0026-895X/03/6403-707–713$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 64:707–713, 2003

Vol. 64, No. 3
2490/1085115
Printed in U.S.A.

5-Fluorouracil Blocks Transforming Growth Factor-β–Induced α_2 Type I Collagen Gene (*COL1A2*) Expression in Human Fibroblasts via c-Jun NH_2-Terminal Kinase/Activator Protein-1 Activation

JEANNE WENDLING, AIMÉ MARCHAND, ALAIN MAUVIEL, and FRANCK VERRECCHIA

Institut National de la Santé et de la Recherche Médicale U532, Skin Research Institute, Saint-Louis Hospital, Paris, France

Received February 28, 2003; accepted May 21, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

5-Fluorouracil (5-FU), a pyrimidine analog widely used in cancer chemotherapy and in glaucoma surgery, has recently shown some efficacy in the treatment of keloids, scars that overgrow the boundaries of original wounds. Given the physiopathological importance of transforming growth factor-β (TGF-β) in keloid and scar formation, we have examined whether the clinical benefits from 5-FU treatment may result from its capacity to interfere with TGF-β signaling and resulting activation of type I collagen gene expression. Using various molecular approaches to study the mechanisms underlying 5-FU effects, we have demonstrated that 5-FU antagonizes TGF-β–driven *COL1A2* transcription and associated type I collagen production by dermal fibroblasts. In addition, 5-FU inhibits both SMAD3/4-specific transcription and formation of SMAD/DNA complexes induced by TGF-β. 5-FU induces c-Jun phosphorylation and activates both AP-1-specific transcription and DNA binding. Overexpression of an antisense *c-jun* expression vector, or that of a dominant-negative form of MKK4 that interferes with c-Jun N-terminal kinase (JNK) activation, blocks the inhibitory activity of 5-FU on TGF-β–induced *COL1A2* transcription. Furthermore, in a cellular context devoid of JNK activity (i.e., $JNK^{-/-}$ fibroblasts), 5-FU inhibits neither formation of SMAD/DNA complexes nor SMAD-driven *COL1A2* transcription in response to TGF-β. Together, these results identify 5-FU as a potent inhibitor of TGF-β/SMAD signaling, capable of blocking TGF-β–induced, SMAD-driven up-regulation of *COL1A2* gene expression in a JNK-dependent manner. We thus provide a molecular explanation to the observed clinical benefits of 5-FU in the treatment of keloids and hypertrophic scars.

Regulation of connective tissue formation during development and repair processes is under the rigorous control of a number of soluble mediators acting in concert to ensure tissue integrity and homeostasis. Disruption of the fragile equilibrium between anabolic and catabolic cytokines may lead to excessive extracellular matrix synthesis and deposition, the hallmark of several fibrotic conditions, including keloids and hypertrophic scars (Jimenez and Saitta, 1999; Uitto and Kouba, 2000). For example, affected skin areas from patients with keloids or hypertrophic scars exhibit abnormal accumulation of type I collagen (Ehrlich et al., 1994; Uitto and Kouba, 2000).

The role of the transforming growth factor-β (TGF-β) as the main factor inducing collagen gene expression leading to tissue fibrosis has been suggested by the observation that 1) TGF-β expression often parallels increased type I collagen gene expression in fibrotic lesions (Nakatsukasa et al., 1990; Peltonen et al., 1990; Broekelmann et al., 1991) and 2) TGF-β is a potent activator of extracellular matrix gene expression both in vitro and in vivo (reviewed in Verrecchia and Mauviel, 2002).

The TGF-βs signal via specific serine/threonine kinase transmembrane receptors (TβRI and TβRII) that phosphorylate cytoplasmic mediators of the SMAD family (Massagué and Chen, 2000; Massagué and Wotton, 2000). The receptor-associated SMADs (R-SMADs), such as SMAD2 and SMAD3, interact directly with and are phosphorylated by activated TβRI. Upon phosphorylation, they form heteromeric complexes with SMAD4, a common mediator for all SMAD pathways. These R-SMAD/SMAD4 complexes are translocated into the nucleus, where they may function as transcription factors, directly or in association with other DNA binding factors such as Fast and Sp1 (reviewed in Massagué and Chen 2000; Massagué and Wotton, 2000). Recently, using a combined cDNA microarray/promoter transactivation ap-

This work was supported by Institut National de la Santé et de la Recherche Médicale and by grants from the Association pour la Recherche contre le Cancer and Electricité de France (Service de Radioprotection) (to A.M.).

ABBREVIATIONS: TGF-β, transforming growth factor-β; R-SMAD, receptor-associated SMAD; 5-FU, 5-fluorouracil; JNK, c-Jun N-terminal kinase; wt, wild type; CAT, chloramphenicol acetyltransferase; TNF-α, tumor necrosis factor-α; EMSA, electrophoretic mobility shift assay.

proach, we identified several skin fibrillar collagen genes, *COL1A1*, *COL1A2*, *COL3A1*, and *COL5A2*, to be direct SMAD3 targets downstream of TGF-β (Verrecchia et al., 2001a).

5-FU was first used for its antimetabolite activity. In this molecule, the hydrogen atom in position 5′ of uracil is replaced by the similarly sized atom of fluoride, and the molecule was designed to occupy the active sites of enzyme targets, thereby blocking metabolism. In the early 1980s, 5-FU was investigated as an adjunct to glaucoma filtering surgery, a procedure in which inhibition of wound healing is desirable to achieve surgical success (Gressel et al., 1984; Skuta et al., 1987). More recently, local 5-FU application has shown some success in the treatment and prevention of hypertrophic scars and keloids (Fitzpatrick 1999; Uppal et al., 2001).

In this work, we investigated the effects of 5FU on TGF-β–induced type I collagen gene expression in human fibroblasts. We identify c-Jun N-terminal kinase (JNK) activation and subsequent c-Jun phosphorylation in response to 5-FU as critical for the antagonism exerted by 5FU against TGF-β–induced SMAD signaling and resulting *COL1A2* transactivation.

Materials and Methods

Cell Cultures. Human dermal fibroblasts were established by explanting neonatal foreskins. Wild-type (wt) and $JNK_1^{-/-}$-$JNK_2^{-/-}$ ($JNK^{-/-}$) immortalized fibroblasts were derived, respectively, from wild-type and $JNK_1^{-/-}$-$JNK_2^{-/-}$ mouse embryos in which simultaneous targeted disruption of the *jnk1* and *jnk2* genes had been performed (Sabapathy et al., 1999). All fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 μg/ml streptomycin-G, and 25 μg/ml Fungizone). TGF-β1 and 5-FU were purchased from R&D Systems Inc. (Minneapolis, MN) and Sigma Chemical (St. Louis, MO), respectively. In all experiments, TGF-β was used at a concentration of 10 ng/ml, which maximally induces SMAD3/4 signaling in fibroblasts (F. Verrecchia and A. Mauviel, unpublished results). Unless noted otherwise, 5-FU was used at a concentration of 10 μM.

Transient Cell Transfections and Reporter Assays. Transient cell transfections were performed with Fugene-6 according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). pRSV–β-galactosidase was cotransfected in every experiment to monitor transfection efficiencies. CAT activity was measured using [^{14}C]chloramphenicol as substrate followed by thin-layer chromatography and quantitation with a PhosphorImager (Amersham Biosciences, Uppsala, Sweden). Luciferase activity was determined with a commercial assay kit (Promega, Madison, WI).

Plasmid Constructs. 3500COL1A2/CAT (gift from Francesco Ramirez, Mt. Sinai School of Medicine, New York, NY), and pRSV/*AS-c-jun* have been described previously (Boast et al., 1990; Chung et al., 1996). pAP1-TA-lux (Mercury pathway profiling vector; BD Biosciences Clontech, Palo Alto, CA) was used to evaluate AP-1–driven transcription. Dominant-negative MKK4 expression vector was used to modulate JNK activity (Verrecchia et al., 2003). To track JNK activity, we used a commercial reporter assay based on the modification of the mammalian one-hybrid system, consisting of a reporter plasmid, Gal4-Lux, bearing five Gal4 binding sites driving luciferase expression, and a transactivator plasmid encoding a chimeric protein, Gal4BD-c-Jun, consisting of the DNA binding domain of Gal4 (Gal4BD) fused to the transactivation domain of c-Jun, the latter requiring phosphorylation by JNK to fully transactivate Gal4-lux (Stratagene, La Jolla, CA).

Northern Blotting. Total RNA was obtained using RNeasy (Qiagen GmbH, Hilden Germany) and analyzed by Northern hybridization with ^{32}P-labeled cDNA probes for *COL1A2* and *GAPDH* as described previously (Verrecchia et al., 2002). Quantitations were performed with a Storm 840 PhosphorImager (Amersham Biosciences).

Western Blot Analyses. Total protein cell extract (50 μg) in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) were denatured by heating at 95°C for 3 min before resolution by SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose filters (Amersham Biosciences), immunoblotted with either rabbit anti-type I collagen (Southern Biotech, Birmingham, AL), anti-phospho-c-Jun (Upstate Biotechnologies, Lake Placid, NY), anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-actin (Sigma) antibodies, all at a dilution of 1:1000 in 1× phosphate-buffered saline/5% nonfat milk for 1 h. After incubation, filters were washed and incubated with a horseradish peroxidase-conjugated goat-anti-rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology) for 1 h. Filters were then washed, developed according to chemiluminescence protocols (ECL, Amersham Biosciences), and revealed with a PhosphorImager.

Electrophoresis Mobility Shift Assays. A 3×CAGA SMAD3/4-specific oligonucleotide (Dennler et al., 1998) and a consensus AP-1 binding oligonucleotide (Promega Corp., Madison, WI) were used as probes. For supershift experiments, nuclear extracts (5–7 μg) were incubated overnight at 4°C with antisera before the binding reaction and separated electrophoretically on native 4% acrylamide gels.

Results

5-FU Does Not Affect Cell Viability at 24 and 48 h at a Concentration of 10 μM. Because high concentrations of 5-FU are cytotoxic, we first had to determine which concentrations of the drug would not induce significant cell death. This was particularly important because cellular toxicity may interfere significantly with cellular signaling outcomes and resulting gene expression. For this purpose, human dermal fibroblasts were incubated for 24 to 72 h with 5-FU concentrations ranging from 5 to 100 μM, at which point a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay was performed. As shown in Fig. 1, no significant cell mor-

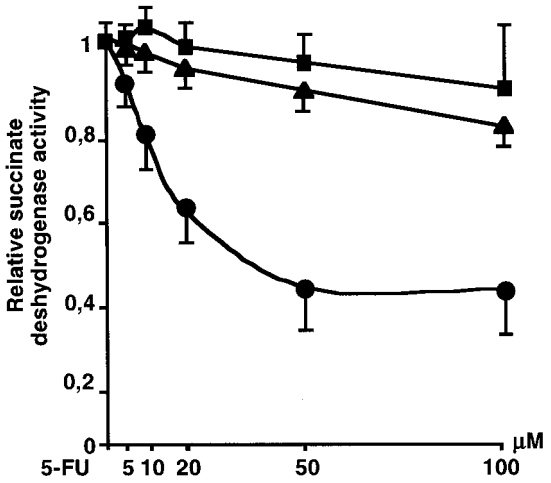

Fig. 1. 5-FU does not affect cell viability at 24 and 48 h with concentration of 10 μM. Succinate dehydrogenase activity was determined according to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay protocol (Sigma). The cells were incubated for 24 (■), 48 (▲), and 72 h (●) with 5-FU concentrations ranging from 5 to 100 μM. Symbols indicate mean ± S.D. of three independent experiments performed, each with triplicate samples.

tality was observed at 24 and 48 h with concentrations of 5 and 10 μM. At 72 h, however, cytotoxicity was more than 30% for concentrations of 5-FU above 10 μM. We thus performed all subsequent experiments with 5-FU at a concentration of 10 μM, which exhibited no detectable cytotoxicity at either the 24- or 48-h time-point and induced less than 20% cell mortality at 72 h.

5-FU Prevents TGF-β–Induced COL1A2 Gene Transactivation in Human Fibroblasts. We first wanted to determine whether 5-FU antagonized TGF-β–driven *COL1A2* promoter transactivation in human dermal fibroblasts. As shown in Fig. 2A, and as expected from the literature, TGF-β enhanced −3500COL1A2/CAT activity 4- to 5-fold above control levels. Addition of 5-FU had a negligible effect on basal *COL1A2* promoter but completely abolished TGF-β response. To determine the physiological relevance of such findings obtained with promoter reporter constructs in transient cell transfection experiments, we next examined whether endogenous *COL1A2* expression followed the same pattern of regulation. As a first approach to address this point, the effect of 5-FU on TGF-β–induced type I collagen gene expression was examined, taking the *COL1A2* mRNA steady-state levels as an endpoint. As shown in Fig. 2B, Northern analysis of total RNA with a specific *COL1A2* probe revealed an expected potent up-regulation by TGF-β (lane 2 versus lane 1, top), that was completely prevented by 5-FU (lane 4 versus lane 2), whereas 5-FU alone did not modify basal *COL1A2* expression (lane 3 versus lane 1). *GAPDH* mRNA steady-state levels were unaffected by treatments (Fig. 2B, bottom), attesting to the specificity of *COL1A2* regulation.

The modulation of TGF-β–induced elevation of *COL1A2* mRNA steady-state levels by 5-FU translated into altered type I collagen production, as determined by Western analysis of whole-cell lysates with an anti-type I collagen antibody (Fig. 2C). Specifically, elevated type I collagen production in response to TGF-β (top, lane 2 versus lane 1) was prevented by addition of 5-FU (lane 4 versus lane 2). Actin levels (bottom) remained unchanged throughout the experiments. Together, these results provide evidence that 5-FU antagonizes TGF-β induced type I collagen production, and this effect of 5-FU occurs at the level of *COL1A2* transcription.

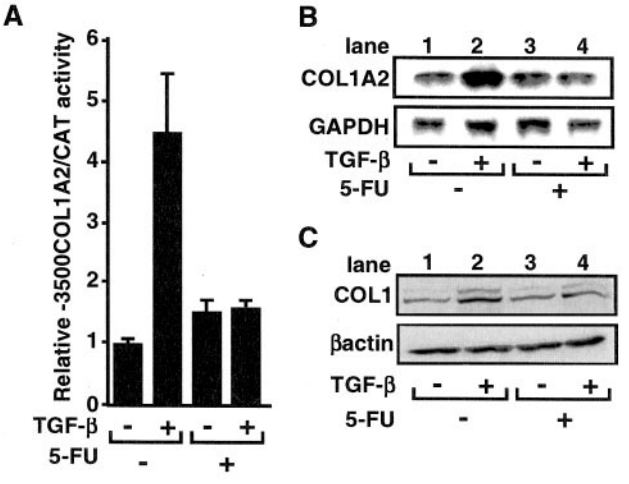

Fig. 2. 5-FU inhibits TGF-β–induced COL1A2 gene transactivation. A, subconfluent fibroblasts, placed in medium supplemented with 1% FCS, were transfected with −3500*COL1A2*/CAT. Three hours later, 5-FU (10 μM) was added, followed by TGF-β (10 ng/ml) 1 h later, and incubations continued for 24 h before CAT assays. Bars indicate mean ± S.D. of at least three independent experiments performed, each with duplicate samples. B, subconfluent fibroblast cultures were treated with TGF-β (10 ng/ml) and 5-FU (10 μM) for 24 h in medium containing 1% serum. After incubations, *COL1A2* mRNA levels was detected by Northern blot analysis (top). Specificity of the modulation was confirmed using a specific GAPDH probe (bottom). C, 24 h after treatment of fibroblasts with TGF-β (10 ng/ml) and 5-FU (10 μM) in medium containing 1% serum. Type I collagen production was detected by Western blot analysis of whole cell lysates (top). Specificity of the modulation was confirmed with an anti-actin antibody (bottom).

5-FU Reduces SMAD-DNA Interactions. The SMAD pathway is critical for activation of the human *COL1A2* promoter by TGF-β (Chen et al., 1999; Zhang et al., 2000; Verrecchia et al., 2001a). Therefore, one possibility to explain the inhibitory activity of 5-FU against TGF-β would be that it interferes with the SMAD pathway. To test this hypothesis, two approaches were chosen. Firstly, we tested the capacity of 5-FU to alter SMAD3/4-specific transcription in a transient cell transfection assay with the SMAD3/4-specific reporter construct $(CAGA)_9$-lux (Dennler et al., 1998). As shown in Fig. 3A, TGF-β up-regulated $(CAGA)_9$-lux activity about 12-fold above control values. 5-FU added 1 h before TGF-β completely abolished such transactivation. To determine whether the blockade of SMAD3/4-dependent gene transcription by 5-FU was caused by a capacity of the latter to interfere with SMAD/DNA complex formation, EMSA experiments were carried out using nuclear extracts from fibroblasts treated with TGF-β for 30 min, a time point at which maximal amounts of SMAD/DNA complexes are formed in response to TGF-β (Vindevoghel et al., 1998a,b), pretreated or not with 5-FU for 60 min. As shown in Fig. 3B, SMAD/DNA complex formation induced by TGF-β (lane 2 versus lane 1) was efficiently prevented when 5-FU was added 60 min before TGF-β addition (lane 4 versus lane 2). Therefore, a good correlation exists between the inhibitory effect of 5-FU on TGF-β/SMAD-dependent *COL1A2* gene transactivation, described in Fig. 2, and on the formation of SMAD/DNA complexes.

5-FU Stimulates the cJun/AP-1 Signaling Pathway. We previously reported the ability of c-Jun to block TGF-β-induced *COL1A2* promoter transactivation and its critical role in mediating the antagonistic effects of TNF-α on SMAD-dependent gene transactivation induced by TGF-β (Chung et al., 1996; Verrecchia et al., 2000, 2003). Furthermore, it has been shown that 5-FU stimulates JNK activity and subsequent c-Jun transcriptional activity (Eichhorst et al., 2000). We therefore investigated whether activation of the cJun/AP-1 signaling pathway by 5-FU may account for its antagonistic activity against TGF-β signaling and resulting collagen gene modulation. First, the AP-1–specific reporter construct pAP1-TA-lux was used in transient cell transfection experiments to determine the effects of 5-FU on AP-1–dependent transcription. As shown in Fig. 4A, 5-FU (10 μM) enhanced pAP-1-TA-lux activity approximately 3.8-fold above control levels. Second, EMSAs were carried out with a radiolabeled consensus AP-1 oligonucleotide as a probe to determine whether 5-FU has the ability to induce AP-1/DNA complexes. As shown in Fig. 4B, a strong retarded band was detected (lane 2 versus lane 1) with nuclear extracts from fibroblasts treated with 5-FU for 1 h, which was supershifted with an anti-c-Jun antibody (lane 4). Together, these results

demonstrate that 5-FU stimulates the cJun/AP-1 signaling pathway. To determine whether c-Jun is directly responsible for the antagonistic activities exerted by 5-FU against TGF-β/SMAD signaling, we examined the effect of 5-FU in the presence of an antisense *c-jun* expression vector shown previously to efficiently antagonize TNF-α–induced *c-jun* expression (Mauviel et al., 1996). As shown in Fig. 4C, overexpression of antisense *c-jun* prevented the inhibitory effect of 5-FU on TGF-β–driven *COL1A2* transactivation in human fibroblasts. These experiments provide evidence that c-Jun plays a critical role in mediating the inhibitory effect of 5-FU on TGF-β/SMAD signaling and subsequent up-regulation of *COL1A2* gene expression by TGF-β.

A Role for JNK in 5-FU–Driven Inhibition of SMAD-Dependent COL1A2 Gene Transcription Downstream of TGF-β. We recently demonstrated that JNK-mediated phosphorylation of c-Jun is critical for c-Jun to antagonize TGF-β/SMAD signaling and subsequent collagen gene transcription (Verrecchia et al., 2002, 2003). We therefore examined the capacity of 5-FU to induce c-Jun phosphorylation using a reporter assay based on the mammalian one-hybrid system (see *Materials and Methods*). As shown in Fig. 5A, 5-FU efficiently transactivated the Gal4-lux construct in the presence of Gal4BD-c-Jun, suggesting that 5-FU activates JNK and subsequent c-Jun phosphorylation. The ability of 5FU to activate c-Jun phosphorylation was further confirmed by Western blot analysis of phospho-c-Jun content in response to 5-FU. As shown in Fig. 5B, 5-FU stimulates c-Jun phosphorylation (lanes 2 versus lanes 1) to levels comparable with those achieved by TNF-α (lane 3).

Second, transient transfection experiments using human dermal fibroblasts showed that expression of a dominant-negative mutant form of MKK4, blocking JNK activation, prevented in part the antagonistic effect of 5-FU against TGF-β–driven *COL1A2* transactivation (Fig. 6A).

Third, we examined the effect of 5-FU on TGF-β–driven *COL1A2* transactivation in fibroblasts devoid of JNK activity, namely double JNK1-JNK2 knock-out fibroblasts ($JNK^{-/-}$ fibroblasts). As shown in Fig. 6B, 5-FU inhibitory

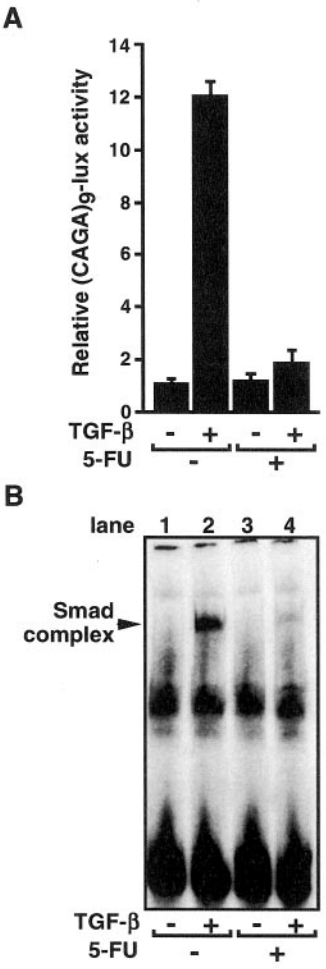

Fig. 3. 5-FU inhibits SMAD3/4-dependent gene transcription and reduces SMAD-DNA interactions. A, subconfluent fibroblasts, placed in medium supplemented with 1% FCS, were transfected with $(CAGA)_9$-lux. Three hours later, 5-FU (10 μM) was added, followed by TGF-β (10 ng/ml) 1 h later, and incubations continued for 24 h before luciferase assays. Bars indicate mean ± S.D. of at least three independent experiments performed, each with duplicate samples. B, EMSAs were performed using the SMAD3/4-specific 3× CAGA oligonucleotide as a probe, together with nuclear extracts from fibroblast cultures treated for 30 min with TGF-β (10 ng/ml) pretreated or not with 5-FU (10 μM, 60 min).

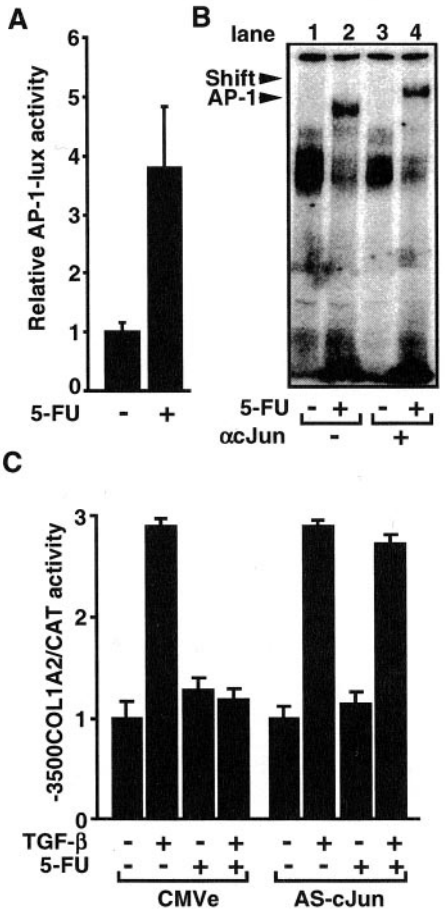

Fig. 4. 5-FU stimulates the cJun/AP-1 signaling pathway. A, cells were transfected with pAP-1-Lux in presence or absence of 5-FU (10 μM, 24 h). B, EMSA was performed using the AP-1–specific oligonucleotide as a probe, together with nuclear extracts from control (lane 1, 3) and 5-FU–treated (10 μM, 1 h, lanes 2 and 4) fibroblast cultures. Supershift assay was carried out with an anti c-Jun antibody (lanes 3 and 4). C, subconfluent human fibroblasts were transfected with −3500*COL1A2*/CAT construct in presence or absence of an antisense c-Jun expression vector. Three hours after transfections, 5-FU (10 μM) was added, followed by TGF-β (10 ng/ml) 1 h later. CAT assays were determined 24 h later. Bars indicate mean ± S.D. of at least three independent experiments performed, each with duplicate samples.

activity against TGF-β–induced COL1A2 promoter activity was readily observed in wild-type fibroblasts but was completely absent in JNK$^{-/-}$ fibroblasts providing definitive evidence for a crucial role of JNK in mediating 5-FU inhibitory effect against TGF-β–induced type I collagen gene expression.

Next, EMSA experiments were carried out using nuclear extracts from wt and JNK$^{-/-}$ fibroblasts treated with TGF-β (30 min), preteated or not with 5-FU for 60 min. As shown in Fig. 7, SMAD/DNA complex formation was equally induced by TGF-β in both wt and JNK$^{-/-}$ fibroblasts (lanes 2 and 6). 5-FU efficiently reduced TGF-β–induced SMAD/DNA complexes in wt (lane 4 versus lane 2); however, no effect of 5-FU on SMAD/DNA complex formation could be observed in JNK$^{-/-}$ fibroblasts. Thus, it seems that a good correlation exists between the role of JNK on the inhibitory effect of 5-FU on SMAD-dependent *COL1A2* gene transactivation, described in Fig. 6, and on the formation of SMAD/DNA complexes.

Dose-Response Relationship of Effects of 5-FU. To ascertain the relationship between JNK activation and repression of TGF-β–induced *COL1A2* gene expression by 5-FU, we next performed a 5-FU dose-response experiment, in which collagen expression, JNK activation, and c-Jun phosphorylation were measured in parallel. As shown in Fig. 8, 5-FU at a concentration of 0.5 μM neither antagonized TGF-β–induced *COL1A2* gene expression (A, lane 3) nor activated JNK (as measured using a Gal4-c-Jun–based mammalian one-hybrid system) (B, lane 2) or c-Jun phosphorylation (C, lane 2). At 2.5 μM, 5-FU had little effect on *COL1A2* expression, JNK activity. and c-Jun phosphorylation (Fig. 8, A, lane 4, and B and C, lanes 3.). Maximal JNK activity and c-Jun phosphorylation levels were observed at 5-FU concentrations of 5 and 10 μM, resulting in significant down-regulation of TGF-β–induced *COL1A2* gene expression (lanes 4 and 5).

Together, these results demonstrate a direct correlation between the capacity of 5-FU to activate JNK at concentrations above 2.5 μM and the antagonistic activity of 5-FU against TGF-β–induced *COL1A2* expression.

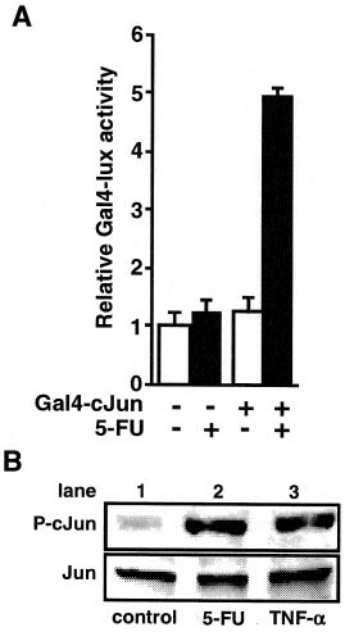

Fig. 5. 5-FU stimulates the AP-1 signaling pathway. A, cells were cotransfected with Gal4-Lux and a transactivator plasmid encoding a chimeric transactivator protein, Gal4BD-c-Jun, in the presence or absence of 5-FU (10 μM). Luciferase activity was determined 24 h later. Bars indicate mean ± S.D. of at least three independent experiments performed, each with duplicate samples. B, Western blot analysis of total protein extracts from fibroblasts treated with 5-FU (10 μM, lane 2) or TNF-α (10 ng/ml, lane 3) for 1 h, using anti phospho-c-Jun and anti-c-Jun antibodies.

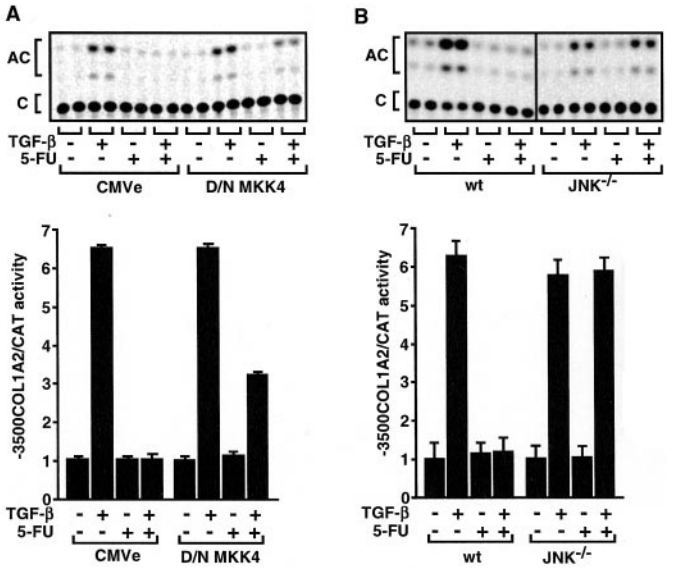

Fig. 6. Critical role for JNK/cJun in 5-FU-driven inhibition of SMAD-dependent COL1A2 gene transcription downstream of TGF-β. A, subconfluent human dermal fibroblasts were transfected with −3500COL1A2/CAT construct in presence or absence of a dominant-negative mutant form of mitogen-activated protein kinase kinase 4 (D/N MKK4). Three hours after transfections, 5-FU (10 μM) was added followed by TGF-β (10 ng/ml) 1 h later. CAT assays was determined 24 h later. Top, autoradiogram of a representative experiment. Bottom, bars indicate mean ± S.D. of at least three independent experiments performed, each with duplicate samples. B, subconfluent wild-type and *JNK*$^{-/-}$ fibroblast cultures were transfected with −3500COL1A2/CAT construct. Three hours after transfections, 5-FU (10 μM) was added followed by TGF-β (10 ng/ml) 1 h later. CAT assays were determined 24 h later. Top, autoradiogram of a representative experiment. Bottom, bars indicate mean ± S.D. of at least three independent experiments, each performed with duplicate samples.

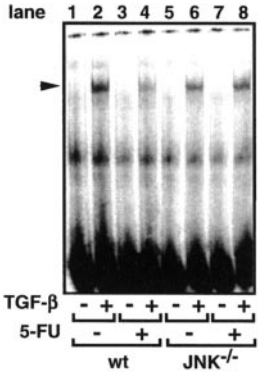

Fig. 7. Critical role for JNK in mediating 5-FU–driven inhibition of SMAD-DNA interactions. EMSAs were performed using the 3× CAGA oligonucleotide as a probe, together with nuclear extracts from wt and *JNK*$^{-/-}$ fibroblast cultures treated for 30 min with TGF-β, pretreated or not with 5-FU (10 μM, 60 min).

Discussion

In this study, in accordance with a previous work (Hendricks et al., 1993), human skin fibroblasts grown to confluence were exposed for 24 h to 5-FU at pharmacologically relevant drug concentrations. This treatment did not affect basal type I collagen synthesis, whereas TGF-β–induced type I collagen synthesis was inhibited. We specifically demonstrated that 5-FU inhibits TGF-β-SMAD-driven *COL1A2* transactivation, providing a molecular basis toward the understanding of the molecular mechanisms underlying the antagonistic activities of 5-FU with TGF-β/SMAD.

SMAD signaling may be blocked by distinct mechanisms: 1) the inhibitory SMADs (SMAD6 or SMAD7) prevent R-SMAD phosphorylation and subsequent nuclear translocation of R-SMAD/SMAD4 heterocomplexes and 2) corepressors such as the viral oncoprotein E1A (Nishihara et al., 1999), the proto-oncogenes c-Ski and SnoN (Akiyoshi et al., 1999), TGIF (Wotton et al., 1999), Snip-1 (Kim et al., 2000), and SIP1 (Verschueren et al., 1999), which compete for R-SMAD/SMAD4 binding to CBP or p300. On the other hand, induction of Jun proteins by cytokines have been shown to directly interfere with the SMAD pathway either by preventing SMAD3 binding to cognate DNA sequences or by sequestering the transcriptional coactivator p300 (Dennler et al., 2000; Verrecchia et al., 2000, 2001b,c).

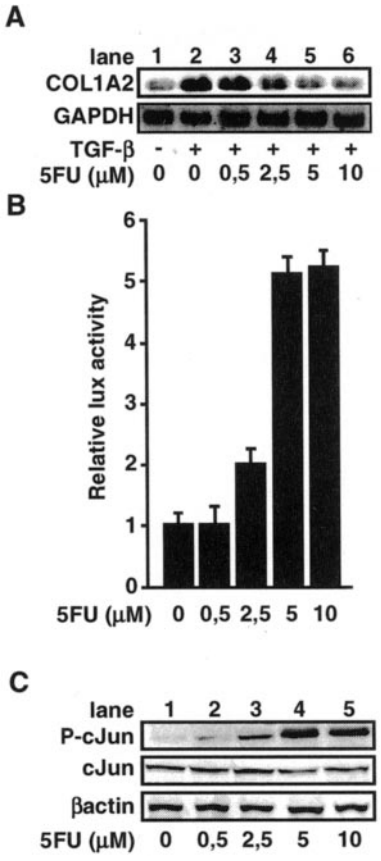

Fig. 8. Dose-response relationship of effects of 5-FU on type I collagen gene expression, JNK activation, and c-Jun phosphorylation. A, subconfluent fibroblast cultures were treated with TGF-β (10 ng/ml) and various concentrations of 5-FU (0.5, 2.5, 5, and 10 μM) for 24 h in medium containing 1% serum. After incubations, *COL1A2* mRNA levels were determined by Northern blot analysis (top). Specificity of the modulation was confirmed using a specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (bottom). B, cells were cotransfected with Gal4-Lux, and a transactivator plasmid encoding a chimeric transactivator protein, Gal4BD-c-Jun, in the presence or absence of various concentration of 5-FU (0.5, 2.5, 5, and 10 μM). Luciferase activity, representative of JNK activity, was determined 24 h later. Bars indicate mean ± S.D. of at least three independent experiments performed, each with duplicate samples. C, Western blot analysis of total protein extracts from fibroblasts treated with 5-FU (lane 2, 0.5 μM; lane 3, 2.5 μM; lane 4, 5 μM; and lane 5, 10 μM) for 1 h, using anti phospho-c-Jun (top) and anti-c-Jun (bottom) antibodies.

In accordance with a recent work showing that an AP-1–*cis*-element in the CD65L promoter are required for CD95L up-regulation by 5-FU (Eichhorst et al., 2000), we provide evidence that 5-FU stimulates c-Jun phosphorylation in dermal fibroblasts. In agreement with the original observation that c-Jun overexpression blocks TGF-β–induced *COL1A2* transactivation (Chung et al., 1996), we established that JNK/c-Jun play a central role in allowing the antagonistic activity of 5-FU against TGF-β–induced type I collagen gene expression. The data establish a link between previous reports about the interference of c-Jun and JNK with SMAD signaling (Dennler et al., 2000) and the inhibition of TGF-β–induced type I collagen gene expression by TNF-α (Verrecchia et al., 2000, 2002). Specifically, one of the proposed mechanisms by which Jun proteins interfere with the SMAD pathway involves direct SMAD-Jun interaction, not compatible with SMAD/DNA complex formation (Verrecchia et al., 2001b,c). Also, 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).

Several different mechanisms could explain the excessive deposition of collagen in the fibrotic skin diseases. First, local expansion of synthetically active fibroblast populations, even with a normal rate of collagen production per cell, could lead to tissue accumulation of collagen. Second, the accumulation of collagen could result from accelerated production of collagen by fibroblasts, and such increase could reflect enhanced collagen gene expression at the transcriptional level (Uitto and Kouba, 2000). In this work, we established that 5-FU effects on type I collagen expression and subsequent collagen deposition could result from inhibition of TGF-β–driven type I collagen transcription.

The basis of keloid formation is not fully understood. Some authors reported that keloid-derived fibroblasts produce increased amounts of collagen per cell compared with normal fibroblasts in culture (English and Shenefelt, 1999). They seem to function autonomously and demonstrate continued collagen synthesis in vitro. These fibroblasts and the collagen produced by them have been the target of antikeloid therapies (Low and Moy, 1992). Our work provides a molecular explanation for the observed clinical benefits of 5FU in the treatment of keloids.

Acknowledgments

Drs. A. Atfi (Paris, France), S. Dennler, and J.-M. Gauthier (Glaxo-Wellcome, Les Ulis, France), provided us with cellular and molecular reagents essential for these studies. Their generosity is gratefully acknowledged.

References

Akiyoshi S, Inoue H, Hanai J, Kusanagi K, Nemoto N, Miyazono K, and Kawabata M (1999) c-Ski acts as a transcriptional co-repressor in transforming growth factor-β signaling through interaction with SMADs. *J Biol Chem* **274:**35269–35277.

Broekelmann TJ, Limper AH, Colby TV, and McDonald JA (1991) Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* **88:**6642–6646.
Boast S, Su MW, Ramirez F, Sanchez M, and Avvedimento EV (1990) Functional analysis of cis-acting DNA sequences controlling transcription of the human type I collagen genes. *J Biol Chem* **265:**13351–13356.
Chen SJ, Yuan W, Mori Y, Levenson A, Trojanowska M and Varga J (1999) Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of SMAD3. *J Investig Dermatol* **112:**49–57.
Chung KY, Agarwal A, Uitto J, and Mauviel A (1996) An AP-1 binding sequence is essential for regulation of the human $\alpha 2_I$ collagen (COL1A2) promoter activity by transforming growth factor-β. *J Biol Chem* **271:**3272–3278.
Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S and Gauthier JM (1998) Direct binding of SMAD3 and SMAD4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO (Eur Mol Biol Organ) J* **17:**3091–3100.
Dennler S, Prunier C, Ferrand N, Gauthier JM, and Atfi A (2000) c-Jun inhibits transforming growth factor β-mediated transcription by repressing SMAD3 transcriptional activity. *J Biol Chem* **275:**28858–28865.
Ehrlich HP, Desmouliere A, Diegelmann RF, Cohen IK, Compton CC, Garner WL, Kapanci Y, and Gabbiani G (1994) Morphological and immunochemical differences between keloid and hypertrophic scar. *Am J Pathol* **145:**105–113.
Eichhorst ST, Muller M, Li-Weber M, Schulze-Bergkamen H, Angel P, and Krammer PH (2000) A novel AP-1 element in the CD95 ligand promoter is required for induction of apoptosis in hepatocellular carcinoma cells upon treatment with anticancer drugs. *Mol Cell Biol* **20:**7826–7837.
English RS and Shenefelt PD (1999) Keloids and hypertrophic scars. *Dermatol Surg* **25:**631–638.
Fitzpatrick RE (1999) Treatment of inflamed hypertrophic scars using intralesional 5-FU. *Dermatol Surg* **25:**224–232.
Gressel MG, Parrish RK, and Folberg R (1984) 5-fluorouracil and glaucoma filtering surgery: I. An animal model. *Ophthalmology* **91:**378–383.
Hendricks T, Martens MF, Huyben CM, and Wobbes T (1993) Inhibition of basal and TGF beta-induced fibroblast collagen synthesis by antineoplastic agents. Implications for wound healing. *Br J Cancer* **67:**545–550.
Jimenez SA and Saitta B (1999) Alterations in the regulation of expression of the alpha 1(I) collagen gene (COL1A1) in systemic sclerosis (scleroderma). *Springer Semin Immunopathol* **21:**397–414.
Kim RH, Wang D, Tsang M, Martin J, Huff C, de Caestecker MP, Parks WT, Meng X, Lechleider RJ, Wang T, et al. (2000) A novel SMAD nuclear interacting protein, SNIP1, suppresses p300-dependent TGF-beta signal transduction. *Genes Dev* **14:**1605–1616.
Low SQ and Moy RL (1992) Scar wars strategies. Target collagen. *J Dermatol Surg Oncol* **18:**981–986.
Nakatsukasa H, Nagy P, Evarts RP, Hsia CC, Marsden E, and Thorgeirsson SS (1990) Cellular distribution of transforming growth factor-beta 1 and procollagen types I, III and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. *J Clin Investig* **85:**1833–1843.
Nishihara A, Hanai J, Imamura T, Miyazono K, and Kawabata M (1999) E1A inhibits transforming growth factor-β signaling through binding to SMAD proteins. *J Biol Chem.* **274:**28716–28723.
Mauviel A, Chung KY, Agarwal A, Tamai K, and Uitto J (1996) Cell-specific induction of distinct oncogenes of the Jun family is responsible for differential regulation of collagenase gene expression by transforming growth factor-β in fibroblasts and keratinocytes. *J Biol Chem* **271:**10917–10923.
Massagué J and Chen YG (2000) Controlling TGF-beta signaling. *Genes Dev* **14:**627–644.
Massagué J and Wotton D (2000) Transcriptional control by the TGF-beta/SMAD signaling system. *EMBO (Eur Mol Biol Organ) J* **19:**1745–1754.
Peltonen J, Kähäri L, Jaakkola S, Kähäri VM, Varga J, Uitto J, and Jimenez SA (1990) Evaluation of transforming growth factor beta and type I procollagen gene expression in fibrotic skin diseases by in situ hybridization. *J Investig Dermatol* **94:**365–371.
Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M, and Wagner EF (1999) Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev* **89:**115–124.
Skuta GL, Assil K, Parrish RK, Folberg R, and Weinreb RN (1987) Filtering surgery in owl monkeys treated with the antimetabolite 5-fluorouridine 5′-monophosphate entrapped in multivesicular liposomes. *Am J Ophthalmol* **103:**714–716.
Uitto J and Kouba D (2000) Cytokine modulation of extracellular matrix gene expression: relevance to fibrotic skin diseases. *J Dermatol Sci* 24:S60–S69.
Uppal RS, Khan U, Kakar S, Talas G, Chapman P, and McGrouther AD (2001) The effects of a single dose of 5-fluorouracil on keloid scars: a clinical trial of timed wound irrigation after extralesional excision. *Plast Reconstr Surg* **108:**1218–1224.
Verrecchia F, Chu ML, and Mauviel A (2001a) Identification of novel TGF-β/SMAD gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J Biol Chem* **276:**17058–17062.
Verrecchia F and Mauviel A (2002) Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. *J Investig Dermatol* **118:**211–215.
Verrecchia F, Pessah M, Atfi A, and Mauviel A (2000) Tumor necrosis factor-α inhibits transforming growth factor-β/SMAD signaling in human dermal fibroblasts via AP-1 activation. *J Biol Chem* **275:**30226–30231.
Verrecchia F, Tacheau C, Schorpp-Kistner M, Angel P, and Mauviel A (2001b) Induction of the AP-1 members c-Jun and JunB by TGF-beta/SMAD suppresses early SMAD-driven gene activation. *Oncogene* **20:**2205–2211.
Verrecchia F, Tacheau C, Wagner EF and Mauviel A (2003) A central role for the Jun-N-terminal kinase pathway in mediating the antagonistic activity of pro-inflammatory cytokines against transforming growth factor-β-driven SMAD3/4 specific gene expression. *J Biol Chem* **278:**1585–1593.
Verrecchia F, Vindevoghel L, Lechleider RJ, Uitto J, Roberts AB, and Mauviel A (2001c) SMAD3/AP-1 interactions control transcriptional responses to TGF-beta in a promoter-specific manner. *Oncogene* **20:**3332–3340.
Verrecchia F, Wagner EF, and Mauviel A (2002) Distinct involvement of the Jun-N-terminal kinase and NFkB pathways in the repression of the human COL1A2 gene by TNF-alpha. *EMBO (Eur Mol Biol Organ) Rep* **3:**1069–1074.
Verschueren K, Remacle JE, Collart C, Kraft H, Baker BS, Tylzanowski P, Nelles L, Wuytens G, Su MT, Bodmer R, et al. (1999) SIP1, a novel zinc finger/homeodomain repressor, interacts with SMAD proteins and binds to 5′-CACCT sequences in candidate target genes. *J Biol Chem* **274:**20489–20498.
Vindevoghel L, Kon A, Lechleider RJ, Uitto J, Roberts AB, and Mauviel A (1998a) Smad-dependent transcriptional activation of human type VII collagen gene (COL7A1) promoter by transforming growth factor-β. *J Biol Chem* **273:**13053–13057.
Vindevoghel L, Lechleider RJ, Kon A, de Caestecker MP, Uitto J, Roberts AB, and Mauviel A (1998b). SMAD3/4-dependent transcriptional activation of the human type VII collagen gene (COL7A1) promoter by transforming growth factor beta. *Proc Natl Acad Sci USA* **95:**14769–14774.
Wotton D, Lo RS, Lee S, and Massague J (1999) A SMAD transcriptional corepressor. *Cell* **97:**29–39.
Zhang W, Ou J, Inagaki Y, Greenwel P, and Ramirez F (2000) Synergistic cooperation between Sp1 and SMAD3/SMAD4 mediates transforming growth factor beta1 stimulation of $\alpha 2_I$-collagen (COL1A2) transcription. *J Biol Chem* **275:**39237–39245.

Address correspondence to: Dr. Franck Verrecchia, INSERM U532, Institut de Recherche sur la Peau, Pavillon Bazin, Hôpital Saint-Louis, 1 avenue Claude Vellefaux, 75475 Paris cedex 10, France. E-mail: verrecchia@chu-stlouis.fr