P = 0.06$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

primary human fibroblasts survived poorly lipofectamine-based transfections. Promoter experiments performed in quadruplicates showed that IL-1α and β inhibit TGF-β-induced CTGF promoter activity; TGF-β increased CTGF promoter activity by about threefold, and this induction was reduced to near basal levels by IL-1α and β (Fig. 3A). In order to investigate the effect of IL-1 on TGF-β-induced Smad pathway activity, we used a TGF-β-inducible promoter construct composed of twelve CAGA boxes (Smad binding repeats) [Dennler et al., 1998b]. IL-1α and β inhibited significantly TGF-β-induced activity of the TGF-β responsive promoter construct (Fig. 3B). In summary, IL-1α and β inhibited TGF-β-induced Smad 3 phosphorylation, reduced the TGF-β-induced activity of the CTGF promoter as well as of a TGF-β/Smad responsive promoter construct in NIH 3T3 fibroblasts.

IL-1α AND β STIMULATE Smad 7 GENE EXPRESSION IN HUMAN DERMAL FIBROBLASTS

Smad 7 is a well-described inhibitor of Smad 3 phosphorylation, and part of a negative feedback loop activated by TGF-β [Stopa et al., 2000]. Since pre-incubation of fibroblasts with IL-1 was observed to inhibit TGF-β-mediated Smad 3 phosphorylation (Fig. 2), we investigated the expression of Smad 7 in our system. TGF-β strongly induced the expression of Smad 7 (Fig. 4). Further, Smad 7 mRNA was also upregulated by incubation with IL-1α and β, albeit to a lesser degree than TGF-β. There was no additive effect observed on the expression of Smad 7 mRNA by the addition of IL-1 and TGF-β.

INHIBITION OF TGF-β-STIMULATED CTGF mRNA BY IL-1 INVOLVES TAK1

Based on recent reports [Benus et al., 2005] we hypothesized that TAK1 may be a linkbetween IL-1 receptor activation and inhibition of TGF-β signaling in our fibroblasts. Knock-down of TAK1 mRNA in fibroblasts was performed as described in the Materials and Methods Section. Residual TAK1 mRNA was $18 \pm 4.3\%$ at the time of CTGF gene expression analysis (Fig. 5A). Transfected cells were incubated with or without TGF-β and/or pretreatment with IL-1. CTGF mRNA expression in TAK1 siRNA transfected fibroblasts responded equally well to TGF-β, indicating that TAK1 is not necessary for the effect of TGF-β on CTGF expression (Fig. 5B). Cells were incubated with IL-1α for 3 or 20 h prior to the addition of TGF-β as indicated in Figure 5B. The effect of IL-1α on CTGF expression was less pronounced after TAK1 knock-down in fibroblasts compared to fibroblasts treated with negative control siRNA (Fig. 5B). The downregulation of CTGF expression in samples

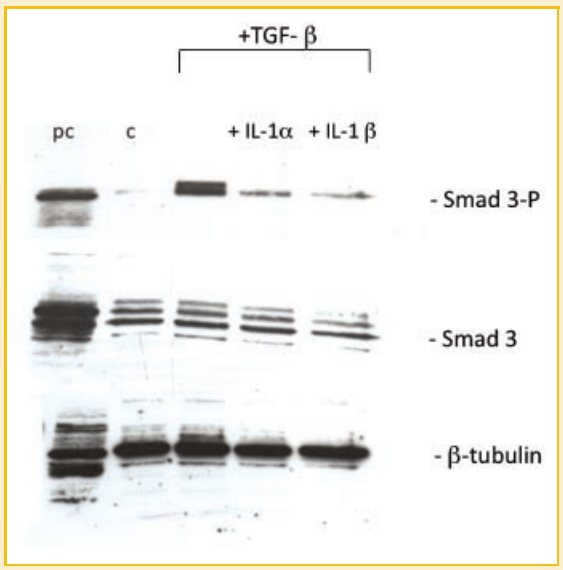

Fig. 2. IL-1α and β inhibit TGF-β-stimulated Smad 3 phosphorylation. Fibroblasts were serum-starved for 24 h. Medium was changed for fresh DMEM, 0.5 ng/ml IL-1α or β was added, and after an additional 3 h 100 pM TGF-β was added. Levels of total and phosphorylated Smad 3 were analyzed 60 min after the addition of TGF-β. Multiple bands are likely to be the result from cross-reactivity with other Smads that are more abundant than Smad 3. Results are representative of three independent experiments. pc, positive control (Mv1Lu + TGF-β whole cell lysate); c, control (untreated fibroblasts).

treated with 0.2 ng/ml IL-1α 3 h before addition of TGF-β, and with 1 ng/ml 20 h prior to TGF-β-treatment were significantly reduced ($P < 0.05$) by TAK1 knock-down. Reduced CTGF downregulation was near significant ($P = 0.07$) in cells treated with 1 ng/ml IL-1α 3 h before TGF-β. In addition, TAK1 knock-down abrogated IL-1β-inhibited CTGF expression to a similar extent as IL-1α in two independent experiments (not shown). We conclude that TAK1 is mediating a significant part of the inhibitory effect of IL-1 on CTGF expression.

DISCUSSION

We demonstrate that IL-1 is a potent inhibitor of CTGF mRNA and protein expression. This can be ascribed to inhibition at the level of the CTGF promoter. Moreover, IL-1α and β both suppress the activity of a TGF-β-inducible promoter construct containing Smad-binding elements, as well as the phosphorylation of Smad 3. Knock-down experiments demonstrate that TAK1 is mediating a significant part of IL-1 inhibition of TGF-β-induced CTGF expression.

The findings are consistent with our previous results that IL-1 is the major factor responsible for keratinocyte-mediated CTGF suppression in cocultures with fibroblasts [Nowinski et al., 2002]. Stimulation of CTGF gene expression by TGF-β involves activation of the CTGF promoter. Like several other genes targeted by TGF-β, the CTGF promoter harbors Smad-binding elements composed of multiple CAGA-boxes [Holmes et al., 2001]. In this study, both IL-1 isoforms were demonstrated to inhibit phosphorylation of Smad 3. Furthermore, IL-1 inhibited the activity of a CTGF promoter construct and of a synthetic promoter construct composed of Smad-binding elements transfected in NIH 3T3 fibroblasts. This strongly indicates that IL-1 inhibits TGF-β-induced CTGF expression through inhibition of the Smad cascade. IL-1 suppression of CTGF mRNA in absence of added TGF-β (Fig. 1 A) may also involve the Smad cascade if autocrine TGF-β signaling is operative. If not, then IL-1 is acting via undisclosed pathways outside the TGF-β context. We could not detect CTGF protein in absence of added TGF-β by Western blotting, but this is likely to be due to imitations in the detection procedure.

Antagonism between TGF-β and IL-1 is believed to be important during the process of wound healing. This balance is controlled by the availability of ligands and receptors, as well as by antagonistic

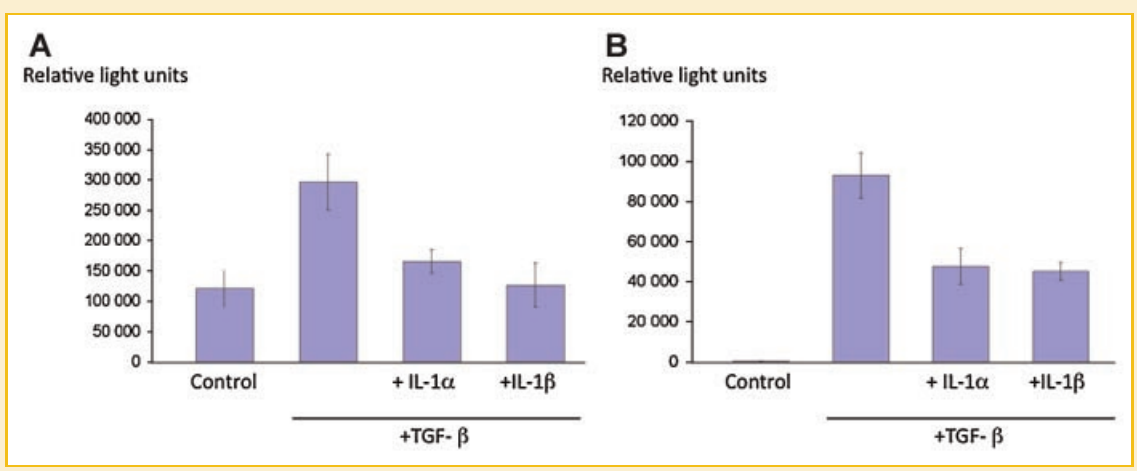

Fig. 3. IL-1α and β inhibit TGF-β-stimulated CTGF promoter activity and inhibit the activity of a TGF-β-responsive promoter construct after TGF-β1 stimulation in NIH 3T3 cells. NIH 3T3 cells were transfected with cDNA constructs as described in the Materials and Methods Section. Transfected 3T3 cells were cultured with or without 0.2 ng/ml IL-1α or β for 24 h. 100 pM TGF-β1 was added, and after an additional 20 h CTGF promoter (A) or TGF-β-responsive promoter (B) activity was analyzed. Relative light units are expressed as mean ± standard deviation of quadruplicates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

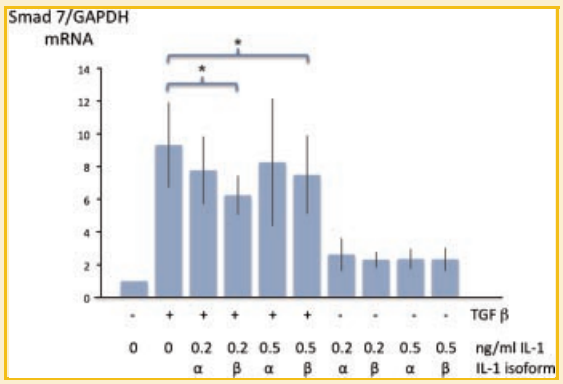

Fig. 4. TGF-β, IL-1α and β stimulate Smad 7 gene expression. Fibroblasts were serum-starved for 24 h. Medium was changed for fresh DMEM, IL-1 was added and after an additional 3 h TGF-β was added. Levels of Smad 7 were analyzed 1 h after the addition of TGF-β with real-time PCR (n = 4). The upregulation of Smad 7 in all samples compared to control was statistically significant ($P < 0.05$). *Smad7 expression was significantly lower in samples treated with TGF-β and IL-1-β compared to TGF-β only. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

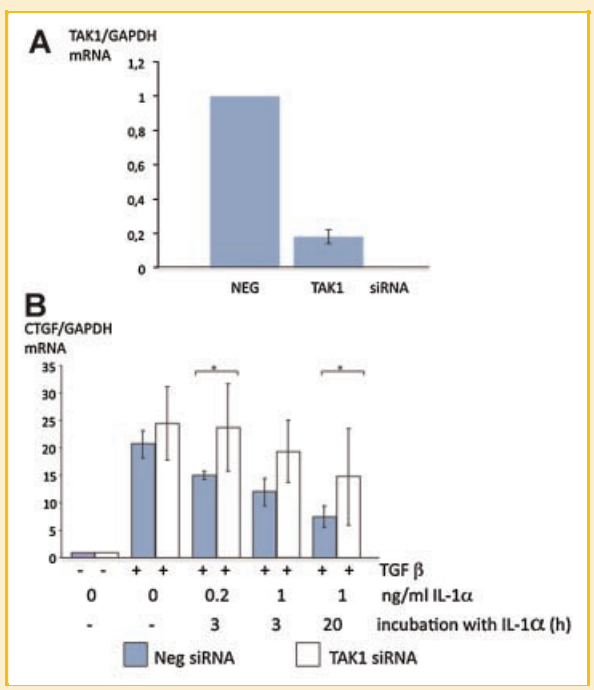

Fig. 5. Knock-down of TAK1 partially reverses IL-1-mediated suppression of TGF-β-stimulated CTGF. Fibroblasts were transfected with siRNA molecules as described in the Materials and Methods Section. Transfected cells were cultured in serum-containing medium for 20 h. Cells were subjected to serum-free medium for 24 h and TGF-β added. After an additional 3 h RNA was extracted and CTGF and TAK1 mRNA were analyzed by real-time PCR. IL-1α was added at different time-points before the addition of TGF-β as indicated in the figure. Results of 3 (0.2 ng/ml IL-1α) or 4 (1 ng/ml IL-1α) independent experiments are presented. A: TAK1 mRNA levels (n = 4). B: CTGF mRNA levels. NEG, negative control. *Statistically significant inhibited down-regulation ($P < 0.5$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cross talk between the intracellular transduction pathways activated by the two cytokines. TAK1 has been demonstrated to be an important intermediate between transduction pathways activated by TGF-β and IL-1. Notably, previous reports have demonstrated that TAK1 can mediate direct inhibitory effects of IL-1 on TGF-β-induced Smad 3 activity [Benus et al., 2005]. In accordance with this, we demonstrate in this report that TAK1 RNA interference significantly reverses the inhibitory effect of IL-1 on CTGF mRNA expression. TAK1 knock-down had no effect on basal or TGF-β-stimulated CTGF expression, which is in accordance with previous data demonstrating that TAK1 is not necessary for the activation of Smad 3 by TGF-β [Shim et al., 2005]. The fact that not all of the effect of IL-1 was blocked by TAK1 knock-down may be due to residual (15–20%) TAK1 activity in the siRNA approach.

The inhibitory Smad7 interferes with activation of the Smad cascade by TGF-β. TGF-β in turn induces Smad7 transcription through the Smad cascade, thus establishing a negative feedback loop. Interference with TGF-β signaling by Smad7 may occur at multiple levels. Smad7 inhibits phosphorylation of Smad3 and thereby complex formation with Smad4 [Hayashi et al., 1997]. In addition, Smad7 may promote degradation of the TGF-β receptor I (TGF-βRI), or mediate deactivation of the TGF-βRI by dephosphorylating enzymes [Dai and Liu, 2004; Valdimarsdottir et al., 2006]. Further, Smad7 has been shown to interfere with binding of the Smad3/4 complex to DNA [Zhang et al., 2007]. Smad7 has also been shown to be upregulated by pro-inflammatory cytokines such as IL-1 and TNF-α in some systems [Park, 2005]. Thus, Smad7 is another potential mediator of the cross-talk between TGF-β and IL-1 signaling. We found that Smad7 mRNA was increased by both isoforms of IL-1, as well as by TGF-β. Addition of both IL-1 and TGF-β was not additive, but rather an anti-synergistic effect could be detected on Smad7 mRNA, indicating self-limitation by its effect on the TGF-β signaling. Interestingly, it has been demonstrated that TGF-β may induce Smad7 transcription through a pathway involving TAK1, independent of the Smad cascade [Kim et al., 2009]. Moreover, Smad7 has been shown to interfere with complex formation between TRAF2, TAK1, and TAB2/3, implying a role as negative regulator of signaling induced by pro-inflammatory cytokines [Hong et al., 2007]. Thus, Smad7 serves as a regulator of several pathways with multiple roles in pathology and physiology in a complex and highly context-dependant manner. For these reasons, IL-1-mediated transcription of Smad7 may, therefore, constitute an important inhibitor, in addition to TAK1, of TGF-β signaling in our system. Another possibility of IL-1-mediated suppression of TGF-β signaling is via c-Jun N-terminal kinase (JNK), which have been shown to sequester Smad 3 in mouse fibroblasts [Verrecchia et al., 2003]. Pertaining to the latter, however, we could see no reversion of the IL-1 effect by adding chemical inhibitors of JNK to human dermal fibroblasts (not shown). Further studies are underway to more precisely elucidate the contribution of different intracellular pathways downstream of IL-1 in this process.

Previous studies have suggested that the constitutive over-expression of CTGF seen in fibroblasts from scleroderma tissues is independent of Smad signaling [Holmes et al., 2001]. However, other investigations support a role for Smad 3 in mediating the

upregulated expression of CTGF in fibroblasts from keloids [Khoo et al., 2006]. Interestingly, the level of IL-1 has been shown to be lower in the epidermis of keloids than in normal scars [Niessen et al., 2001]. An intriguing possibility following this is that a relative deficit in pro-inflammatory cytokines such as IL-1 could be a mechanism in the pathogenesis of hypertrophic scarring. Thus, although CTGF is implicated in most fibrosis conditions, the mechanism leading to its increase may be different depending on the cellular and tissue context. Our finding that TAK1 is involved in the effect of IL-1 on CTGF expression may have important implications for the understanding of the pathogenesis of fibrosis. A better knowledge of how CTGF and other profibrotic factors are regulated may become important for the development of new approaches on how to limit disfiguring and disabling scarring.

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