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Distinct Involvement of the Jun-N-Terminal Kinase and NF-κB Pathways in the Repression of the Human *COL1A2* Gene by TNF-α

Franck Verrecchia[1], Erwin F. Wagner[2], and Alain Mauviel[1]*

[1]INSERM U532, Institut de Recherche sur la Peau, Hôpital Saint-Louis, Pavillon Bazin, 75010 Paris, France, and [2]Research Institute for Molecular Pathology, University of Vienna, Vienna, Austria

*Corresponding author:
Tel: 33+ 1 53 72 20 69
Fax: 33+ 1 53 72 20 51
mauviel@chu-stlouis.fr

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ABSTRACT

We used a gene knockout approach to elucidate the specific roles played by the Jun-N-terminal kinase (JNK) and NF-κB pathways downstream of TNF-α in the context of α(2) type I collagen gene (*COL1A2*) expression. In $JNK_1^{-/-}$-$JNK_2^{-/-}$ ($JNK^{-/-}$) fibroblasts, TNF-α inhibited basal *COL1A2* expression but had no effect on TGF-β-driven gene transactivation unless *jnk1* was introduced ectopically. Inversely, in NF-κB Essential Modulator$^{-/-}$ ($NEMO^{-/-}$) fibroblasts, the lack of NF-κB activation did not influence the antagonism exerted by TNF-α against TGF-β, but prevented repression of basal *COL1A2* gene expression. Similar signaling mechanisms are utilized by TNF-α to regulate type I collagen gene expression in human dermal fibroblasts, as evidenced using transfected dominant-negative forms of MKK4 and IKK-α, critical kinases upstream of the JNK and NF-κB pathways, respectively. These results represent the first demonstration for alternate usage of distinct signaling pathways by TNF-α to inhibit the expression of a given gene, *COL1A2*, depending on its activation state.

KEYWORDS: TNF-α, Signal transduction, NF-κB, Jun-N-terminal kinase, Gene expression, Collagen

INTRODUCTION

Through their ability to modulate the expression of extracellular matrix (ECM) components and ECM-degrading enzymes, cytokines and growth factors orchestrate the balance between ECM destruction and neosynthesis, and therefore play an important role in the control of tissue homeostasis and repair (Mutsaers et al., 1997; Uitto and Kouba, 2000). Disruption of the fragile equilibrium between anabolic and catabolic cytokines in favor of TGF-β may lead to excessive collagen deposition, the hallmark of fibrotic conditions.

TGF-β signals via serine/threonine kinase transmembrane receptors that phosphorylate cytoplasmic mediators of the Smad family. Phospho-Smads are translocated into the nucleus where they function as transcription factors, binding DNA either directly or in association with other proteins (Attisano and Wrana, 2000). Smad signaling may be blocked by TNF-α, via mechanisms that implicate either c-Jun (Verrecchia et al., 2000) or NF-κB (Bitzer et al., 2000).

Jun-N-terminal kinases (JNKs), members of the family of Mitogen Activated Protein Kinases (MAPKs), are activated upon exposure of cells to cytokines, growth factors, and environmental stresses (Davis, 2000). Three distinct genes encode JNKs: *jnk1*, *jnk2* and *jnk3*, the former two being ubiquitously expressed. Dual Thr and Tyr phosphorylation of JNK by the MAPK kinases MKK4 and MKK7, results in its activation and nuclear translocation, and subsequent phosphorylation of transcription factors, such as c-Jun or ATF2 (Davis, 2000).

In the human *COL1A2* promoter, a Smad3/4 gene target downstream of TGF-β (Chen et al., 1999; Verrecchia et al., 2001a), a region between nucleotides –313 and 235 and containing AP-1, NF-κB, Sp1, Smad3/4, and C/EBP binding sites, has been shown to participate in the regulatory programs activated by TGF-β and TNF-α to control type I collagen expression (Ghosh, 2002).

In this study, we demonstrate the critical role of JNK activation for TNF-α in antagonizing TGF-β-induced *COL1A2* transactivation, while NF-κB activation is essential for inhibition of basal *COL1A2* expression. These results provide the first demonstration of an alternate usage of distinct signaling mechanisms by TNF-α to repress the *COL1A2* gene depending on its activation state.

RESULTS AND DISCUSSION

Modulation of JNK and NF-κB activities by TNF-α in JNK$^{-/-}$ and NEMO$^{-/-}$ fibroblasts. We examined the endogenous modulation of the *COL1A2* gene in immortalized fibroblast lines derived from *JNK*$^{-/-}$ and *NEMO*$^{-/-}$ mouse embryos (Sabapathy et al., 1999; Schmidt-Supprian et al., 2000). First, to validate our experimental system, we examined the modulation of the JNK and NF-κB pathways by TNF-α in these cell lines. Using NF-κB- and AP-1- pathway-specific reporter constructs in transient cell transfection experiments, together with Western analysis of endogenous phospho-c-Jun content, we determined that *JNK*$^{-/-}$ fibroblasts are devoid of JNK activity but exhibit "normal" NF-κB response, whereas *NEMO*$^{-/-}$ fibroblasts, lack NF-κB activity, and exhibit a functional JNK pathway, allowing efficient c-Jun phosphorylation and AP-1-dependent transactivation in response to TNF-α (not shown). Of note, TGF-β neither activated the JNK nor the NF-κB pathways in any fibroblast line tested (not shown).

Respective roles of JNK and NF-κB in the regulation of COL1A2 gene expression by TNF-α in mouse embryo fibroblasts. The effect of TNF-α on TGF-β-induced *COL1A2* expression was examined by measuring the modulation of *COL1A2* steady-state mRNA levels by TGF-β and TNF-α. Experiments were carried out in medium supplemented with 1% fetal calf serum, a condition that allows potent *COL1A2* upregulation by TGF-β (Chung et al., 1996), whereas higher serum concentrations may interfere with TGF-β, due to the ability of α2-macroglobulin to complex and inhibit growth factors (O'Connor-McCourt and Wakefield, 1987). Strong enhancement of *COL1A2* mRNA steady-state levels (3.5-fold) was observed in response to TGF-β in the three cell types (Fig. 1A, lanes 2, 6 and 10). TNF-α antagonized TGF-β in both wt and *NEMO*$^{-/-}$ fibroblasts (lanes 4 vs. 2 and 12 vs. 10, respectively), but not

in *JNK*$^{-/-}$ fibroblasts (lanes 8 vs. 6). Type I collagen production paralleled the modulation of *COL1A2* mRNA steady-state levels (Fig. 1B, upper panel). Specifically, TGF-β treatment resulted in increased type I collagen production by wt, *JNK*$^{-/-}$ and *NEMO*$^{-/-}$ fibroblasts (lanes 2, 6 and 10). TNF-α efficiently blocked TGF-β-induced accumulation of immunoreactive type I collagen in wt and *NEMO*$^{-/-}$ fibroblasts (lanes 4 and 12, respectively), but not in *JNK*$^{-/-}$ fibroblasts (lane 8). Actin levels showed no modulation by cytokines in either cell type (lower panel). These data indicate a critical role of JNK, but not NF-κB, in mediating the antagonistic effect of TNF-α against TGF-β-induced type I collagen gene expression. Noteworthy, despite the lack of NF-κB activation, no apoptosis was detected in TNF-α-treated (0, 1, 10 and 100 ng/ml up to 24h) immortalized *NEMO*$^{-/-}$ fibroblasts (not shown).

Under experimental conditions most favorable for high basal *COL1A2* expression, e.g. medium containing 10% serum (Chung et al., 1996,) adequate for studying inhibitory mechanisms by cytokines (Czuwara-Ladykowska et al., 2001; Higashi et al., 1998; Kouba et al., 1999), TNF-α inhibited *COL1A2* mRNA levels (Fig. 2A) and type I collagen production (Fig. 2B) in both wt and *JNK*$^{-/-}$ fibroblasts, but had no effect in *NEMO*$^{-/-}$ fibroblasts.

Next, we performed transient transfection experiments with pMS3.5CAT, a plasmid that contains 3.5 kb of 5' regulatory sequences of the *COL1A2* gene, driving the expression of the CAT gene. Under low serum conditions, TNF-α induced (a), a 30% reduction in *COL1A2* promoter activity in wt and *JNK*$^{-/-}$ fibroblasts exclusively, and (b), a marked inhibitory activity against TGF-β in wt and *NEMO*$^{-/-}$ fibroblasts, but not in the *JNK*$^{-/-}$ fibroblasts (Fig. 3A). In contrast, in medium containing 10% serum, a 70% repressory effect of TNF-α on basal *COL1A2* promoter activity was observed in wt and *JNK*$^{-/-}$ fibroblasts, but not in *NEMO*$^{-/-}$ fibroblasts (Fig. 3B). These results, consistent with the data presented in Fig. 2 for endogenous collagen type I expression, indicate that JNK and NF-κB exert distinct effects on *COL1A2* expression, at the transcriptional level.

c-Jun requires functional JNK to antagonize TGF-β-induced COL1A2 promoter transactivation. c-Jun, the prototypic JNK substrate, is a key effector of TNF-α inhibitory activity on Smad signaling (Verrecchia et al., 2000), and blocks TGF-β-induced *COL1A2* promoter transactivation (Chung et al., 1996). In addition, an antisense *c-jun* expression vector completely prevents TNF-α antagonism against TGF-β-induced *COL1A2* promoter transactivation (not shown). To determine whether c-Jun inhibitory potential against TGF-β required functional JNK, c-Jun and pMS3.5CAT were co-transfected into wt, *JNK*$^{-/-}$ and *NEMO*$^{-/-}$ fibroblasts subsequently treated with TGF-β. c-Jun expression abolished TGF-β effect in wt and *NEMO*$^{-/-}$ fibroblasts but had no effect in *JNK*$^{-/-}$ fibroblasts (Fig. 4A). The lack of c-Jun effect in *JNK*$^{-/-}$ fibroblasts was actually due to the *jnk* gene knockout and not from non-specific altered cytokine responsiveness in this cell type, as ectopic expression of *jnk1*, which allowed for c-jun phosphorylation in response to TNF-α (Supplementary figure 1A,B), entirely rescued the antagonistic effect of c-Jun against TGF-β□ □ □ □ □ □4□ □. Similarly, *jnk1* expression restored the ability of TNF-α to interfere with TGF-β induced collagen gene expression in *JNK*$^{-/-}$ fibroblasts (Supplementary figure 1C).

Respective roles of JNK and NF-κB in the regulation of COL1A2 gene expression by TNF-α in human dermal fibroblasts. The JNK and NF-κB cascades were targeted in human dermal fibroblasts by means of transfected dominant-negative mutant forms of the upstream kinases MKK4 and IKK-α, respectively (Supplementary figure 2). As shown in Fig. 5A, and as expected from our previous work using this cell type (Chung et al., 1996), TNF-α prevented 90% of the TGF-β response. D/N-MKK4 blocked the inhibitory effect of TNF-α against TGF-β, whereas D/N IKK-α had no effect, indicating that the MKK4/JNK cascade is critical for TNF-α to interfere with TGF-β□ □ □ □ □ □ □ □*COL1A2* promoter activation, whereas the NF-κB cascade is not. Indeed, in *NEMO*$^{-/-}$ fibroblasts, transfection of D/N MKK4, and

that of an antisense *c-jun* vector, prevented TNF-α antagonism against TGF-β-induced *COL1A2* promoter transactivation (not shown).

Under high serum conditions, and consistent with the effects observed with the knockout fibroblasts, inhibition of *COL1A2* promoter activity by TNF-α was abolished by D/N IKK-α, not by D/N MKK4 (Fig. 5B). Of note, the slight inhibitory effect exerted by TNF-α on basal promoter activity under low serum conditions (Fig. 5A) was abolished by D/N IKK-α but not by D/N MKK4.

SPECULATION

Using independent approaches to interfere with the JNK and NF-κB pathways, we have provided compelling evidence for a central role of JNK in allowing the antagonistic activity of TNF-α and c-Jun against TGF-β-induced type I collagen gene expression, whereas NF-κB activity, although critical for the inhibitory effect of TNF-α on basal *COL1A2* expression, plays no role in mediating the former phenomenon. A schematic representation summarizing our findings is provided in Fig. 6: TNF-α activates distinct signaling pathways that regulate a given target gene independently, according to its activation state. Direct NF-κB-driven repression occurs via a proximal NF-κB binding site within the *COL1A2* promoter (Kouba et al., 1999). *COL1A2* is a direct Smad3/4 gene target (Chen et al., 1999; Verrecchia et al., 2001a). Our data indicate that JNK blocks TGF-β-induced type I collagen gene expression via c-Jun phosphorylation, certainly by preventing Smad signaling. Mechanisms underlying c-Jun inhibitory activity include (a) physical association of Jun and Smad3, not compatible with Smad/DNA complex formation (Shi et al., 1998; Verrecchia et al., 2001b), and (b), sequestration of the shared transcriptional co-activator p300 (Verrecchia et al., 2000). In accordance with our findings, overexpression of constitutively active forms of either MEKK1

or MKK4, kinases involved in JNK activation, enhance Smad-Jun associations and repress Smad-dependent transcription (Dennler et al., 2000).

With regard to JNK function in the context of ECM turnover, our findings are complementary to a recent study indicating that a synthetic inhibitor of JNK, SP600125, suppresses interleukin-1-induced phospho-Jun accumulation, Jun/DNA interactions, and interstitial collagenase (MMP-1) gene expression in synovial fibroblasts (Han et al., 2001). It appears therefore that the benefit of JNK targeting in degenerative inflammatory diseases such as rheumatoid arthritis results not only from blocking degradative events induced by interleukin-1 or TNF-α, but also by maintaining the anabolic functions of TGF-β on ECM deposition, otherwise inhibited by these cytokines. Conversely, means to activate the JNK pathway may be of interest in pathological situations where interfering with TGF-β signaling is critical, such as in fibrosis.

MATERIALS AND METHODS

Cell Cultures. Immortalized fibroblast cell lines were derived from wild-type (wt), $JNK_1^{-/-}$-$JNK_2^{-/-}$ (Sabapathy et al., 1999) and $NEMO^{-/-}$ (Schmidt-Supprian et al., 2000) mouse embryos, in which targeted disruption of the *jnk1* and *jnk2* or the *nemo* genes has been performed, respectively. Human dermal fibroblasts were established by explanting neonatal foreskins. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 μg/ml streptomycin-G and 0.25 μg/ml Fungizone™). Human recombinant TGF-β1 (R&D Systems Inc., Minneapolis, MN), referred to as TGF-β throughout the text, and human recombinant TNF-α (Roche Diagnostic, Indianapolis, IN) were both used at a concentration of 10 ng/ml.

Northern Blotting. Total RNA was obtained using an RNeasy kit (Qiagen GmbH, Hilden Germany) and analyzed by Northern hybridization (20 μg/lane) with ^{32}P-labeled cDNA probes for *COL1A2* and *GAPDH*, as described previously (Mauviel et al., 1991). Hybridization signal was quantified with a phosphorimager (Storm 840, Amersham-Pharmacia).

Western Blotting. Whole cell lysates from fibroblasts were prepared by washing cells twice in 1x PBS, followed by scraping into Laemmli buffer (62.5 mM TRIS-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.5 mM PMSF). 25-100 μg of protein were denatured by heating at 95˚C for 3 min prior to resolution by SDS-PAGE. After electrophoresis, proteins were transferred to Hybond ECL nitrocellulose filters (Amersham-Pharmacia Biotech, Uppsala, Sweden), immunoblotted with either anti-type I collagen (Southern Biotech, Birmingham, AL), or anti-

actin (Sigma Chemical Co., St-Louis, MO) antibodies, incubated with a horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), developed according to ECL protocols (Amersham-Pharmacia), and revealed with a phosphorimager (Storm 840, Amersham-Pharmacia).

Plasmid Constructs. pMS3.5/CAT, a plasmid containing approximately 3.5 kb of human *COL1A2* promoter linked to the CAT reporter gene has been described previously (Boast et al., 1990). pRSV-β-galactosidase (Promega Corp., Madison, WI) was used to control transfection efficiency. pNF-κB-lux and pAP1-TA-lux (Clontech, Palo Alto, CA) were used to determine NF-κB-, and AP-1-driven transcription, respectively. For c-Jun expression, we used a full-length human cDNA cloned into pRSV (Chiu et al., 1989). Dominant-negative MKK4 (D/N MKK4), D/N-IKK-α and *jnk1* expression vectors have been described previously (Atfi et al., 1997; Sabapathy et al., 2001).

Transient Cell Transfections and Reporter Assays. Transient cell transfections were performed with the calcium phosphate/DNA co-precipitation procedure using a commercial assay kit (Promega, Madison, WI). Reporter activities were determined as described previously (Verrecchia et al., 2000).

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FIGURE LEGENDS

Figure 1: *Role of JNK in the downregulation of TGF-β-induced type I collagen gene expression by TNF-α.* Sub-confluent wt, *JNK*$^{-/-}$, and *NEMO*$^{-/-}$ fibroblast cultures were treated with TGF-β and TNF-α for 24 h in medium containing 1% serum. *A*, Northern hybridizations of total RNA with *COL1A2* and *GAPDH* probes. A representative autoradiogram is shown. *B*, Western analysis of whole cell lysates for type I collagen and actin levels.

Figure 2: *NF-κB is required for inhibition of basal collagen production by TNF-α.* Sub-confluent wt, *JNK*$^{-/-}$, and *NEMO*$^{-/-}$ fibroblast cultures were treated with TNF-α for 24 h in medium containing 10% serum. *A*, Total RNA was analyzed by Northern hybridizations with *COL1A2* and *GAPDH* probes. A representative autoradiogram is shown. *B*, after incubations, levels of type I collagen and actin production were determined by Western analysis of whole cell lysates with specific antibodies.

Figure 3: *Regulation of COL1A2 promoter activity in JNK*$^{-/-}$ *and NEMO*$^{-/-}$ *mouse immortalized fibroblasts. A*, sub-confluent immortalized wt, *JNK*$^{-/-}$, and *NEMO*$^{-/-}$ fibroblast cultures were transfected with pMS3.5CAT, consisting of 3.5 kb of the human *COL1A2* promoter linked to the CAT gene, and treated with TGF-β and TNF-α in medium containing 1% serum. Promoter activity was determined 24 h later. *B*, wt, *JNK*$^{-/-}$, and *NEMO*$^{-/-}$ fibroblast cultures were transfected with pMS3.5CAT and treated with TNF-α for 24 h in the presence of 10% serum before promoter activity was determined.

Figure 4: *JNK is required for c-Jun to antagonize TGF-β-induced COL1A2 promoter transactivation. A*, wt, *JNK*$^{-/-}$, and *NEMO*$^{-/-}$ fibroblast cultures were transfected with

pMS3.5CAT in the presence of either empty pRSV or pRSV-c-Jun expression vectors. Eighteen hours later, TGF-β was added and incubations continued for 24 h in medium containing 1% serum before promoter activity was determined. *B*, Sub-confluent wt and *JNK*$^{-/-}$ fibroblast cultures were transfected with pMS3.5CAT, in the absence or presence of *jnk1* expression vector and placed in medium supplemented with 1% serum. Six hours later, TGF-β and TNF-α were added, and incubations continued for 24 h before CAT assays. Bars indicate mean ± S.D. of 3 independent experiments.

Figure 5: *Respective roles of the JNK and NF-κB pathways downstream of TNF-α-driven regulation of COL1A2 promoter activity in human dermal fibroblasts*. Sub-confluent human dermal fibroblasts were transfected with pMS3.5CAT, together with either D/N-IKK-α or D/N-MKK4 expression vectors. Empty pCMV was used to maintain equivalent amounts of transfected DNA in each plate. *A*, after glycerol shock, the cells were placed in medium supplemented with 1% serum. Six hours later, TGF-β and TNF-α were added and incubations were continued for 24 h before promoter activity was determined. *B*, after glycerol shock, cells were placed in medium supplemented with 10% serum. TNF-α was added 6 hours later, and incubations continued for 24 h before promoter activity was determined. Results are mean ± S.D. of at least 3 independent experiments performed with duplicate samples.

Figure 6: *Schematic diagram depicting the mechanisms underlying COL1A2 gene modulation by TNF-α.* TNF-α activates both the NF-κB and JNK pathways. The former exerts a direct repression on basal *COL1A2* transcription. On the other hand, activated JNK phosphorylates c-Jun, allowing the latter to block TGF-β-induced *COL1A2* gene expression by interfering with the Smad pathway.