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Cooperation of H_2O_2-mediated ERK activation with Smad pathway in TGF-β1 induction of $p21^{WAF1/Cip1}$

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Abstract

Although it has been demonstrated that $p21^{WAF1/Cip1}$ could be induced by transforming growth factor-β1 (TGF-β1) in a Smad-dependent manner, the cross-talk of Smad signaling pathway with other signaling pathways still remains poorly understood. In this study, we investigated a possible role of hydrogen peroxide (H_2O_2)-ERK pathway in TGF-β1 induction of $p21^{WAF1/Cip1}$ in human keratinocytes HaCaT cells. Using pharmacological inhibitors specific for MAP kinase family members, we found that ERK, but not JNK or p38, is required for TGF-β1 induction of $p21^{WAF1/Cip1}$. ERK activation by TGF-β1 was significantly attenuated by treatment with *N*-acetyl-L-cysteine or catalase, indicating that reactive oxygen species (ROS) generated by TGF-β1, mainly H_2O_2, stimulates ERK signaling pathway to induce the $p21^{WAF1/Cip1}$ expression. In support of this, TGF-β1 stimulation caused an increase in intracellular ROS level, which was completely abolished by pretreatment with catalase. ERK activation does not appear to be associated with nuclear translocation of Smad-3, because ERK inhibition did not affect nuclear translocation of Smads by TGF-β1, and H_2O_2 treatment alone did not cause nuclear translocation of Smad-3. On the other hand, ERK inhibition ablated the phosphorylation of Sp1 by TGF-β1, which was accompanied with the disruption of interaction between Smad-3 and Sp1 as well as of the recruitment of Sp1 to the $p21^{WAF1/Cip1}$ promoter induced by TGF-β1, indicating that ERK signaling pathway might be necessary for their interaction. Taken together, these results suggest that activation of H_2O_2-mediated ERK signaling pathway is required for $p21^{WAF1/Cip1}$ expression by TGF-β1 and led us to propose a cooperative model whereby TGF-β1-induced receptor activation stimulates not only a Smad pathway but also a parallel H_2O_2-mediated ERK pathway that acts as a key determinant for association between Smads and Sp1 transcription factor.
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Keywords: $p21^{WAF1/Cip1}$; TGF-β; ERK; H_2O_2; Smad

1. Introduction

Transforming growth factor-β (TGF-β) is a secreted multifunctional signaling molecule, which play pivotal roles in a broad array of cellular processes, including cell proliferation and differentiation, apoptosis, deposition of extracellular matrix, and cell adhesion [1–3]. During development, TGF-β also regulates cell fate decisions and pattern formation in species from nematodes to vertebrates [4,5]. Many of the effects induced by TGF-β results from its ability to regulate transcription of specific sets of genes. Among the genes, induction of $p21^{WAF1/Cip1}$ causes growth inhibition by TGF-β [6,7]. Increased $p21^{WAF1/Cip1}$ leads to an increase in its association with cyclin D-CDK4 and cyclin E-CDK2 and a decrease in the activity of cyclin-CDK complex [8].

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TGF-β signals are transduced through transmembrane serine/threonine kinase receptors, the type II and type I receptors [2,9]. In response to TGF-β stimulation, Smad-2 and/or Smad-3 are phosphorylated by type I receptor which is activated by type II receptors, allowing them to heteromerize with Smad-4. The heteromeric complexes of receptor-activated Smads and Smad-4 are then translocated into the nucleus, where they exert ligand-induced changes in transcription of a variety of genes [10–12]. The heteromeric Smad complex regulates transcription through its ability to cooperate functionally with several promoter-specific transcription factors and/or to bind specific DNA sequences [9]. Like other TGF-β target genes, induction of $p21^{WAF1/Cip1}$ also requires nuclear translocation of Smads complexes. However, some reports show that nuclear translocation of Smad complexes alone is not sufficient for induction of $p21^{WAF1/Cip1}$. Overexpression of Smad-3 and/or Smad-4 does not cause the induction of $p21^{WAF1/Cip1}$ transcription [13], but are sufficient to activate transcription of plasminogen activator inhibitor, other target of TGF-β [12], indicating that other pathways may also be needed for the transcriptional induction of $p21^{WAF1/Cip1}$.

There are emerging evidences that reactive oxygen species (ROS) at low concentration may function as a signaling intermediator of cellular responses [14]. The generation of ROS by external stimuli is associated with various cellular processes, such as cell proliferation or apoptosis. ROS has been shown to stimulate signaling pathways implicated in growth factor and cytokine effects through activating their important components, such as transcription factors, NF-κB [15,16] and AP-1 [17], and especially also to stimulate ERK1/2 [14,18,19]. TGF-β also stimulates ROS production in various cell lines including bovine pulmonary artery endothelial cells [20], vascular endothelial cells [21], mouse osteoblastic cells [22], and human lung fibroblast cells [23,24]. In addition, ROS, mainly hydrogen peroxide (H_2O_2), has been demonstrated to be implicated in the TGF-β induction of its target gene in various cellular systems [22,24].

In light of the emerging evidence for the interaction between the Smad and the H_2O_2-mediated signaling pathway, it was of interest to investigate the possible cross-talk between these two pathways for the regulation of $p21^{WAF1/Cip1}$ induction. The current study investigated the hitherto unexplored mechanisms of TGF-β1 signaling pathway by studying the possible role of ROS-mediated signaling events in Smad-dependent induction of $p21^{WAF1/Cip1}$ in response to TGF-β1. Our data provide evidence that TGF-β1 treatment results in the production of ROS, mainly H_2O_2, leading to the stimulation of ERK activity, which is required for TGF-β1 induction of $p21^{WAF1/Cip1}$. ERK activation does not affect the signaling pathway for Smads nuclear translocation, but might be necessary for their interaction between Smad-3 and Sp1 induced by TGF-β1. Taken together, these results suggest that H_2O_2-mediated ERK signaling pathway might play as a key mediator for the interaction between Smads and Sp1 transcription factor to induce the $p21^{WAF1/Cip1}$ expression by TGF-β1.

2. Materials and methods

2.1. Cell culture and reagents

Human keratinocytes HaCaT cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), and 1% penicillin/streptomycin (Gibco, Grand Island, NY). TGF-β1 was obtained from Calbiochem (La Jolla, CA), and *Aspergillus niger* catalase was from Sigma (St. Louis, MO). Pharmacological inhibitors such as U0126, PD98059, SB203580, SP600125, and mithramycin were from BioMol (Plymouth Meeting, PA). Expression plasmid encoding human catalase was prepared as previously described [25], and transfected to HaCaT cells with SuperFect™ transfection system (Qiagen).

2.2. Immunoblotting

HaCaT cells were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin, 1 mM sodium orthovanadate, and 1 mM NaF). Proteins were separated on SDS-polyacrylamide gel electrophoresis (15%) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in TBS containing 0.1% tween 20 (TBS-T) and 5% (w/v) dry skim milk powder, and incubated overnight with specific antibodies for $p21^{WAF1/Cip1}$ (Santa Cruz Biotechnology), Sp1 (Abcam), ERK/phospho-ERK (Cell Signaling), phosphoserine (Zymed), and Smad-3/-4(Santa Cruz Biotechnology). The membranes were then washed with TBS-T and incubated for 1 h with a secondary antibody. Bound antibodies were visualized with the Enhanced Chemiluminescence detection kit (Amersham Life Sciences, IL).

2.3. Immunoprecipitation

Appropriate amounts of proteins from cells lysed in lysis buffer were incubated with anti-Smad-3 (Santa Cruz Biotechnology) or anti-Sp1 (Abcam) and protein A-Sepharose bead (Amersham Pharmacia Biotech) for immunoprecipitation experiments. Binding reactions were for 5 h at 4 °C with continual rotation. The beads were collected and washed three times (3 min/wash) with lysis buffer. Bound proteins were eluted by boiling in 1 × Laemmli sample buffer, separated by SDS-PAGE, and subjected to immunoblot analysis.

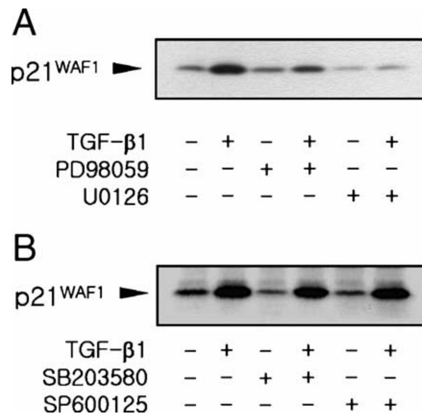

Fig. 1. Requirement of ERK signaling pathway for TGF-β1 induction of p21$^{WAF1/Cip1}$ HaCaT cells were pretreated with (A) PD98059 (20 μM) or U0126 (20 μM) and (B) SB203580 (20 μM) or SP600125 (20 μM) for 1 h, and further incubated in the absence or presence of TGF-β1 (100 pM) for 24 h. Whole lysates (30 μg proteins) of the HaCaT cells were examined by 15% SDS-PAGE and analyzed with immunoblotting using antibodies for p21$^{WAF1/Cip1}$ as described under Materials and methods.

2.4. Measurement of intracellular reactive oxygen species

Cells were grown on coverslips for 24 h and made quiescent in serum-free media for additional 24 h. The serum deprived cells were stabilized in serum-free media without phenol red for at least 30 min and stimulated with TGF-β1 for different times. Sometimes, cells were preincubated with antioxidants for 30 min before treatment with TGF-β1. 5 μM of 2′,7′-dichlorofluorescein (DCF) was added to monitor intracellular H_2O_2. Then, the cells were immediately observed with a laser scanning confocal microscope (Carl Zeiss LSM 410). The images of samples excited by a 488 nm argon laser were filtered by a long pass 515 nm filter.

2.5. Immunostaining

Cells were plated on coverslips and grown overnight. The next day, cells were treated with TGF-β for indicated times, fixed in methanol for 10 min at 4 °C, and permeabilized in 0.5% Triton X-100 for 5 min. Slips were then incubated in primary (anti-Smad-3, Santa Cruz Biotechnology) and secondary antibody for 1 h at 37 °C with frequent washes. Slips were then mounted and photographs taken on a fluorescent microscope (Olympus).

2.6. Chromatin Immunoprecipitation

Analysis was performed by means of a kit protocol (Upstate Biotechnology). Chromatin from sonicated 1×10^6 HaCaT cells was precleared with salmon-sperm DNA-saturated protein G sepharose and was precipitated using Sp1 antibody. Samples were analyzed by PCR using ExTaq polymerase (Takara, Japan) and primer to amplify the p21 promoter region including Sp1 binding sites [26].

3. Results

3.1. TGF-β1 induction of p21$^{WAF1/Cip1}$ requires ERK signaling pathway

It has been demonstrated that the MEK pathway is required for stimulation of p21$^{WAF1/Cip1}$ by TGF-β1 [27]. To obtain more direct evidence for the potential involvement of ERK signaling pathway in the induction of p21$^{WAF1/Cip1}$ in response to TGF-β1, we examined the effect of PD98059 or U0126, which are well known pharmacological inhibitors of MEK, on the induction of p21$^{WAF1/Cip1}$ by TGF-β1. As shown in Fig. 1A, pretreatment of human keratinocytes HaCaT cells with PD98059 or U0126 dramatically inhibited TGF-β1 induction of p21$^{WAF1/Cip1}$. To investigate whether the other ERK family members, p38 and JNK, are involved in TGF-β1 induction of p21$^{WAF1/Cip1}$, we next examined the effect of SB203580 and SP600125 which are specific inhibitor of p38 and JNK, respectively. Pretreatment of cells with these inhibitors did not affect the induction of p21$^{WAF1/Cip1}$ by TGF-β1 (Fig. 1B). These data indicate that induction of p21$^{WAF1/Cip1}$ by TGF-β1 requires the activation

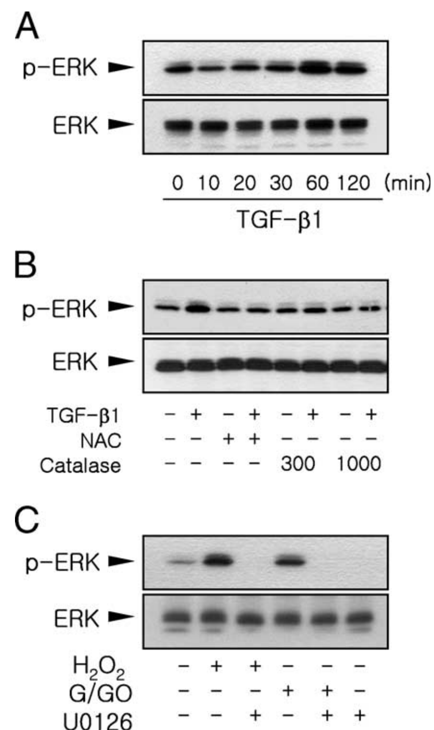

Fig. 2. ROS-mediated activation of ERK by TGF-β1. (A) After serum starvation for 24 h, HaCaT cells were stimulated with TGF-β1 (100 pM) for the indicated times. Whole lysates (30 μg protein) were examined by 15% SDS-PAGE and analyzed with immunoblotting using antibodies for phospho-ERK or ERK as described under Materials and methods. (B) After pretreatment with NAC (30 mM) or catalase for 1 h, HaCaT cells were stimulated with TGF-β1 (100 pM) for another 1 h. Phosphorylation of ERK was analyzed by immunoblotting as described above. (C) HaCaT cells were treated with H_2O_2 (200 μM) or G/GO (0.33U) for 10 min, and phosphorylation of ERK was analyzed. In some experiments, U0126 (20 μM) was pretreated before treatment of H_2O_2 or G/GO.

of ERK signaling pathway, but not of p38 or JNK signaling pathway.

3.2. ERK activation by TGF-β1 is dependent of reactive oxygen species

Next, we determined whether ERK signaling pathway could be stimulated by treatment of HaCaT cells with TGF-β1. The increase in phosphorylation of ERK following TGF-β1 treatment was apparent with maximum activity at 60 min, which was sustained longer than 120 min (Fig. 2A). This sustained activation of ERK with TGF-β1 differed from the transient activation of other growth factors such as EGF [28]. Recent accumulating evidences suggest that ROS is an important intracellular messenger to mediate the activation of various signaling molecules including ERK and S6K1 in various cells [14,25,29,30]. Therefore, we examined the possibility of ROS to act as an intracellular messenger for TGF-β1 activation of ERK. Indeed, treatment with either antioxidant *N*-acetyl-L-cysteine (NAC) or catalase was accompanied by the inhibition of ERK stimulation by TGF-β1 (Fig. 2B). To obtain more direct evidence for the potential involvement of ROS in ERK signaling in response to TGF-β1, we examined the effect of exogenous H_2O_2 on the activation of ERK. H_2O_2 treatment caused an increase in phosphorylation of ERK within 10 min. In addition, treatment with glucose/glucose oxidase, which could generate extracellularly ROS, also led to an activation of ERK. This activation was completely inhibited by pretreatment with U0126, indicating that stimulation of ERK in response to TGF-β1 was mediated by ROS.

We then asked whether ROS was actually generated by TGF-β1 treatment in HaCaT cells. Microfluorometric studies with laser scanning confocal microscopy revealed that exposure of HaCaT cells to TGF-β1 resulted in a significant increase of DCF fluorescence. The TGF-β1-induced increase of DCF fluorescence appeared from 20 min, with maximal production at 60 min, which was slightly decreased at 120 min (Fig. 3). This kinetics was faster than that of ERK activation (see Fig. 2A), indicating that H_2O_2

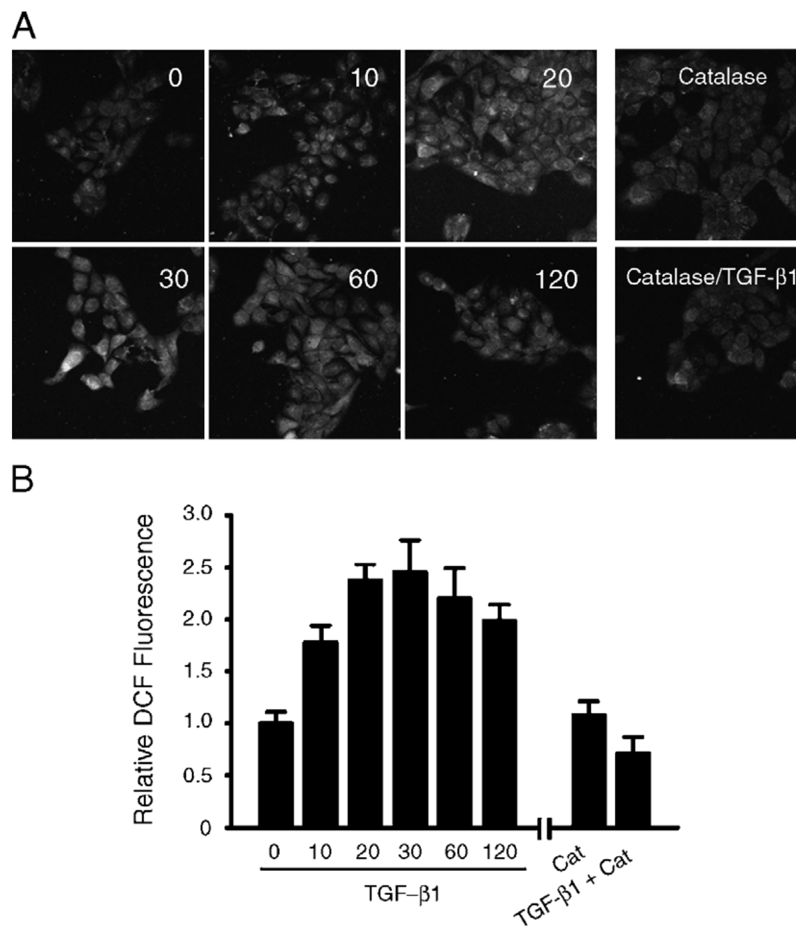

Fig. 3. ROS production by TGF-β1 in HaCaT cells. (A) After incubation of serum-starved HaCaT cells in the presence of TGF-β1 (100 pM) for the indicated times, DCF fluorescence was measured with a confocal laser-scanning microscope as described under Materials and methods Alternatively, cells were pretreated with *Aspergillus niger* catalase (300 U/ml) for 1 h, and further incubated following treatment with TGF-β1 for 1 h. (B) Thirty cells were randomly selected from the above each experiment, and the fluorescence intensity of each group of cells was then measured by Karl Zeiss vision system (KS400, version 3.0) and averaged. The fluorescence intensities of each group were expressed as fold increase over control value. Data shown represent the means ± S.E.M. of fluorescence intensities of each group.

signaling seems to be a prerequisite for the activation of ERK. Furthermore, DCF fluorescence induced by TGF-β1 treatment was completely abolished by catalase treatment (Fig. 3), suggesting that the predominant species of ROS produced was H_2O_2. Taken together, these results suggest that H_2O_2 production by TGF-β1 might contribute to the activation of ERK.

3.3. H_2O_2-mediated ERK activation is required for TGF-β1 induction of $p21^{WAF1/Cip1}$

To examine the possible link between H_2O_2 signaling pathway and $p21^{WAF1/Cip1}$ induction, we first examined the effect of NAC and catalase on the expression of $p21^{WAF1/Cip1}$ by TGF-β1. As shown in Fig. 4A, the expression of $p21^{WAF1/Cip1}$ in response to TGF-β1 was significantly attenuated by pretreatment with either NAC or catalase. To further confirm that TGF-β1 induction of $p21^{WAF1/Cip1}$ requires H_2O_2-mediated signaling, we examined the effect of ectopic expression of catalase in HaCaT cells on the expression of $p21^{WAF1/Cip1}$ by TGF-β1. Transfection of the cells with recombinant catalase completely abrogated the TGF-β1 induction of $p21^{WAF1/Cip1}$ (Fig. 4B), suggesting that H_2O_2-mediated ERK activation is necessary for TGF-β1 induction of $p21^{WAF1/Cip1}$ in HaCaT cells.

3.4. Nuclear translocation of Smads by TGF-β1 does not require H_2O_2-ERK signaling pathway

It has been previously reported that Smads were translocated into the nucleus compartment in response to TGF-β1 stimulation, which was essential for induction of TGF-β1-target genes [9,31]. To determine whether inhibition of ERK signaling pathway can alter the subcellular localization of Smads, we first used immunofluorescence microscopy with Smad-3 specific antibody. As expected, Smad-3 in unstimulated cells showed a diffuse, mainly cytoplasmic staining. When cells were stimulated with TGF-β1, most of cells exhibited a predominantly nuclear staining of Smad-3 with maximal translocation at 60 min treatment (Fig. 5A). However, inhibition of ERK signaling pathway with U0126 did not alter the nuclear translocation of Smad-3 in response to TGF-β1. In addition, H_2O_2 treatment did not cause Smad-3 nuclear translocation (Fig. 5A), although ERK signaling was fully activated (Fig. 2C), indicating that ERK may act in parallel with signaling pathway for nuclear translocation of Smads. To further support this result, nuclear fraction was prepared and analyzed using immunoblot with specific antibodies of Smad-3 and Smad-4, respectively. Smad-3 and Smad-4 were significantly translocated into the nucleus following treatment with TGF-β1, however, inhibition of ERK signaling pathway did not affect the nuclear translocation of Smads (Fig. 5B). This result is in

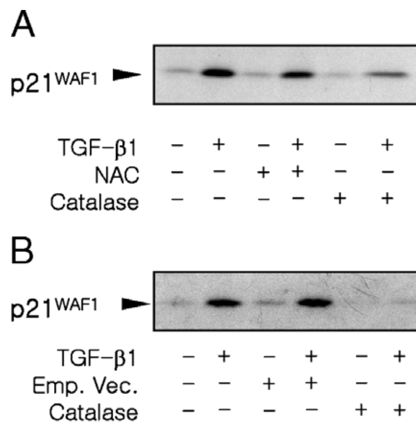

Fig. 4. Requirement of H_2O_2-mediated signaling pathway for TGF-β1 induction of $p21^{WAF1/Cip1}$. (A) After pretreatment with NAC (30 mM) or catalase (300 U/ml) for 1 h, HaCaT cells were stimulated with TGF-β1 (100 pM) for another 24 h. Whole lysates (30 μg protein) of the HaCaT cells were examined by 15% SDS-PAGE and analyzed with immunoblotting using antibodies for $p21^{WAF1/Cip1}$ as described under Materials and methods. (B) HaCaT cells were transiently transfected with the catalase plasmid for 24 h, and incubated for an additional 24 h following treatment with TGF-β1 (100 pM). The expression level of $p21^{WAF1/Cip1}$ was analyzed with immunoblotting as described above.

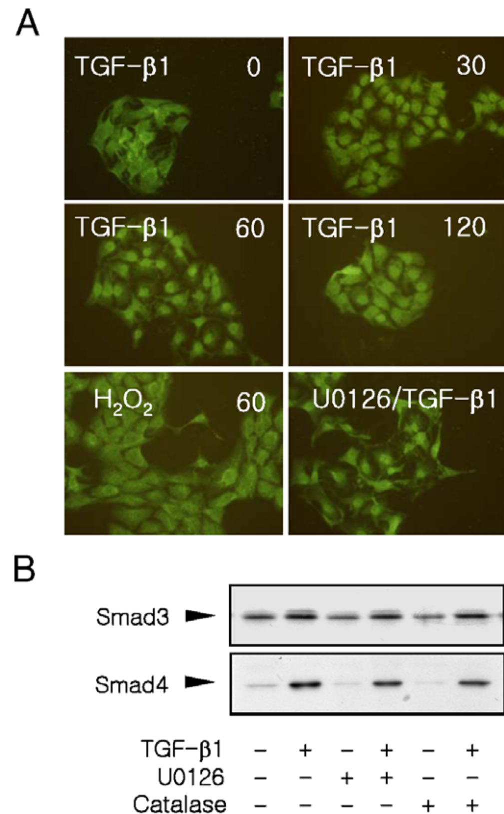

Fig. 5. Role of H_2O_2-ERK activation on nuclear translocation of Smads by TGF-β1. (A) After treatment with TGF-β1 for indicated times, cells were fixed, permeabilized, stained with anti-Smad-3, and then visualized with secondary antibody coupled to fluorescein isothiocyanate by fluorescent microscopy as described under Materials and methods. Alternatively, cells were pretreated with U0126 (20 μM) for 1 h, and further incubated with TGF-β1 for 1 h. In some experiments, HaCaT cells was stimulated with H_2O_2 (200 μM) alone for 60 min. (B) After pretreatment with U0126 (20 μM) or catalase (300 U/ml), nuclear fractions were prepared and analyzed using immunoblot with anti-Smad-3 and anti-Smad-4, respectively.

good agreement with that of immunostaining. Furthermore, elimination of H_2O_2 by catalase also did not alter the Smads nuclear translocation (Fig. 5B). This result strongly indicates that H_2O_2-mediated ERK signaling pathway could be a distinct, parallel pathway from Smad signaling pathway.

3.5. Interaction between Smads and Sp1 requires ERK signaling pathway

It has been demonstrated that $p21^{WAF1/Cip1}$ induction by TGF-β1 was mediated through binding of Sp1 transcription factor to Sp1 sites in the region of promoter [32], and interaction between Sp1 transcription factor and Smad proteins was critical for the induction of TGF-β1 target genes [13,33,34]. We reinvestigated previous reports that TGF-β1 induction of $p21^{WAF1/Cip1}$ was dependent of Sp1 transcription factors. When the transcription activity of Sp1 family was inhibited by pretreatment with mithramycin, the expression of $p21^{WAF1/Cip1}$ protein by TGF-β1 was significantly attenuated (Fig. 6A). Next, we determined whether physical interaction between Sp1 and Smads is required for the $p21^{WAF1/Cip1}$ induction by TGF-β1 in HaCaT cells. After treatment with TGF-β1, cell lysates were prepared and subjected to anti-Smad-3 immunoprecipitation followed by immunoblotting with anti-Sp1 antibody. As shown in Fig. 6B (lane 2), Sp1 was detected specifically in an immune complex with Smad-3, indicating that Sp1 could interact with Smad proteins in TGF-β1 induction of $p21^{WAF1/Cip1}$. However, inhibition of ERK signaling pathway with U0126 disrupted the formation of Sp1-Smad complex (Fig. 6B, lane 4), which was enough to suppress the expression of $p21^{WAF1/Cip1}$ by TGF-β1 as shown in Fig. 1A. Consistent with the results, the recruitment of Sp1 to the promoter of $p21^{WAF1/Cip1}$ including Sp1 binding sites was disrupted by inhibition of ERK signaling as determined using chromatin immunoprecipitation assay (Fig. 6D). In addition, Sp1 phosphorylation by TGF-β1 was also inhibited by treatment with U0126 (Fig. 6C). Collectively, our data suggest that H_2O_2-mediated ERK signaling pathway is required for the interaction between Smads and Sp1 to induce TGF-β1 induction of $p21^{WAF1/Cip1}$ through Sp1 sites.

4. Discussion

Our data presented herein show that TGF-β1 treatment stimulates not only a Smads pathway but also a parallel H_2O_2-ERK pathway, which are required for the induction of $p21^{WAF1/Cip1}$. This notion was supported by the observation that the elimination of ROS with either antioxidant or catalase was accompanied by the inhibition of ERK activation by TGF-β1, resulting in attenuation of $p21^{WAF1/Cip1}$ expression without any alteration of nuclear translocation of Smads.

Although the involvement of ROS in TGF-β1 induction of $p21^{WAF1/Cip1}$ is not clearly unraveled, possible evidences have been raised; treatment of human lung cancer cells and mouse fibroblast with exogenous H_2O_2 could induce the expression of $p21^{WAF1/Cip1}$ in a p53-independent manner

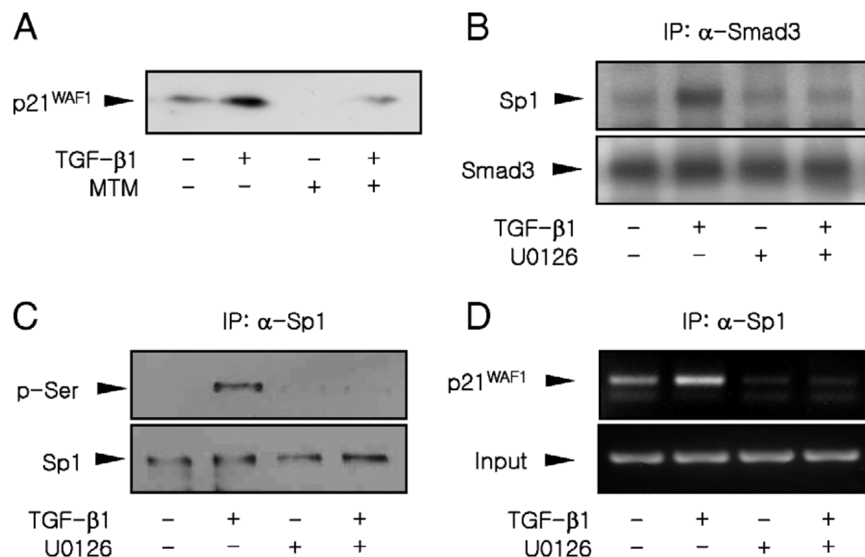

Fig. 6. Involvement of ERK signaling pathway in interaction between Smads and Sp1. (A) After pretreatment of HaCaT cells with mithramycin (200 nM), the expression level of $p21^{WAF1/Cip1}$ was analyzed with immunoblotting as described under Materials and methods. (B) After pretreatment of HaCaT cells with U0126 (20 μM), cell lysates (200 μg of proteins) were incubated with anti-Smad-3 and protein A-Sepharose bead for 5 h at 4 °C with continual rotation. Immunoprecipitated complexes were separated by SDS-PAGE, and subjected to immunoblot analysis with Sp1 specific antibody. (C) Cell lysates (1 mg of proteins) were incubated with anti-Sp1 and protein A-Sepharose bead for 5 h at 4 °C with continual rotation. Immunoprecipitated complexes were separated by SDS-PAGE, and subjected to immunoblot analysis with anti-phosphoserine or anti-Sp1 specific antibody. (D) HaCaT cells were pretreated with or without U0126 (20 μM) for 1 h, and further incubated with TGF-β1 for 1 h. Soluble chromatin was immunoprecipitated with anti-Sp1 from the cell lysate. PCR primer for the promoter region of the $p21^{WAF1/Cip1}$ gene was used to amplify the DNA isolated from the immunoprecipitated chromatin.

[35,36]. In addition, H_2O_2 production by TGF-β1 has been shown to mediate the expression of plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase 3 in mesangial cells and chondrocytes primary cultures, respectively [37,38]. Indeed, H_2O_2 was sufficiently produced in HaCaT cells in response to TGF-β1, and TGF-β1-induced $p21^{WAF1/Cip1}$ expression was antagonized by pretreatment with either NAC or catalase, suggesting the requirement of ROS-mediated signaling pathway for $p21^{WAF1/Cip1}$ induction. Consistent with these observations, the production of ROS has been shown to be stimulated by other growth factors such as platelet-derived growth factor [14] and basic fibroblast growth factor [17], which leads to transcriptional activation of their target genes. ERK signaling pathway was not only stimulated by H_2O_2 produced in response to growth factors [14,29], but also required for TGF-β1 induction of $p21^{WAF1/Cip1}$ [27]. These observations led to an assumption that H_2O_2-induced ERK activation might mediate the induction of $p21^{WAF1/Cip1}$ in response to TGF-β1. Production of H_2O_2 by TGF-β1 preceded the activation of ERK activation, and elimination of H_2O_2 led to the suppression of ERK activation as well as $p21^{WAF1/Cip1}$ induction, supporting the assumption.

Since Smads have been implicated in TGF-β1 signal transduction and transcriptional activation in various cellular systems [10–12,39], TGF-β stimulation of the Smad signaling pathway in HaCaT cells is of particular significance. Indeed, we provided the evidence for requirement of Smads nuclear translocation to induce $p21^{WAF1/Cip1}$ in TGF-β1-treated HaCaT cells. Although Smads signaling pathway is critical for TGF-β1-induced gene transcription, other signaling molecules including ERK appear to be necessary for the gene transcription. Recent reports demonstrated the cross-talk between ERK and Smads signaling pathway in TGF-β1-induced gene transcription, which was evidenced by that inhibition of ERK blocks nuclear translocation of Smad by TGF-β1 in HepG2 cells [40]. In contrast to this report, our findings that exogenous H_2O_2 failed to induce Smad-3 nuclear translocation, and neither ERK inhibitor nor catalase, a H_2O_2 scavenger, was able to inhibit nuclear translocation of Smad-3/-4 in response to TGF-β1 strongly suggest that these two signaling pathways are independent and functionally coupled in the nucleus but not in the cytosol of HaCaT cells. The exact reasons for these discrepancies remain unknown, but it is clear that multiple interactions between ERK and Smad pathways can occur depending on the cell type and possibly the extent of ERK activation.

It has been demonstrated that the ability of Smads to induce specific transcription programs in response to TGF-β1 resulted from a functional cooperativity with other transcription factors in multiprotein complexes in nucleus [31,41]. In addition, the requirement of functional and physical interaction between Smad proteins and Sp1 transcription factors for the induction of $p21^{WAF1/Cip1}$ in response to TGF-β1 has been reported [13,33]. Consistent with this report, the requirement of Sp1 transcription factor for TGF-β1 induction of $p21^{WAF1/Cip1}$ was confirmed by examining the effect of mithramycin, an inhibitor of Sp1-DNA binding, on $p21^{WAF1/Cip1}$ expression. Furthermore, we demonstrated the importance of physical interaction between Smads and Sp1 for $p21^{WAF1/Cip1}$ gene expression using coimmunoprecipitation assay. In addition, it was interesting to observe the requirement of ERK signaling pathway for formation of multiprotein complexes between Smads and Sp1 transcription factor. Interaction of Smad with Sp1 seems to be mediated by Sp1 phosphorylation via ERK signaling pathway, because Sp1 phosphorylation by TGF-β1 was antagonized by inhibition of ERK signaling. Furthermore, the recruitment of Sp1 to the $p21^{WAF1/Cip1}$ promoter by TGF-β1 was dependent of ERK signaling. Consistent with the results, Sp1 transcription factor has been shown to be phosphorylated by active ERK, which led to an increase in its DNA binding activity and in transcriptional activity [42,43]. However, we do not also exclude the possibility that nuclear-translocated Smads might be regulated by ERK in the nucleus.

In conclusion, our data herein demonstrate that the H_2O_2-ERK signaling pathway appears to be a critical mediator for TGF-β1 induction of $p21^{WAF1/Cip1}$. Furthermore, H_2O_2-ERK signaling pathway might be required for physical and/or functional interaction between Smads and Sp1 transcription factors but not for TGF-β1 stimulation of Smads nuclear translocation. Better understanding of the molecular mechanisms of transcriptional regulation of $p21^{WAF1/Cip1}$ through cross-talk between signaling pathways induced by TGF-β1 could provide novel insights into its role in normal physiological and pathological states.

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References

[1] R. Derynck, X.H. Feng, Biochim. Biophys. Acta 1333 (1997) F105.
[2] J. Massague, Annu. Rev. Biochem. 67 (1998) 753.
[3] A.B. Roberts, Miner. Electrolyte Metab. 24 (1998) 111.
[4] B.L. Hogan, Curr. Opin. Genet. Dev. 6 (1996) 432.
[5] M. Whitman, Genes Dev. 12 (1998) 2445.
[6] M.B. Datto, Y. Li, J.F. Panus, D.J. Howe, Y. Xiong, X.F. Wang, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 5545.
[7] C.Y. Li, L. Suardet, J.B. Little, J. Biol. Chem. 270 (1995) 4971.
[8] C.J. Sherr, J.M. Roberts, Genes Dev. 9 (1995) 1149.
[9] R. Derynck, Y. Zhang, X.H. Feng, Cell 95 (1998) 737.
[10] F. Liu, A. Hata, J.C. Baker, J. Doody, J. Carcamo, R.M. Harland, J. Massague, Nature 381 (1996) 620.
[11] M. Macias-Silva, S. Abdollah, P.A. Hoodless, R. Pirone, L. Attisano, J.L. Wrana, Cell 87 (1996) 1215.
[12] Y. Zhang, X. Feng, R. We, R. Derynck, Nature 383 (1996) 168.

[13] K. Pardali, A. Kurisaki, A. Moren, P. ten Dijke, D. Kardassis, A. Moustakas, J. Biol. Chem. 275 (2000) 29244.
[14] M. Sundaresan, Z.X. Yu, V.J. Ferrans, K. Irani, T. Finkel, Science 270 (1995) 296.
[15] V. Lakshminarayanan, E.A. Drab-Weiss, K.A. Roebuck, J. Biol. Chem. 273 (1998) 32670.
[16] R. Schreck, P. Rieber, P.A. Baeuerle, EMBO J. 10 (1991) 2247.
[17] Y.Y.C. Lo, T.F. Cruz, J. Biol. Chem. 270 (1995) 11727.
[18] K.Z. Guyton, Y. Liu, M. Gorospe, Q. Xu, N.J. Holbrook, J. Biol. Chem. 271 (1996) 4138.
[19] Y.Y.C. Lo, J.M.S. Wong, T.F. Cruz, J. Biol. Chem. 271 (1996) 15703.
[20] V.J. Thannickal, P.M. Hassoun, A.C. White, B.L. Fanburg, Am. J. Physiol. 265 (1993) L622.
[21] Y.H. Hong, H.B. Peng, V. La Fata, J.K. Liao, J. Immunol. 159 (1997) 2418.
[22] M. Ohba, M. Shibanuma, T. Kuroki, K. Nose, J. Cell Biol. 126 (1994) 1079.
[23] V.J. Thannickal, B.L. Fanburg, J. Biol. Chem. 270 (1995) 30334.
[24] E. Junn, K.N. Lee, H.R. Ju, S.H. Han, J.Y. Im, H.S. Kang, T.H. Lee, Y.S. Bae, K.S. Ha, Z.W. Lee, S.G. Rhee, I. Choi, J. Immunol. 165 (2000) 2190.
[25] G.U. Bae, Y.K. Kim, H.K. Kwon, J.W. Park, E.K. Lee, S.J. Paek, W.S. Choi, I.D. Jung, H.Y. Lee, E.J. Cho, H.W. Lee, J.W. Han, Exp. Cell Res. 300 (2004) 476.
[26] Y.K. Kim, J.W. Han, Y.N. Woo, J.K. Chun, J.Y. Yoo, E.J. Cho, S. Hong, H.Y. Lee, Y.W. Lee, H.W. Lee, Oncogene 22 (2003) 6023.
[27] P.P. Hu, X. Shen, D. Huang, Y. Liu, C. Counter, X.F. Wang, J. Biol. Chem. 274 (1999) 35381.
[28] M. Susa, A.R. Olivier, D. Fabbro, G. Thomas, Cell 57 (1989) 817.
[29] G.U. Bae, D.W. Seo, H.K. Kwon, H.Y. Lee, S. Hong, Z.W. Lee, K.S. Ha, H.W. Lee, J.W. Han, J. Biol. Chem. 274 (1999) 32596.
[30] D.K. Jung, G.U. Bae, Y.K. Kim, S.H. Han, W.S. Choi, H. Kang, D.W. Seo, H.Y. Lee, E.J. Cho, H.W. Lee, J.W. Han, Exp. Cell Res. 290 (2003) 144.
[31] J. Massague, D. Wotton, EMBO J. 19 (2000) 1745.
[32] M.B. Datto, Y. Yu, X.F. Wang, J. Biol. Chem. 270 (1995) 28623.
[33] A. Moustakas, D. Kardassis, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6733.
[34] X.H. Feng, X. Lin, R. Derynck, EMBO J. 19 (2000) 5178.
[35] Y.W. Chung, D.W. Jeong, J.Y. Won, E.J. Choi, Y.H. Choi, I.Y. Kim, Biochem. Biophys. Res. Commun. 293 (2002) 1248.
[36] K. Barnouin, M.L. Dubuisson, E.S. Child, S. Fernandez de Mattos, J. Glassford, R.H. Medema, D.J. Mann, E.W. Lam, J. Biol. Chem. 277 (2002) 13761.
[37] Z. Jiang, J.Y. Seo, H. Ha, E.A. Lee, Y.S. Kim, D.C. Han, S.T. Uh, C.S. Park, H.B. Lee, Biochem. Biophys. Res. Commun. 309 (2003) 961.
[38] W.Q. Li, H.Y. Qureshi, A. Liacini, F. Dehnade, M. Zafarullah, Free Radic. Biol. Med. 37 (2004) 196.
[39] R. Derynck, Y.E. Zhang, Nature 425 (2003) 577.
[40] F. Blanchette, N. Rivard, P. Rudd, F. Grondin, L. Attisano, C.M. Dubois, J. Biol. Chem. 276 (2001) 33986.
[41] P.K. Datta, M.C. Blake, H.L. Moses, J. Biol. Chem. 275 (2000) 40014.
[42] J.L. Merchant, M. Du, A. Todisco, Biochem. Biophys. Res. Commun. 254 (1999) 454.
[43] S. Chupreta, M. Du, A. Todisco, J.L. Merchant, Am. J. Physiol. Cell Physiol. 278 (2000) C697.