A Central Role for the JNK Pathway in Mediating the Antagonistic Activity of Pro-inflammatory Cytokines against Transforming Growth Factor-β-driven SMAD3/4-specific Gene Expression*

We have focused our attention on the molecular events underlying the antagonistic activities of pro-inflammatory cytokines against transforming growth factor-β (TGF-β)/SMAD signaling. Using jnk1/2-knockout (jnk−/−) and 1xκ kinase-γ/nemo−/− fibroblasts, we have determined the specific roles played by the JNK/AP-1 and NF-κB/Rel pathways in this phenomenon. We demonstrate that, in a cellular context devoid of JNK activity (i.e. jnk−/− fibroblasts), interleukin-1 and tumor necrosis factor-α (TNF-α) did not inhibit the formation of SMAD-DNA complexes and the resulting SMAD-driven transcription in response to TGF-β. On the other hand, lack of NF-κB activity in nemo−/− fibroblasts did not affect the antagonistic effect of pro-inflammatory cytokines against TGF-β. In the latter cell type, overexpression of antisense c-jun mRNA or of a dominant-negative form of MKK4 blocked the inhibitory activity of TNF-α, similar to what was observed in normal human dermal fibroblasts. Among JNK substrates, c-Jun and JunB (but not activating transcription factor-2) antagonized TGF-β/SMAD signaling in a JNK-dependent manner. Overexpression of JNK1 in jnk−/− fibroblasts restored the ability of cytokines and Jun proteins to interfere with SMAD signaling. In junaA mouse embryonic fibroblasts, in which c-Jun can no longer be phosphorylated by JNK, JunB substituted for c-Jun in mediating the cytokine effect against SMAD-driven transcription in a JNK-dependent manner. These results suggest a critical role for JNK-mediated c-Jun and JunB phosphorylation in transmitting the inhibitory effect of pro-inflammatory cytokines against TGF-β-induced SMAD signaling. In addition, we demonstrate that such a JNK-dependent regulatory mechanism underlies the antagonistic activity of TNF-α against TGF-β-induced up-regulation of type I and III collagens in fibroblasts.

Transforming growth factor-β (TGF-β)1 is a potent anabolic factor for fibroblasts, stimulating extracellular matrix compo-

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The abbreviations used are: TGF-β, transforming growth factor-β; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; M KK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; ATF2, activating transcription factor-2; WT, wild-type; FCS, fetal calf serum; Lux, luciferase; VP16AD, VP16 activation domain; Gal4BD, Gal4 DNA-binding domain; EMSA, electrophoretic mobility shift assay.
JNK Interference with TGF-β Signaling

and MKK7 results in its activation and nuclear translocation, after which it phosphorylates several transcription factors such as c-Jun and ATF2 (16). Phosphorylation of c-Jun by JNK is thought to be critical for its maximal transcriptional activity (17). On the other hand, overexpression of constitutively active MEKK1 (MAP3K8/RK kinase kinase-1), which, in turn, activates several MAPKs including JNK, has been shown to inhibit TGFinductive SMAD signaling through stabilization of SMAD-Jun interactions (12).

In this study, using genetically modified jnk1−/−, nemo−/−, and junAA mouse embryo fibroblasts, we have further refined the understanding of the molecular mechanisms underlying the antagonistic activities of pro-inflammatory cytokines against TGF-β. Our study establishes a key role for JNK activation by TNF-α in blocking TGF-β-induced SMAD signaling and SMAD-dependent transactivation of fibrillar collagen genes, whereas the NF-κB pathway, also activated by these cytokines, plays little role (if any) in this regulation. In addition, we provide novel evidence that JunB is a substrate for JNK that is able to substitute for c-Jun in mediating the JNK-dependent inhibitory effect of pro-inflammatory cytokines against TGF-β/SMAD signaling.

MATERIALS AND METHODS

Cell Cultures—Immortalized fibroblast cell lines were derived from wild-type (WT), jnk1−/− (referred to as jnk−/−; targeted disruption of the jnk1 and jnk2 genes) (18), and nemo gene (19) mouse embryos. junAA immortalized fibroblasts were derived from mouse embryos carrying a mutant c-jun allele in which the JNK phosphoacceptor Ser63 and Ser73 residues are mutated to alanines (20). Human dermal fibroblasts were derived by explanting neonatal foreskins. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 μg/ml penicillin, 50 μg/ml streptomycin, 6.8 μg/ml funginomycin and subsequent nuclear translocation, was without effect on the inhibitory activity of TNF-α against TGF-β/SMAD signaling (6). Conversely, c-Jun overexpression was shown to block TGF-β/SMAD signaling. Possible mechanisms underlying c-Jun inhibitory effects were identified: (α) interference with SMAD-DNA complex formation and (b) sequestration of the shared transcriptional coactivator p300. Also, it was previously reported that inhibition or activation of JNK activity in HepG2 cells could activate or inhibit TGF-β/SMAD signaling, respectively (12).

We first wanted to determine whether JNK activity is required for pro-inflammatory cytokines to antagonize TGF-β/SMAD signaling. As shown in Fig. 1A, expression of both an antisense vector against c-Jun and a dominant-negative MKK4 vector prevented the inhibitory effect of TNF-α on TGF-β-driven (CAGA)_3-Lux transactivation in human dermal fibroblasts. On the other hand, overexpression of a dominant-negative form of IκB kinase-α, known to block NF-κB activation and subsequent nuclear translocation, was without effect on the inhibitory effect exerted by TNF-α. Similar results were obtained when IL-1β (10 units/ml) was used instead of TNF-α (data not shown).

To further understand the mechanisms by which pro-inflammatory cytokines (known inducers of JNK activity) are capable of interfering with the SMAD pathway, we next investigated the potential implication of another MAPK kinase, MKK7, in mediating TNF-α inhibition of SMAD signaling. As shown in Fig. 1B, when MKK4 activity was blocked by expression of its dominant-negative mutant form, MKK7 overexpression was able to significantly rescue the inhibitory activity of TNF-α. A similar rescue mechanism was observed when IL-1β was used instead of TNF-α (data not shown), indicating that both MKK4 and MKK7 may contribute to the signaling cascade activated by pro-inflammatory cytokines to counteract TGF-β/SMAD signaling.

JNK Activity Is Required for TNF-α to Antagonize TGF-β/SMAD Signaling—To ascertain the role played by the JNK pathway and to definitely rule out the possible implication of NF-κB, the antagonistic activity of TNF-α against TGF-β/SMAD signaling was blocked by expression of the dominant-negative mutant form, MKK7. In addition, overexpression of a dominant-negative MKK4 vector prevented the inhibitory effect of TNF-α on TGF-β-driven (CAGA)_3-Lux transactivation in human dermal fibroblasts. On the other hand, overexpression of a dominant-negative form of IκB kinase-α, known to block NF-κB activation and subsequent nuclear translocation, was without effect on the inhibitory effect exerted by TNF-α. Similar results were obtained when IL-1β (10 units/ml) was used instead of TNF-α (data not shown).

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Western blot analysis of phospho-c-Jun content in response to JNK activity in the various cell types was further confirmed by expression in these cells, which would be consistent with its activity. This is likely representative of an absence of JNK3 inhibition of jnk1 not in nemo presence of Gal4BD-c-Jun in WT and supplemented with 1% FCS. Six hours later, TGF-α was added and incubations were continued for 24 h before luciferase activity was determined. B, subconfluent human dermal fibroblasts were cotransfected with 2 μg of (CAGA)9-Lux together with 4 μg of dominant-negative MKK4 and/or MKK7 expression vector. Empty pCMV was used to maintain equivalent amounts of transfected DNA in each plate. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Six hours later, TNF-α and NF-κB-dependent gene transactivation downstream of TNF-α was determined. Bars indicate mean ± S.D. of three independent experiments, each performed with duplicate samples.

SMAD signaling was examined in immortalized fibroblast lines derived from jnk<sup>−/−</sup> and nemo<sup>−/−</sup> mouse embryos. To fully exploit the analytical power of such a cellular system, it was important to determine how the various signaling pathways to be investigated were regulated by cytokines in the different cell lines. First, as shown in Fig. 2A and consistent with our expectations from the literature, NF-κB-dependent gene transactivation in response to TNF-α was absent in nemo<sup>−/−</sup> fibroblasts, but normal in jnk<sup>−/−</sup> fibroblasts. Inversely, AP-1-dependent gene expression downstream of TNF-α was normal in nemo<sup>−/−</sup> fibroblasts, but absent in jnk<sup>−/−</sup> fibroblasts (Fig. 2B). Second, we examined the capacity of pro-inflammatory cytokines to activate JNK in the various cell lines. As shown in Fig. 2C, TNF-α efficiently transactivated the Gal4-Lux construct in the presence of Gal4BD-c-Jun in WT and nemo<sup>−/−</sup> fibroblasts, but not in jnk<sup>−/−</sup> fibroblasts, suggesting that simultaneous targeting of jnk1 and jnk2 is sufficient to completely eliminate JNK activity. This is likely representative of an absence of JNK3 expression in these cells, which would be consistent with its known strict tissue-specific expression (15). The specificity of JNK activity in the various cell types was further confirmed by Western blot analysis of phospho-c-Jun content in response to TNF-α. As shown in Fig. 2D, TNF-α stimulation of both WT and nemo<sup>−/−</sup> fibroblasts resulted in a dramatic induction of c-Jun phosphorylation (lanes 2 and 6 versus lanes 1 and 5), the latter not being detected in the jnk<sup>−/−</sup> fibroblasts (lane 4 versus lane 3).

To complete our characterization of these cell lines in terms of growth factor response, we next examined the transactivation of (CAGA)<sub>9</sub>-Lux by TGF-β in jnk<sup>−/−</sup>, nemo<sup>−/−</sup>, and junAA immortalized fibroblasts. As shown in Fig. 3A, full SMAD3/4-dependent responsiveness downstream of TGF-β was observed in all cell lines, indicating that neither JNK nor NF-κB activity nor the c-Jun phosphorylation state plays a role in TGF-β-driven SMAD3/4 responses. Of note, using the Gal4-based transactivation assay system, we determined that TGF-β did...
protein-DNA complex was equally induced by TGF-β over the (CAGA)₉ oligonucleotide (21) as a probe. EMSAs were performed using the SMAD3/4-specific jnk antibody together with nuclear extracts from WT, jnk⁻/⁻, and nemo⁻/⁻ fibroblast cultures treated for 30 min with TGF-β and/or TNF-α. The SMAD content of the TGF-β-induced complex (arrow) was verified by supershift with an anti-SMAD3 antibody (right panel). Note that the TGF-β-induced SMAD-DNA complex was not reduced by TNF-α in jnk⁻/⁻ fibroblasts. C, EMSAs were performed using a consensus NF-κB oligonucleotide as a probe together with nuclear extracts from WT, jnk⁻/⁻, and nemo⁻/⁻ fibroblast cultures as described for B. Note the complete dissociation of SMAD and NF-κB binding from their respective DNA recognition sites.

not activate JNK in any cell type (data not shown). Together, these results validate our model system for further investigation of the functional aspects of cytokine/TGF-β transcrip- tional antagonism in a cellular context exhibiting normal TGF-β SMAD responsiveness, but devoid of either JNK or NF-κB activity or in which c-Jun phosphorylation by JNK is impossible. As shown in Fig. 3A, TNF-α-mediated inhibition of SMAD signaling, as measured using the (CAGA)₉-Lux vector as a reporter system, was consistently observed in immortalized WT fibroblasts, as well as in nemo⁻/⁻ and junAA fibroblasts, but not in jnk⁻/⁻ fibroblasts, suggesting (a) a critical role for JNK and (b) the existence of alternative mechanisms not requiring c-Jun phosphorylation to allow the inhibitory activity of pro-inflammatory cytokines against TGF-β.

To determine whether this cellular context-specific inhibi- tory activity of TNF-α correlates with the known ability of TNF-α to interfere with SMAD-DNA complex formation in normal human dermal fibroblasts (6), EMSA experiments were carried out using nuclear extracts from WT, jnk⁻/⁻, and nemo⁻/⁻ fibroblasts treated with TGF-β and/or TNF-α for 30 min, a time point previously shown to be ideal for the detection of SMAD-DNA complexes after TGF-β stimulation (28, 29). As shown in Fig. 3B (left panel), in the three cell types, a unique protein-DNA complex was equally induced by TGF-β (second, sixth, and tenth bars). Supershift experiments with an anti-SMAD3 antibody confirmed that this complex is indeed a SMAD-DNA complex (Fig. 3B, right panel). TNF-α efficiently reduced TGF-β-induced SMAD-DNA complex formation in both WT (lane 4 versus lane 2) and nemo⁻/⁻ (lane 12 versus lane 10) mouse fibroblast extracts, whereas no reduction in SMAD-DNA complex formation could be observed in jnk⁻/⁻ fibroblasts (lane 8 versus lane 6). No change in total SMAD3 content (measured by Western blot analysis of whole cell extracts) was observed in WT fibroblasts treated with TNF-α for 30 min to 24 h (data not shown), indicating that the reduced amount of SMAD-DNA complexes observed in EMSAs and the reduced SMAD-dependent gene transactivation when TNF-α was added concomitantly with TGF-β (see above) are not due to reduced SMAD3 levels in response to TNF-α. Parallel EMSA with an NF-κB-specific probe (Fig. 3C) highlighted the lack of correlation between TNF-α-dependent reduction in SMAD-DNA complexes seen in Fig. 3B and the induction of NF-κB DNA-binding activity.

Because c-Jun is a key effector of TNF-α inhibitory activity in SMAD signaling (6), we overexpressed c-Jun instead of adding exogenous TNF-α and examined its inhibitory potential on SMAD signaling in WT, jnk⁻/⁻, and nemo⁻/⁻ fibroblasts. As shown in Fig. 4A, c-Jun inhibitory activity against SMAD-driven TGF-β-induced (CAGA)₉-Lux transactivation was readily observed in WT and nemo⁻/⁻ fibroblasts, but absent in jnk⁻/⁻ fibroblasts, suggesting a critical role for c-Jun N-termi- nal phosphorylation in the inhibitory activity of c-Jun against the SMAD pathway. To determine the origin of such JNK activity in WT cells, several approaches were taken. First,
potential activation of JNK by TGF-β was examined. As shown in Fig. 4B (upper panel), no JNK phosphorylation in response to TGF-β (detected by Western blot analysis of endogenous phospho-JNK proteins) was observed 15 min to 24 h after TGF-β addition. An antibody directed against JNK verified the total JNK content was identical in each sample (middle panel). These data are consistent with our previous demonstration that TGF-β is able to transactivate AP-1-dependent genes only in epithelial cells, but not in fibroblasts (7). Second, to determine the possibility of basal JNK activity in unstimulated cultured fibroblasts, we used a Gal4-based transactivation system. Specifically, a Gal4BD-c-Jun fusion protein expression vector injection was cotransfected with the Gal4-Lux reporter construct and Gal4BD-c-Jun fusion protein expression vector in the presence of either empty pRSVe or the dominant-negative MKK4 expression vector.

Results from three separate experiments are shown as means ± S.D. of three independent experiments, each performed with duplicate samples. B, WT fibroblast cultures were treated with TGF-β, and whole cell lysates were prepared at various time points for Western blot analysis of phospho-JNK (P-JNK), JNK, and β-actin contents. C, WT fibroblast cultures were cotransfected with the Gal4-Lux reporter construct and Gal4BD-c-Jun fusion protein expression vector in the presence of either empty pRSVe or the dominant-negative MKK4 expression vector.

From the results presented above, it appears that JunB is strictly due to the absence of JNK activity. C-Jun and JunB (but not ATF2) Inhibit TGF-β/SMAD Signaling in a JNK-dependent Manner—Our next aim was to determine which JNK substrate(s) may be able to interfere with TGF-β/SMAD signaling. For this purpose, the effect of overexpression of c-Jun, JunB, and ATF2, three known JNK substrates (16), on (CAGA)9-Lux transactivation was examined in WT and jnk-/- fibroblasts was effectively due to the knockout of JNK activity, the effect of jnk1 expression rescue was examined. As shown in Fig. 5, ectopic expression of jnk1, which had no significant effect on SMAD signaling per se in WT fibroblasts, entirely rescued the inhibitory effect of TNF-α in jnk-/- fibroblasts, attesting that the lack of inhibitory effect of TNF-α is strictly due to the absence of JNK activity.

Further Evidence That JunB Function Is Dependent on JNK—from the results presented above, it appears that JunB may act as a substrate for JNK, an issue that has been somewhat controversial (30–32). To confirm this hypothesis, we compared the effect of JunB on TGF-β/SMAD signaling in WT and jnk-/- fibroblasts in the absence or presence of exogenously added jnk1 expression vector. As shown in Fig. 7A, JunB overexpression efficiently blocked TGF-β-driven SMAD-dependent trans-
activation in WT fibroblasts. The inhibitory effect of JunB was lost in \textit{jnk}^{--} fibroblasts, but rescued by ectopic \textit{jnk}1 expression in the latter cell type, attesting for a direct role of JNK activity in controlling the JunB effect against TGF-\(\beta\)/SMAD signaling. Of note, the same results were obtained when \textit{c-Jun} was used instead of JunB in the same experimental setting (data not shown).

We have determined that TGF-\(\alpha\)-efficiently blocks TGF-\(\beta\) signaling in \textit{junAA} fibroblasts (Fig. 3). Together with our data indicating the critical role for \textit{c-Jun} phosphorylation by JNK in mediating the TGF-\(\alpha\)-effect in human and mouse fibroblasts, these observations led us to investigate (a) whether JNK plays a role downstream of TGF-\(\alpha\) in \textit{junAA} cells and (b) whether JunB may substitute for \textit{c-Jun} in the latter cell type. To this end, \textit{junAA} fibroblasts were transfected with either a dominant-negative MKK4 or an antisense \textit{junB} expression vector, and the antagonism between TGF-\(\alpha\)- and TGF-\(\beta\)-on (CAGA)_9-Lux was determined. As shown in Fig. 7B, dominant-negative MKK4 efficiently blocked the effect of TGF-\(\alpha\) against TGF-\(\beta\), indicating that this inhibitory mechanism in \textit{junAA} fibroblasts is also dependent on JNK function. Furthermore, overexpression of the antisense \textit{junB} vector resulted in almost complete abolishment of the TGF-\(\alpha\)-effect, indicating that JunB substitutes for \textit{c-JunAA} in mediating the inhibitory activity of TGF-\(\alpha\) against SMAD signaling in a JNK-dependent manner.

One of the mechanisms by which Jun proteins interfere with the SMAD pathway involves direct SMAD-Jun interaction, not compatible with SMAD-DNA complex formation (13, 33, 34). To determine whether JNK activity may play a role in controlling SMAD3-JunB interactions, we adapted the mammalian one-hybrid Gal4-based transactivation assay in \textit{jnk}^{--} fibroblasts. As shown in Fig. 7C, in the absence of the JNK1 expression vector, expression of VP16AD-JunB only slightly enhanced Gal4BD-SMAD3-mediated transactivation, representative of weak interactions between SMAD3 and JunB. No effect of TGF-\(\alpha\)-on this interaction could be observed. On the other hand, when \textit{jnk}1 was coexpressed, VP16AD-JunB expression resulted in a dramatic enhancement of the Gal4BD-SMAD3 effect, representative of JNK-dependent SMAD3-JunB interactions, which were further enhanced by exogenous TGF-\(\alpha\), reflecting activation of the MEK1 (MAPK/ERK kinase-1)/MKK4/JNK cascade by the latter. Together, these results provide strong evidence for a role of JNK in enhancing SMAD-Jun direct interactions that are not compatible with SMAD-DNA complex formation (i.e., no transactivation).

**TNF-\(\alpha\)-Prevents TGF-\(\beta\)-induced COL1A1 and COL3A1 Gene Expression in a JNK-dependent Manner**—The data presented above demonstrate that JNK plays a crucial role downstream of pro-inflammatory cytokines in interfering with the SMAD pathway. To determine the role of JNK in a physiologically relevant gene context, we examined the antagonistic activity of TGF-\(\alpha\)-against TGF-\(\beta\)-induced up-regulation of the endogenous extracellular matrix genes \textit{COL1A1} and \textit{COL3A1}. These fibroblast collagen genes were previously identified as SMAD3/4 targets (35). As shown in Fig. 8, strong enhancement of both \textit{COL1A1} and \textit{COL3A1} mRNA steady-state levels (6–8-fold) was observed in response to TGF-\(\beta\) in the three cell types (lanes 2, 6, and 10, respectively). TGF-\(\alpha\)-antagonized the TGF-\(\beta\)-effect on type I and type III collagen gene expression in both WT and \textit{nemo}^{--} fibroblasts (lanes 4 versus lanes 2 and 12 versus lane 10, respectively), but not in \textit{jnk}^{--} fibroblasts (lane 8 versus lane 6). Glyceraldehyde-3-phosphate dehydrogenase mRNA steady-state levels showed no modulation by cytokines in any of the cell types (lower panel). These data are in agreement with our observation that TGF-\(\alpha\)-antagonizes TGF-\(\beta\)-induced \textit{COL1A2} expression and promoter transactivation in a \textit{c-Jun}/JNK-dependent manner (36, 37).

**DISCUSSION**

Using both normal human dermal fibroblasts and genetically modified immortalized mouse fibroblast lines established from \textit{jnk}^{--}, \textit{nemo}^{--}, and \textit{junAA} embryos, we have provided definite evidence for a central role for JNK in allowing the inhibitory activity of pro-inflammatory cytokines against TGF-\(\beta\)/SMAD signaling, whereas NF-kB activity plays no role in the phenomenon. We have identified \textit{c-Jun} and JunB (but not ATF2) as JNK substrates responsible for this effect and determined that JNK activity allows for strong protein-protein interactions between JunB and SMAD3, not compatible with SMAD-DNA complex formation (33) and leading to reduced SMAD-dependent gene transcription (13).
transfected with (CAGA)9-Lux together with either the pRSV/AS-junB expression vector or dominant-negative JNK1 expression vector. Six hours following glycerol shock, the cells were placed in medium supplemented with 1% FCS. Eighteen hours later, TGF-β and TNF-α were added, and incubations were continued for 24 h before luciferase activity was determined. Data from a representative experiment are shown.

**Lack of JNK Activity Does Not Alter the TGF-β/SMAD Response**—The use of cell lines genetically devoid of JNK activity allowed us to rule out any implication of the latter MAPK in the activation of the SMAD cascade by TGF-β. Our results, obtained mainly by transiently transfecting the artificial constructs (CAGA)9-Lux and (SMAD binding element)4-Lux (38) (data not shown), both of them highly specific for the SMAD3/4 pathway, differ from those of Engel et al. (39), who demonstrated that JNK phosphorylation of SMAD3 facilitates its activation by TGF-β receptor type I and subsequent nuclear translocation. Several explanations can be found for these discrepancies. The latter results were obtained using indirect approaches to interfere with Rho and Rac GTPase-driven TGF-β-induced JNK activity. A second reason that may explain the discrepancies is that, in several experiments, Engel et al. utilized the construct 3TP-Lux as a SMAD reporter, and it is now well established, as was somewhat suggested by the authors themselves, that its transactivation is highly AP-1-dependent, in other words, JNK-dependent. It is also worth noting that, in all mouse fibroblast lines we tested, TGF-β itself did not activate JNK, as measured either by the modified one-hybrid transactivation system based on c-Jun phosphorylation (data not shown) or by Western blotting (Fig. 4B), therefore contrasting with the biphasic activation of JNK observed in Mv1Lu and MDA-MB-468 cells (39). This cell type-specific activation of JNK by TGF-β is entirely consistent with our previous observation that TGF-β induces AP-1-dependent gene transactivation in epithelial cells, but not in fibroblasts (7).

**JNK Activity Is Central to the Inhibitory Activity of Pro-inflammatory Cytokines against TGF-β/SMAD Signaling, whereas NF-κB Activity Is Not**—As described above, in some cell types, JNK positively regulates some of the SMAD- and TGF-β-mediated transcriptional responses, yet JNK activators only partially stimulate transcriptional responses characteristic of TGF-β without coincident SMAD pathway activation. It has also been reported that, in some cell types, triggering of the

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**Fig. 7. JNK-dependent JunB functions.** A, JNK1 overexpression restores JunB inhibitory activities against TGF-β/SMAD signaling in jnk−/− fibroblasts. Subconfluent WT and jnk−/− fibroblast cultures were transfected in parallel with (CAGA)9-Lux together with either empty pRSV or pRSV-JunB without or with the JNK1 expression vector. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Eighteen hours later, TGF-β and TNF-α were added, and incubations were continued for 24 h before luciferase activity was determined. B, in junAA fibroblasts, JunB substitutes for c-JunAA and mediates TNF-α-induced inhibition of TGF-β/SMAD signaling in a JNK-dependent manner. Subconfluent junAA fibroblasts were cotransfected with (CAGA)9-Lux together with either the pRSV/AS-junB dominant-negative (D/N) MKK4 expression vector or pCMVe expression vector. Empty pCMVe was used to maintain equivalent amounts of transfected DNA in each plate. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Six hours later, TGF-β and TNF-α were added, and incubations were continued for 24 h before luciferase activity was determined. In A and B, bars indicate means ± S.D. of at least three experiments, each performed with duplicate samples. C, JNK activity increases SMAD3-JunB interactions in the mammalian two-hybrid system. Subconfluent WT and jnk−/− fibroblast cultures were transfected with Gal4-Lux, Gal4BD-SMAD, and/or VP16AD-JunB together with either the empty pCMVe or JNK1 expression vector. Six hours following glycerol shock, TNT-α was added, and luciferase activity was measured 24 h later. Data from a representative experiment are shown.

**Fig. 8. Role of JNK in the down-regulation of TGF-β-induced type I and type III collagen gene expression by TNF-α.** Subconfluent WT, jnk−/−, and nemo−/− fibroblast cultures were treated with TGF-β and TNF-α for 24 h in medium containing 1% serum. Shown are representative Northern hybridization signals of total RNA with COL1A1, COL3A1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Note the absence of down-regulation of TGF-β effect on both COL1A1 and COL3A1 mRNA steady-state levels by TNF-α in jnk−/− fibroblasts.
SAPK/JNK pathway by TGF-β itself could participate in a negative feedback loop controlling TGF-β responses (12). Although they are somewhat opposite with regard to JNK activation by TGF-β itself, these results are in full agreement with the concept of an interdependent relationship between the JNK and SMAD pathways in TGF-β-mediated transcription.

Another interesting and novel observation from our work is that JNK activity is a prerequisite for pro-inflammatory cytokines to interfere with the SMAD pathway, whereas the NF-κB pathway, although critical for numerous inflammatory responses, plays little role (if any) in this phenomenon. Again, the use of genetically altered cell lines devoid of any NF-κB activity allowed us to definitely rule out the involvement of the latter in the antagonistic activity of TNF-α against SMAD signaling.

Both TNF-α and IL-1β, prototypic inflammatory cytokines, inhibited TGF-β-induced SMAD signaling in human dermal fibroblasts and in the various mouse lines tested, except in the jnk−/− fibroblasts. Their inhibitory activity was restored in the latter cell type upon overexpression of jnk1. In human dermal fibroblasts, their inhibitory activity was prevented by a dominant-negative form of MKK4 that blocks JNK activation (17). Concomitant expression of MKK7 together with dominant-negative MKK4 restored most of the inhibitory activity of TNF-α against SMAD signaling, suggesting that both MKK4 and MKK7 are capable of mediating this cytokine effect. Searching for JNK substrates involved in this inhibitory mechanism, we identified c-Jun and JunB as potential candidates, both of which are known to be up-regulated in fibroblasts by several pro-inflammatory cytokines, whether in the presence or absence of TGF-β (3). These two Jun family members are able to interfere with SMAD signaling, but this is the first demonstration that their inhibitory activity can be exerted only through JNK activation downstream of pro-inflammatory cytokines.

ATF2, another known JNK substrate, did not interfere with the SMAD pathway.

Another mechanism by which TNF-α might block SMAD signaling is through NF-κB activation. The latter may, in turn, induce SMAD7 expression, a molecule that interferes with SMAD phosphorylation by TGF-β receptor type I and subsequent translocation into the cell nucleus (10). Activation of SMAD7 expression through the NF-κB cascade appears to be restricted to certain subsets of mouse embryonic fibroblasts, as (a) in human embryonic kidney 293 cells, NF-κB activation inhibits SMAD7 gene expression (11); (b) we did not previously observe any activation of SMAD7 expression by TNF-α in human dermal fibroblasts (6); (c) in several primary and immortalized mouse cell lines tested during this study, transfection of a dominant-negative mutant of IkB kinase-α, which blocks NF-κB activation, did not interfere with TNF-α blockade of SMAD signaling (data not shown); and (d) mouse embryonic fibroblasts devoid of NF-κB activity, e.g. nemo−/−, allowed full inhibitory activity of TNF-α against SMAD signaling (this study). The latter data unequivocally eliminate the NF-κB pathway from playing a role in the interference exerted by pro-inflammatory cytokines with TGF-β/SMAD signaling.

JunB Function Depends on JNK Activity—Several of the experiments described in this study reinforce the idea that JunB is a JNK substrate. First, we found that, in human dermal fibroblasts, JunB inhibitory activity against SMAD signaling was blocked by expression of a dominant-negative mutant form of MKK4 blocking the JNK signal transduction pathway (17). Second, we observed that JunB did not exert its inhibitory activity in jnk−/− fibroblasts unless epistomal expression of jnk1 was allowed by means of a transfected expression vector. Finally, using the mammalian two-hybrid system, we determined that protein-protein interactions between SMAD3 and JunB were very weak in jnk−/− fibroblasts, potentiated by ectopic jnk1 expression and further enhanced by TNF-α. The latter phenomenon is likely representative of JNK1 activation by TNF-α, resulting in enhanced functionality of JunB upon phosphorylation. Together, these data provide ample evidence for JunB as a JNK1 substrate. They also indicate that JNK activity promotes SMAD3-JunB interactions, which result in decreased SMAD-dependent gene transcription, as this association is not compatible with SMAD binding to its cognate DNA sequences (6, 13).

JunB May Substitute for c-Jun in Mediating the Inhibitory Activity of Pro-inflammatory Cytokines against TGF-β/SMAD Signaling—in human dermal and WT mouse fibroblasts, most of the antagonistic activity of TNF-α against TGF-β/SMAD signaling is dependent on c-Jun, as evidenced using antisense c-Jun approaches (Ref. 6 and this study). Earlier studies have shown that c-Jun needs to be phosphorylated at Ser63 and Ser73 to become transcriptionally active (40, 41) and for c-Jun-dependent apoptosis (20, 42). Our data indicate that c-Jun phosphorylation by JNK is also critical for inhibitory activities that do not depend on c-Jun binding to specific DNA sequences. JunAA, a protein in which the phosphoacceptor Ser63 and Ser73 residues have been mutated to alanines, fails to transactivate from AP-1 elements and to cotransform (20). Moreover, a dominant-negative JNK1 mutant was shown to interfere with c-Jun-dependent transformation (20, 42), further emphasizing the central role of Jun phosphorylation in Jun-dependent biological responses.

JunAA fibroblasts allowed us to evaluate the role of JNK in a cellular context in which its main substrate, c-Jun, is not functional as such. In this cellular context, in which JNK was activated upon TNF-α stimulation (see Fig. 2, B and C), we unveiled a novel function of JunB downstream of TNF-α signaling. Specifically, we determined that JunB could substitute for c-Jun in mediating the inhibitory activity of TNF-α against SMAD signaling in a JNK1-dependent manner. This is an important result, as it is too often considered that JNK inactivation and c-Jun targeting are somewhat similar. Furthermore, in several instances, JunB has been shown to exert antagonistic activities against c-Jun (3, 7, 43, 44).

Substitutions of some of the functions of AP-1 family members with JunB have also been described. For example, defects in the placentation of fra-1-knockout embryos can be rescued by a junB transgene, although with a low efficiency (45). Knock-in mice in which c-Jun has been replaced by junB, although dying a few hours after birth because of malformed cardiac outflow tracts, develop normal livers, clearly indicating that JunB is able to complement for c-Jun in hepatic development (46).

Conclusion—We have reported a critical role for JNK in the mechanism of suppression of TGF-β/SMAD3 signaling by pro-inflammatory cytokines, involving the transcription factors c-Jun and JunB. These AP-1 components are key factors in the transmission of signals from various pro-inflammatory cytokines known to antagonize TGF-β in the context of tissue repair and maintenance of tissue homeostasis. Our experiments demonstrate that JNK, which is critical in conferring transcriptional activity to Jun proteins, is also instrumental in allowing these proteins to exert inhibitory activities independent of their DNA-binding ability and associated transactivating capabilities, such as interfering with SMAD-dependent gene transcription downstream of TGF-β. We have also demonstrated that such a JNK-dependent mechanism underlies the inhibitory activity of TNF-α against TGF-β-induced SMAD-dependent fibrillar collagen gene expression.
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