

Signaling Mechanisms of Transforming Growth Factor- β (TGF- β) in Cancer: TGF- β Induces Apoptosis in Lung Cells by a Smad-Dependent Mechanism

Mi Jung Lim, Tiffany Lin and Sonia B. Jakowlew
*National Cancer Institute, Cancer Training Branch,
Bethesda, Maryland,
USA*

1. Introduction

1.1 TGF- β ligands, receptors and smads

Transforming Growth Factor-beta (TGF- β), a cytokine that is expressed in a variety of normal tissues, including the lung (Bartram & Spear, 2004; Jakowlew et al., 1995, 1998; Kang et al., 2000; Montuenga et al., 1998), exerts diverse effects on a wide variety of cellular processes, including proliferation, differentiation, and apoptosis (Elliot & Blobe, 2005; Massagué, 1998). More than sixty different TGF- β family members have been identified in various organisms, with at least 29 of these proteins being encoded in humans. Among the many proteins in the TGF- β superfamily are four TGF- β ligands, five activins, eight bone morphogenetic proteins (BMP), and 15 growth and differentiation factors (GDF). Three TGF- β isoforms have been identified in humans, including TGF- β 1, TGF- β 2, and TGF- β 3, with each being a homodimeric polypeptide with a molecular weight of 25-kDa. All three TGF- β isoforms are initially synthesized as 55-kDa pro-proteins that consist of an amino-terminal pro-region and a carboxy-terminal mature region (Gentry et al., 1988). The pro-region facilitates necessary dimerization of the pro-proteins for future activity. TGF- β is secreted in a latent, inactive form in which the 12.5-kDa carboxyl-terminal 112 amino acid-long mature form is non-covalently associated with the 80-kDa Latency-Associated Peptide (LAP) amino-terminal remainder (Barcellos-Hoff, 1996; Barcellos-Hoff & Ewan, 2000). The LAP forms a complex with the 12.5-kDa TGF- β to keep it inactive (Arndjelovic et al., 2003; Stander et al., 1999). This complex is referred to as the small latent TGF- β complex. The small latent TGF- β complex may associate with members of the latent TGF- β -binding protein (LTBP) family to form the large latent TGF- β complex (Öklü & Hesketh, 2000). The liberation of TGF- β from the latent complexes is referred to as activation (Annes et al., 2003). The precise steps that are involved in liberation of the bioactive dimer are not completely understood, but may involve cleavage of the LTBP or LAP or both (Hyytiäinen et al., 2004).

Active TGF- β exerts its effects with specific high affinity receptors. In mammals, five TGF- β superfamily type I receptors and seven type II receptors have been identified (Derynck et al.,

2001; Massagué, 2000). The TGF- β type I and type II receptors are structurally related transmembrane glycoproteins that consist of an extracellular N-terminal ligand-binding domain with more than ten cysteine residues that regulate the dimeric structure, a transmembrane region, and a C-terminal serine/threonine kinase domain. The type I receptors, but not type II receptors, have a highly conserved region that is rich in glycine and serine residues, referred to as the GS domain, in the juxtamembrane domain next to the N-terminus of the kinase domain. The GS domain is a target for the type II receptor kinase, and upon its phosphorylation on specific serine and threonine residues, the type I receptor becomes activated (Heldin et al., 1997; Massagué, 2000). Being downstream of the type II receptor, the type I receptor plays an important role in determining the specificity of intracellular signals. The type I and II receptors exist as homodimers at the cell surface in the absence of ligands, but have an inherent heteromeric affinity for each other. Only select combinations of type I and II receptors act as ligand-binding signaling complexes. The molecular basis of the selectivity of the type I-type II receptor interactions remains poorly understood, but the structural complement at the interface may help define the selectivity of the receptor combinations. Most of the TGF- β ligands bind with high affinity to the type I receptor, also known as activin receptor-like kinase (ALK), or to the type II receptor, while others bind efficiently only to heteromeric receptor combinations.

The intracellular signal transduction triggered by the kinase activity of TGF- β involves the phosphorylation of Smad family proteins and in turn, complex changes in the transcriptional regulation of various response genes. The Smad family proteins include Smad 1, 2, 3, 4, 5, 7, and 8. The Smads are divided into three subclasses depending on their structure and function: the receptor-regulated Smads (R-Smads), common-mediator Smad (Co-Smad), and inhibitory Smads (I-Smads). In general, the R-Smads, Smads 2 and 3, function downstream of the TGF- β ligands, while Smads 1, 5, and 8 are downstream of members of the BMP and GDF subfamilies of ligands. Smads 1, 2, 3, 5, and 8 are direct substrates for the TGF- β type I receptor kinase, whereas Co-Smad, Smad 4, participates in Smad complex formation. Smads 6 and 7, the I-Smads, interfere with TGF- β -induced Smad-dependent signal transduction (Park, 2005; Whitman, 1997). Activation of cell surface receptors by ligands leads to phosphorylation of the R-Smads at two serine residues in a SSXS motif at their extreme C-termini. This phosphorylation allows the R-Smads to form both homomeric and heteromeric complexes with Smad4 that accumulate in the nucleus. There, they are directly involved in transcriptional regulation of target genes in cooperation with other transcription factors.

Signaling by TGF- β is mediated by a ligand-induced heteromeric complex of two types of transmembrane serine/threonine kinase receptors designated as TGF- β type I receptor (TGF- β RI) and type II receptor (TGF- β RII). Initial ligand binding to constitutively active TGF- β RII is followed by recruitment of TGF- β RI into the heteromeric complex. Subsequent phosphorylation of TGF- β RI at its GS-domain and activation is mediated by TGF- β RII and leads to activation of TGF- β RI. Upon this activating phosphorylation, TGF- β RI phosphorylates the receptor-activated Smad proteins (R-Smads), Smad2 and Smad3, which form a heteromeric complex with the co-Smad, Smad4, and enter the nucleus. In the nucleus, the Smad complex associates with other transcription factors for transcriptional activation of specific target genes (Massagué and Wotton, 2000; ten Dijke et al., 2000; Wrana and Attisano, 2000).

1.2 Tumor suppressor activity of TGF- β

TGF- β was originally called one of the most potent polypeptide growth inhibitors isolated from natural sources (Moses et al., 1985; Tucker et al., 1984). When it was demonstrated that TGF- β could act as an autocrine negative growth regulator in the several different epithelial cell lines, it was hypothesized that TGF- β may act as an inhibitor of tumor progression, a tumor suppressor (Arteaga et al., 1990; Glick et al., 1989). The identification and characterization of the intermediates in the TGF- β signaling pathway, comprised of the genes and proteins for the TGF- β receptors and Smads, has increased our understanding of the role of TGF- β as a tumor suppressor. The involvement of the TGF- β signaling pathway in tumor suppression is shown by mutations in the genes that encode the TGF- β receptors and Smad proteins in human tumors.

The gene for TGF- β RII is frequently mutated in colon carcinoma cells from patients with hereditary non-polyposis colorectal cancer that also show microsatellite instability, as well as in gastric cancers and gliomas (Chung et al., 1996; Izumoto et al., 1997; Markowitz et al., 1995). A specific region of adenine nucleotides in the coding region of TGF- β RII is prone to mutation in these patients from germline defects in their capacity for DNA mismatch repair. The nucleotide deletions or additions result in a shortened version of TGF- β RII that cannot participate in signaling transduction (Lu et al., 1996). However, the TGF- β RII gene is not mutated in other types of carcinoma with microsatellite instability, including breast, liver, pancreatic, and endometrial carcinoma (Abe et al., 1996; Kawate et al., 1999; Vincent et al., 1996), while, a somatic frameshift mutation in the polyadenine tract of the TGF- β RII gene does occur in some endometrial cancer patients (Parekh et al., 2002). Missense and inactivating mutations in TGF- β RII have also been detected in colon cancers that do not exhibit microsatellite instability (Grady et al., 1999). Expression of TGF- β RII can be decreased in some cases of carcinoma, including head and neck squamous carcinoma, breast carcinoma, and laryngeal carcinoma (Eisma et al., 1996; Franchi et al., 2001; Gobbi et al., 1999). Re-expression of TGF- β RII in carcinoma cells that have either lost expression of TGF- β RII or show reduced TGF- β RII expression can inhibit the ability to become malignant.

Although less common than in TGF- β RII, mutations in TGF- β RI also occur in patients with a variety of cancers, including ovarian cancers, metastatic breast cancers, T-cell lymphomas, and head and neck cancer metastases (Chen et al., 1998, 2001; Goggins, 1998; Schiemann et al., 1999). Patients with ovarian cancer show a high frequency of mutations of TGF- β RI (Chen et al., 2001), while expression of TGF- β RI is transcriptionally repressed by DNA methylation in cells from patients with gastric cancer (Kang et al., 1999). Over-expression of TGF- β RI in colon carcinoma cells with low levels of TGF- β RI also inhibits tumor progression as with TGF- β RII (Wang et al., 1996). Mutations in TGF- β RI do not appear to be associated with TGF- β RII mutations; such mutations suggest that these TGF- β receptors may function as tumor suppressors.

Decreased TGF- β receptor expression or availability of TGF- β receptors at the cell surface may allow tumor cells to escape the growth inhibitory function of TGF- β (Kim et al., 2000). Expression of the TGF- β receptors in tumor cells may also be reduced by altered levels or activities of transcription factors that are required for expression of TGF- β RII, such as the Ets transcription factor. Hypermethylation of CpG islands in the promoters of TGF- β RI and TGF- β RII or mutations in the TGF- β RII promoter that interfere with transcription factor binding may also result in transcriptional silencing (Amoroso, et al., 1998). Decreased TGF- β RII function results in resistance to the growth inhibitory activity of TGF- β , but other TGF- β responses may not be affected in a similar fashion because they may require different levels

of signaling (Chen et al., 1993; Fafeur et al., 1993). The tumor suppressor role of TGF- β RII has been demonstrated by expressing wildtype TGF- β RII in cancer cells that lack a functional TGF- β RII allele (Sun et al., 1994; Wang et al., 1995) or by over-expressing TGF- β RII *in vitro* (Turco et al., 1999). Enhanced expression of TGF- β RII seems to confer growth inhibition, to suppress anchorage independent growth, and to significantly reduce tumor formation in experimental mice compared with parental cells. Over-expression of TGF- β RI or TGF- β RII in transgenic mice also shows enhanced tumor suppressor activity (Cui et al., 1996; Minn et al., 2005), while expression of dominant-negative forms of TGF- β RII increases tumor formation (Böttinger et al., 1997; Go et al., 1999).

Some of the genes for Smad proteins that function as mediators of TGF- β signal transduction also have mutations and deletions that occur in human carcinomas. Mutations in the genes encoding Smad2 and Smad3 are relatively rare and seem to occur only in a limited number of lung and colon carcinomas for Smad2 (Riggins et al., 1996; Uchida et al., 1996), and gastric cancer for Smad3 (Han et al., 2004). In contrast, mutational inactivation of Smad4 (DPC4) is prominent in pancreatic cancer (Hahn et al., 1996). Mutations of Smad4 can be detected in human colorectal cancer, especially in those patients with late stage, metastatic disease (Maitra et al., 2000). This suggests that Smad4 may play a central role in TGF- β -mediated tumor suppression. Inactivation of the genes encoding Smad2 and Smad4 occurs by several means, including deletion of entire chromosome segments, small deletions, and frameshift, nonsense, or missense mutations (Massagué & Wotton, 2000; Massagué et al., 2000). Mutations in Smad4 are detected principally in pancreatic carcinomas, and in colon carcinomas, and in other types of carcinomas, although with less frequency. Inactivation of both alleles of Smad4 and haploinsufficiency of the Smad4 locus may contribute to the progression of pancreatic and gastric cancers (Luttges et al., 2000; Xu et al., 2000). The existence of Smad4 mutations in several juvenile polyposis families further supports the suggestion that Smad4 is a tumor suppressor (Howe, 1998). Inactivating mutations in Smad4 are also observed in conjunction with mutations in TGF- β RI and TGF- β RII (Grady et al., 1999). This suggests that Smad4 also has tumor suppressor activities that are not related to TGF- β signaling. Alterations of Smad signaling, and of phosphorylation of Smad2 in particular, are associated with poor prognosis in human breast carcinomas, colon carcinomas, and head and neck squamous cell carcinomas (Xie et al., 2002, 2003a, 2000b). Loss of Smad3 expression in gastric cancer tissues and cell lines increases susceptibility to tumorigenesis (Han et al., 2004). Introduction of Smad3 into human gastric cancer cells that do not express Smad3 restores responsiveness to TGF- β . In addition, loss of Smad4 expression and/or activity may increase the Ras signaling pathway to result in tumor progression (Iglesias et al., 2004). A protein-based strategy has been used to rapidly identify the most common alterations in the TGF- β signaling pathway by combining measurements of the levels and the state of activation of Smad signaling intermediates with DNA-based diagnostic assays (Yan et al., 2000). A mechanism for TGF- β resistance has been identified in TGF- β refractory squamous cell carcinoma cell lines using this protein-based strategy.

1.3 TGF- β and apoptosis

Another mechanism by which the tumor suppressor activity of TGF- β is mediated is through the process of programmed cell death or apoptosis. Unlike the molecular mechanisms by which TGF- β participates in cell proliferation and differentiation that have been well described, the mechanisms by which TGF- β exerts its apoptotic effects are only poorly understood in comparison. TGF- β -dependent apoptosis is important in the

elimination of damaged or abnormal cells from many normal tissues (Schuster and Krieglstein, 2002). For example, TGF- β is implicated to play a role in controlling liver size, and intravenous injection of TGF- β induces apoptosis in normal and regressing liver (Schulte-Hermann et al., 1993). Hepatic over-expression of TGF- β in transgenic mice causes apoptosis, as does treatment of primary hepatocytes with TGF- β . The apoptotic fate of cells after they are treated with TGF- β 1 is often determined by cellular context and experimental conditions. For example, TGF- β acts as a death stimulus inducing apoptotic death in fetal hepatocytes and podocytes (Herrera et al., 2001; Schiffer et al., 2001), whereas it elicits pro-survival activity to protect macrophages against apoptosis (Chin et al., 1999; Schlapbach et al., 2000). A regulated balance of cell division and apoptosis is required for normal morphogenesis, and alterations in these processes can lead to neoplastic transformation. Cell cycle progression and the onset of apoptosis have been connected in DNA-damaged cells through the analysis of the activation of the apoptotic cascade in p21^{Cip1}-deficient HCT116 colorectal cancer cells (Le et al., 2005; Pardali et al., 2005). DNA damage induces a similar level of p53 activation and proapoptotic Bcl-2 family member PUMA induction in p21^{Cip1}-deficient cells compared to wildtype isogenic counterparts. However, only p21^{Cip1}-deficient cells show extensive cytochrome C release, mitochondrial membrane depolarization, and caspase activation. When ectopically expressed in p21^{Cip1}-deficient cells, p21^{Cip1}, p27^{Kip1}, and p16^{Ink4a} are all similarly effective at causing cell cycle arrest and inhibiting DNA damage-induced apoptotic events. Application of TGF- β stimulates apoptosis in various epithelial cells. Preliminary findings show that TGF- β induces apoptosis through the regulation of the expression of various pro- and anti-apoptotic molecules, including p53, Bad, Bax, Bik, Bcl-2, and Bcl-XL (Motyl et al., 1998; Saltzman et al., 1998; Sanchez-Capelo, 2005; Teramoto et al., 1998). TGF- β -induced apoptosis can also be mediated by caspases (Brown et al., 1998; Chen and Chang, 1997; Choi et al., 1998; Saltzman et al., 1998). The mitochondrial septin-like protein, Apoptosis-Related Protein in the TGF- β Signaling Pathway (ARTS), enhances cell death induced by TGF- β through activation of caspase 3 (Larisch et al., 2000). In addition, TGF- β -induced apoptosis is associated with the generation of reactive oxygen species (Albright et al., 2003). Antioxidants can block the TGF- β -induced apoptotic process. Resistance to apoptosis is one of the characteristics of cancer cells during progressive tumorigenesis. Apoptosis of human prostate cancer cells that is induced by TGF- β or over-expression of Smad7 is caused by a specific activation of the p38 MAP kinase pathway that may occur in a TGF- β -activated kinase 1 (TAK1) and mitogen-activated protein kinase kinase 3 (MKK3)-dependent manner (Edlund et al., 2003). Members of the Mixed Lineage Kinase 3 (MLK3) family also mediate TGF- β -induced apoptosis in hepatoma cells (Kim et al., 2004). There is also strong evidence that the stress- and cytokine-inducible Growth Arrest and DNA Damage (GADD) inducible gene 45 protein (Mita et al., 2002; Takekawa & Saito, 1998; Takekawa et al., 2002; Yoo et al., 2003) and GADD153 protein (Park et al., 1992), also known as CCAAT/enhancer-binding Homologous Protein (CHOP) (Ron & Habener, 1992), CCAAT/Enhancer-Binding Protein-zeta (C/EBP- ζ) (Hanson, 1998), and DNA Damage Inducible Transcript-3 (DDIT3) (Fornace et al., 1989), function in the p38 Mitogen-Activated Protein Kinase (MAPK) pathway and induce apoptosis (Corazzari et al., 2003). It has also been shown that Smad-dependent expression of GADD45b is responsible for the delayed activation of p38 MAP kinase by TGF- β 1 in pancreatic carcinoma cells (Takekawa et al., 2002). Activation of GADD45b by the TGF- β receptor/Smad signaling pathway also mediates the induction of proteoglycan biglycan expression by TGF- β , also with the involvement of mitogen activated protein kinase kinase 6 (MKK6) and p38 MAP kinase

(Mita et al., 2005). The p38 MAP kinase and p160/Rho/ROCK pathways have a role in TGF- β -mediated Smad-dependent growth inhibition of breast cancer cells (Kamaraju & Roberts, 2005). Smad3 contributes in a non-redundant manner to the induction of apoptosis in the mammary gland, but is dispensible for TGF- β effects on proliferation and differentiation in this tissue. TGF- β -induced p160/Rho/ROCK activation is also involved in the inhibition of Cdc25A, with resulting cell cycle arrest. TGF- β also regulates radiation-induced apoptosis and this is reduced in TGF- β 1 null mice along with decreased p53 phosphorylation. TGF- β regulates biglycan gene expression through p38 MAP kinase signaling downstream of the Smads that also requires the small GTPase Rac1 (Groth et al., 2005). However, TGF- β -Receptor activated p38 MAP kinase also mediates Smad-independent responses in breast cancer cells (Yu et al., 2002). Non-Smad signal transducers that are under the control of TGF- β provide quantitative regulation of the signaling pathway, and serve as nodes for cross-talk with other signaling pathways, such as Notch, tyrosine kinase, G-protein-coupled receptor kinases, and cytokine receptors (Moustakas & Heldin, 2005). One of the characteristics of cancer cells during progressive tumorigenesis is resistance to apoptosis (Hanahan & Weinberg, 2000). Increasing the sensitivity of tumor cells to anticancer therapy is tightly correlated with the induction of apoptosis by anticancer drugs. Thus, it would be promising for disease treatment, including lung cancer, to activate TGF- β -mediated apoptosis by modulating the function of TGF- β in specific normal and tumor cell types.

1.4 TGF- β and lung

There is accumulating evidence that TGF- β may have a role in lung cancer and in lung disease. For example, elevated levels of TGF- β 1 have been shown in plasma and lung tumors of patients with advanced lung cancer, and the prognosis of lung cancer patients who showed positive TGF- β 1 was poorer than that of patients who were negative for this growth factor (Kong et al., 1996; Takanami et al., 1997). Lung cancer patients who responded to radiation therapy showed a decrease in circulating TGF- β levels compared to patients with no response or stable disease (Vujaskovic and Groen, 2000). One potential function of TGF- β in lungs and airways is regulation of epithelial cell survival through apoptosis. TGF- β 1 treatment of lung bronchial BEAS-2B cells increased apoptosis in cells exhibiting overexpression of Smad2 or Smad 3 (Yanagisawa et al., 1998). TGF- β 1 treatment also enhanced Fas-induced apoptosis of alveolar and airway epithelial cells, and Fas-mediated apoptosis of alveolar epithelial cells was reported to be associated with increased expression of TGF- β 1 (Hagimoto et al., 2002; Hagimoto et al., 1997).

Besides TGF- β 1, interleukin (IL)-6 is a multifunctional cytokine that is produced by a variety of cells during infection, trauma, and immunological challenge (Kishimoto et al., 1995). IL-6 has been shown to mediate many inflammatory processes in the lung (Taga, 1997), and its dysregulated release has been implicated in the pathogenesis of a variety of respiratory conditions, including interstitial lung diseases (Berger, 2002; Bhatia & Moochhala, 2004; Shahr et al., 1996). IL-6 has been reported to have different effects on apoptosis of fibroblasts from normal and fibrotic lungs, with fibrotic lung cells showing enhanced resistance to apoptosis (Moodley et al., 2003) and to induce an increase in expression and activity of cathepsin, a cysteine protease that plays a major role in lysosomal bulk proteolysis, protein processing, matrix degradation, and tissue remodeling in the lung, in A549 lung cells (Gerber et al., 2001). Earlier reports have examined cross-talk between TGF- β and IL-6 in epithelial cells, with TGF- β playing a role in the negative regulation of IL-6 signaling in intestinal epithelial cells (Walia et al., 2003), as well as activating IL-6 expression in

prostate cancer cells (Park et al., 2003). In addition, bronchial epithelial 16 cells that are undergoing apoptosis have been shown to produce significantly more TGF- β , but less IL-6, than non-apoptotic cells (Hodge et al., 2002). This suggests that increased production of TGF- β and decreased expression of IL-6 by lung epithelial cells may contribute to the inhibition of proliferation, squamous metaplasia, and reduction of inflammation in lung injury.

Lung cancer is the most lethal type of lung injury/cancer for both men and women. In the United States, in 2007, the most recent year for which statistics are currently available, lung cancer accounted for more deaths than breast, prostate and colon cancer combined, according to the U.S. Cancer Statistics Working Group. In that year, 109,643 men and 93,893 women were diagnosed with lung cancer, and 88,329 men and 70,354 women died from lung cancer. The National Cancer Institute estimates there were 222,520 new cases of lung cancer and 157,300 deaths from lung cancer in 2010. Currently, 85% of lung cancer patients die within 5 years of diagnosis. This reflects the urgent need for improved therapies. Targeting signal transduction pathways that affect therapeutic resistance is one approach to improve patient outcomes. In normal cells, signaling is tightly regulated and begins with the transduction of signals through growth factor receptors or integrins to intracellular kinase enzymes, culminating in the regulation of cellular processes. Precise regulation of processes like cell division and apoptosis is required for normal morphogenesis, and alterations in these processes can lead to cancer. Increasing the sensitivity of tumor cells to anticancer therapy is tightly correlated with induction of apoptosis by anticancer drugs. Thus, it would be promising for disease treatment, including lung cancer, to activate TGF- β -mediated apoptosis by modulating the function of TGF- β in specific normal and tumor cells.

We reported earlier that apoptosis is significantly decreased in the bronchio-alveolar epithelium of mice that are heterozygous for TGF- β 1 (Tang et al., 1998). This is consistent with a role for endogenous TGF- β 1 in regulating apoptosis in lung. Here, we examined the ability of immortalized normal lung alveolar type II epithelial C10 cells to respond to TGF- β 1 and the functionality of the TGF- β 1 signal transduction pathway in these cells. Our findings show that TGF- β 1-mediated signaling induces apoptosis in C10 cells by a Smad-dependent mechanism that requires activation of p38 MAPK and inhibition of the AKT pathway induced by IL-6. Furthermore, the GADD family members GADD45b and GADD153 are mediators of p38 MAPK activation in the process of TGF- β 1-mediated apoptosis.

2. Materials and methods

2.1 Cell culture and reagents

Previously established non-tumorigenic C10 cells derived from normal mouse lung epithelium were obtained from Dr. A. Malkinson (University of Colorado, Denver, CO). Cells were cultured in CMRL-1066 medium (Invitrogen, San Diego, CA) containing 10% heat-inactivated fetal bovine serum (FBS). Gadd153-deficient and wildtype cells were grown in DMEM with 10% non-heat inactivated FBS (Zinszner et al., 1998). Cells were treated with 5 ng/ml recombinant human TGF- β 1 obtained from R&D Systems (Minneapolis, MN) in a vehicle of 4 mM HCl containing 1 ng/ml BSA or vehicle alone. Antibodies against phospho-specific and total p38 MAPK, ERK, JNK and AKT were from Cell Signaling Technologies (Beverly, MA). Antibodies against SMAD7 and GADD153 were from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-hemagglutinin antibodies were from Covance (Berkeley, CA). The MAPK inhibitor SB203580 was from Calbiochem (San Diego, CA).

2.2 Plasmid constructs

The hamster Gadd153 promoter fragments have been described previously (Luethy et al., 1990). The 5'-deletion constructs of the hamster Gadd153 promoter containing fragments -778 to +21, -225 to +21 and -36 to +21 were used to generate a Luciferase assay system in the pGL3-basic vector (Promega, Madison, WI). The 5'-deletion constructs (-165 to +21 and -105 to +21) of the hamster Gadd153 promoter were generated by PCR using the 5'-primers (GGATATCGTCAGTGCCAGCGTGCCG and GGATATCGTCAGTGCCAGCGTGCCG, respectively) and the 3'-primer (ggaagcttgtgtgagactcaggctactg) and subcloned into the pGL3-basic vector. The mouse Gadd153- expressing plasmid was made by RT-PCR amplification using the 5'-primer (CGAAGCTTCCAGAAGGAAGTGCATC) and the 3'-primer (CGGGATCCGGAGAGACAGACAGG). All constructs were verified by DNA sequencing.

2.3 Transient transfection and Luciferase assays

C10 cells were transfected with 1-2 µg/well of DNAs and 1 ng/well of Renilla Luciferase reporter plasmid pRLTK (Promega) to normalize transfection efficiencies using Lipofectamine 2000 (Invitrogen). After 18-24 hours, cells were treated with TGF-β1 or vehicle. After 18 hours, cells were lysed and Luciferase activity was measured using a dual-luciferase reporter assay system (Promega). All assays were performed in duplicate.

2.4 Generation of stable cell lines

C10 cells were transfected with HA-tagged Gadd45b, antisense Gadd45b, Gadd153 or Smad7 expression plasmids. Twenty-four hours after transfection, and every 4 days thereafter, the medium was replaced with fresh selection medium containing neomycin G418 (Invitrogen) at 800-µg/ml for 2 weeks. Neomycin-resistant clones were then individually transferred and expanded. After two additional passages in selection medium, independent clones were cultured in standard medium. As a mock control, the pcDNA3 empty vector was used to transfect C10 cells and selected in the presence of neomycin.

2.5 Western blot analysis

C10 cells were stimulated in the presence or absence of TGF-β1. Freshly collected cells were homogenized in lysis buffer containing 0.05 mM Tris-HCl pH 7.4, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM EDTA with protease inhibitors. Equal amounts of cell lysates were heated at 70°C for 10 minutes in sample loading buffer, separated by electrophoresis and transferred to membrane filters. Membranes were blocked with 5% nonfat dry milk in TBST buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween 20 for 1 hour at room temperature, washed in TBST and incubated with various primary antibodies overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, washed in TBST buffer and proteins were visualized by enhanced chemiluminescence according to the manufacturer's directions (Pierce, Rockford IL).

2.6 Detection of apoptosis

For DNA fragmentation assays, the Apoptotic DNA Ladder kit (Roche Applied Sciences, Indianapolis, IN) was used according to the manufacturer's directions. Apoptosis was also quantitated by a Cell Death Detection ELISA assay (Roche Applied Sciences). All data points for the Cell Death Detection ELISA assay were determined in triplicate.

2.7 Semi-quantitative and real-time reverse transcription-polymerase chain reaction amplification

Total RNA was isolated from C10 cells using Trizol reagent according to the manufacturer's directions (Invitrogen). Reverse transcription (RT) was performed, followed by amplification by polymerase chain reaction (PCR) with oligonucleotide primers including Gadd45b 5'-primer (gggggatttgcaatctct) and 3'-primer (cggtgaggcgatcctga), Gadd153 5'-primer (ccagtcagagtctatggc) and 3'-primer (catgctgggagcaggctgac) and glucose 6-phosphate dehydrogenase (G6PD) 5'-primer (actgcagttccgagacgtgg) and 3'-primer (cagaagaggcagagtatagtggtg). As an internal control, mRNA for glucose-6-phosphate dehydrogenase (G6pd) was also amplified. Relative quantification of the mRNA levels of the target genes was determined using the DDC_T method (Schmittgen et al., 2008). Results were expressed as N-fold difference in treated relative to untreated sample. All assays were performed twice in duplicate in independent PCR amplification reactions.

2.8 Interleukin-6 immunoassay

The levels of IL-6 in the supernatant of cultured cells were quantitated using a mouse Quantikine system (R&D Systems). Each measurement was performed in triplicate and an average value was recorded as pg/ml.

2.9 Statistics

Results were expressed as means \pm standard error (S.E.) and the differences between means of treated and control groups were analyzed using the Student's t test for paired data. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1 TGF- β 1 mediates apoptosis in mouse lung C10 cells

Mouse lung C10 cells are a stable cell line originally derived from a Balb/c mouse lung explant with characteristics of alveolar type II pneumocytes (Smith et al., 1984). These normal cells are not transformed, are non-tumorigenic, and contain only wildtype K-ras alleles (Malkinson et al., 1997). Here, we examined the responsiveness of TGF- β 1 in C10 cells. TGF- β 1 addition to C10 cells transiently transfected with Smad2 (ARE)- or Smad3 ((SBE)4)-dependent constructs augmented the transcriptional activity of these constructs by 11-fold and 1.6-fold, respectively (Figure 1A), indicating that C10 cells respond to TGF- β 1 and Smad signaling is functional. Dramatic morphological changes characteristic of apoptosis, including large vacuoles, cell shrinkage and cytoplasmic blebbing were observed at 48 hours after TGF- β 1 addition (Fig. 1B). Treatment with TGF- β 1 also induced a 150- to 200-bp internucleosomal DNA cleavage that produced a DNA fragmentation laddering pattern by 48 hours, and that was also detected by ELISA assay (Figure 1C and 1D).

3.2 TGF- β 1 activates p38 MAPK

The MAPK signaling pathway, including p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), has been implicated in many physiological and pathological processes, including apoptosis (Javelaude & Mauviel, 2005; Zavadil & Böttinger, 2005). To study the possible role of the MAPK pathway in TGF- β 1-mediated apoptosis in lung cells, C10 cells were treated with exogenous TGF- β 1, and activation of p38 MAPK, ERK1/ERK2, and JNK was then also examined using antibodies specific for total

and phosphorylated forms of each protein kinase. As shown in Figure 2A, there was a dramatic activation of p38 MAPK that occurred by 48 hours and the timing of activation of p38 MAPK by TGF- β 1 coincided with induction of apoptosis by TGF- β 1 (Figure 1). Activation of ERK1/ERK2 showed complex patterns of reduction and induction that were only modest. No activation of JNK by TGF- β 1 was detected (data not shown).

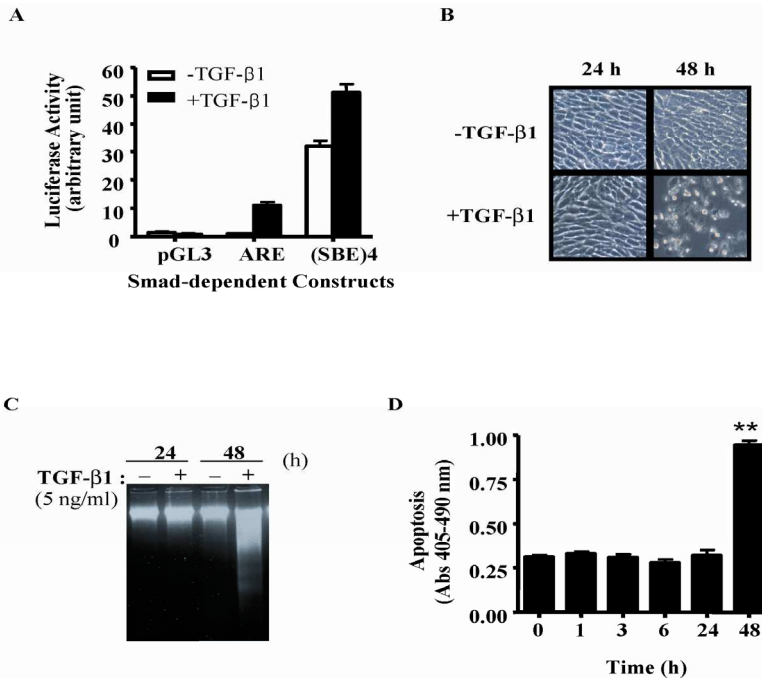


Fig. 1. TGF- β 1 induces apoptosis in mouse lung C10 cells.

A, Effect of TGF- β 1 on basal ARE-Luc and (SBE)4-Luc transcription in C10 cells. C10 cells were transiently transfected with Smad2(ARE)-Luc, Smad3((SBE)4)-Luc or 3TP-Luc Luciferase reporters and cultured in vehicle (open bars) or TGF- β 1 (filled bars) for 18-h. Luciferase activity was normalized to Renilla Luciferase values. The pGL3-basic empty vector control is shown. The results shown are the means standard error (S.E.) of two independent experiments performed in triplicate. B, Morphology of C10 cells by phase contrast microscopy at 24- and 48 hours after addition of TGF- β 1. Magnification: 200X. The apoptotic response was determined by examining the DNA fragmentation pattern by C, gel electrophoresis and D, Cell Death Detection ELISA assay at various times after treatment with TGF- β 1. **, $p < 0.001$ versus control.

Because of the dramatic activation of p38 MAPK and induction of apoptosis by TGF- β 1 in C10 cells, we chose to examine the role of p38 MAPK in this delayed apoptosis with MAPK SB203580, a potent inhibitor of p38 MAPK. Treatment of C10 cells with SB203580 strongly inhibited TGF- β 1-induced apoptosis at 48 hours as determined by DNA fragmentation (Figure 2B). Inhibition of TGF- β 1-induced apoptosis by SB203580 by 66% at 48 hours was also detected by ELISA assay (Figure 2C). Treatment of C10 cells with SB203580 also abrogated the morphological changes characteristic of apoptosis that were observed after 48

hours (Figure 2D). Our findings suggest that p38 MAPK activation by TGF- β 1 is involved in TGF- β 1-mediated apoptosis in C10 cells.

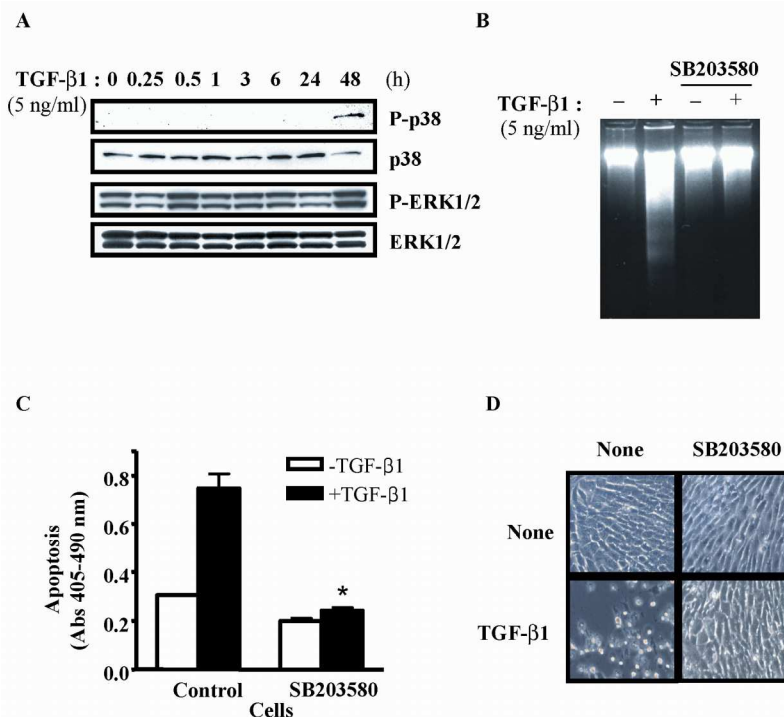


Fig. 2. Activation of p38 MAPK by TGF- β 1 results in apoptosis in C10 cells. A, Time course of phosphorylation of endogenous p38 MAP and ERK kinases after stimulation by TGF- β 1. Total cell lysates were prepared from untreated or TGF- β 1-treated cells and used for immunoblotting. Phosphorylated (P) and non-phosphorylated forms of p38 MAPK and ERK are indicated in the upper and lower panels, respectively. B-D, Cells were treated with TGF- β 1 or vehicle alone in the presence or absence of 10 μ M SB203580. The apoptotic response of C10 cells was determined by examining the DNA fragmentation pattern by B, gel electrophoresis and C, ELISA assay. The results shown are the means S.E. of two independent experiments performed in triplicate. *, $p < 0.001$ versus control. D, Morphology of C10 cells by phase contrast microscopy at 48 hours after treatment with TGF- β 1 and SB203580. Magnification: 200X.

3.3 TGF- β 1 increases Gadd45b mRNA

Since induction of apoptosis by TGF- β 1 and activation of p38 MAPK occurred at a late time after addition of TGF- β 1 in C10 cells, we considered the possibility that additional protein mediators may be needed to activate p38 MAPK in response to TGF- β 1. Previous studies have suggested the stress- and cytokine-inducible GADD45 family proteins (GADD45a, GADD45b, and GADD45g) function as specific activators of MEKK4, a MAPK kinase upstream in the p38 MAPK pathway (Mita et al., 2002), and induce apoptosis (Takekawa & Saito, 1998). To test the possible involvement of GADD family proteins in TGF- β 1-mediated

apoptosis in C10 cells, we first examined expression of Gadd mRNA transcripts following treatment with TGF- β 1 in these cells. Semi-quantitative and real-time RT-PCR amplification of RNA showed that Gadd45b mRNA was rapidly and significantly increased 8- and 11-fold by 30 minutes and 1 hour, respectively, after treatment with TGF- β 1, before decreasing (Figure 3A and B). Expression of transcripts for Gadd45a or Gadd45g was detected in C10 cells by RT-PCR only after extended amplification at much lower levels (data not shown). These results suggest that GADD45b may play a role in TGF- β 1-mediated apoptosis in C10 cells.

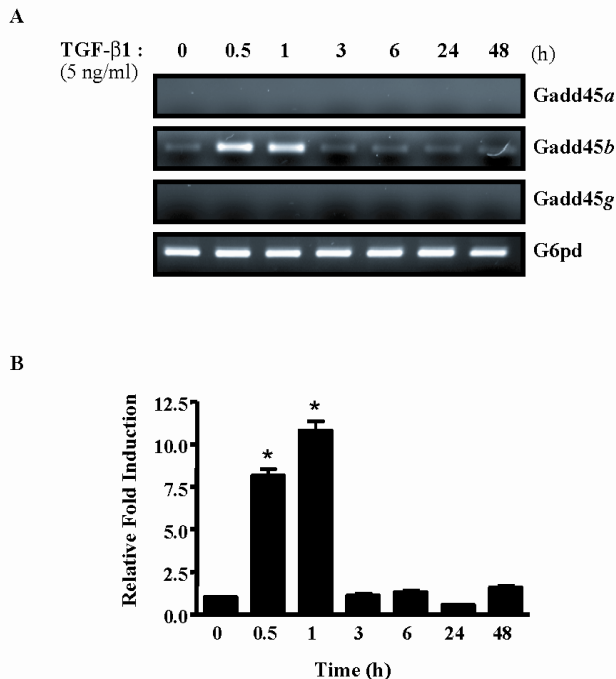


Fig. 3. TGF- β 1 increases expression of Gadd45b mRNA in C10 cells.

Time course of expression of Gadd45 mRNA transcripts after stimulation by TGF- β 1 using A, semi-quantitative and B, quantitative real-time RT-PCR amplification. Total RNA was prepared, reverse-transcribed and equal amounts of first-strand cDNA were amplified by PCR with Gadd45a-, Gadd45b- and Gadd45g -specific primers. G6pd, glucose-6-phosphate dehydrogenase. B, Relative expression of the Gadd45b gene in C10 cells treated with TGF- β 1. The results shown are the means S.E. of two independent experiments performed in duplicate. *, $p < 0.001$ versus control.

3.4 GADD45b enhances the timing and sensitivity of TGF- β 1-mediated apoptosis

Having demonstrated that p38 MAPK is activated and expression of Gadd45b mRNA is increased coordinately with the induction of apoptosis by TGF- β 1 in C10 cells, we next investigated whether overexpression of GADD45b could affect activation of p38 MAPK and lead to apoptosis. To test this, C10 cells were stably transfected with a hemagglutinin (HA)-tagged Gadd45b expression plasmid or empty vector used as control. In GADD45b-expressing cells (G-45b), p38 MAPK was activated in the absence of TGF- β 1 treatment, while TGF- β -induced activation of p38 MAPK occurred in control cells (Figure 4A).

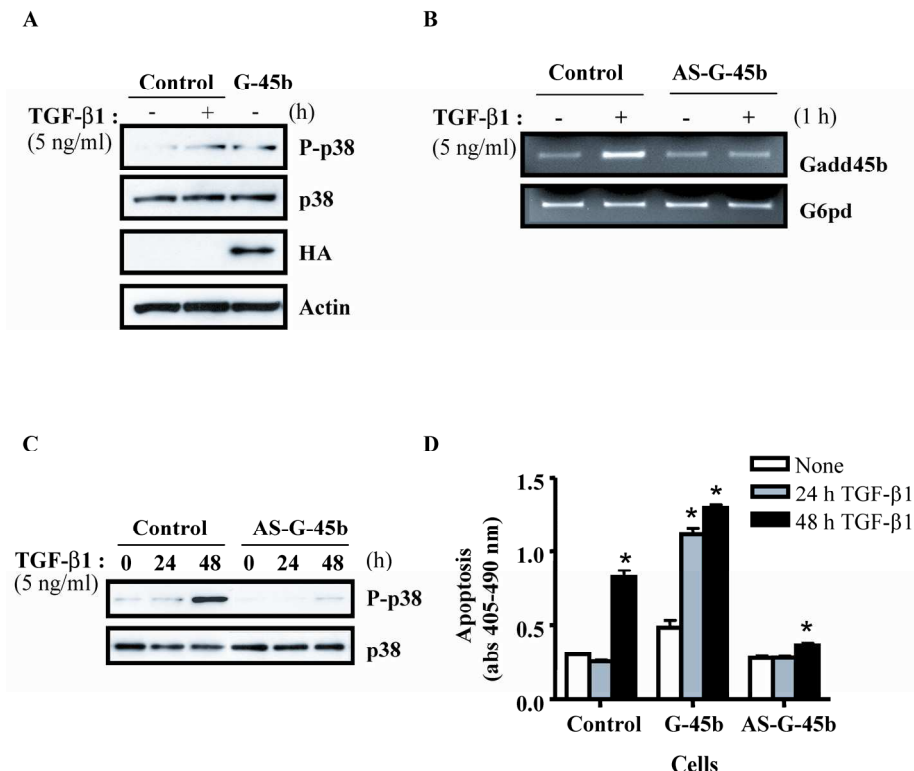


Fig. 4. Overexpression of GADD45b activates p38 MAPK and increases the sensitivity of C10 cells to TGF- β 1-mediated apoptosis.

A, C10 cells were stably transfected with either an empty vector (Control) or a hemagglutinin (HA)-tagged Gadd45b expression vector (G-45b). Total cell lysates prepared from the cells untreated or treated with TGF- β 1 for 48 hours were analyzed by immunoblotting. Phosphorylated (P) and non-phosphorylated forms of p38 MAPK are shown in the upper and middle panels, respectively. Expression of HA-tagged GADD45b and actin is shown in the middle and lower panels, respectively. B, Sense Gadd45b transcripts in C10 cells stably transfected with either an empty vector (Control) or an antisense Gadd45b expression vector (AS-G-45b) were subjected to RT-PCR analysis. Following a 1 hour incubation of the cells in the presence or absence of TGF- β 1, total RNA was prepared and subjected to RT-PCR amplification using primers specific to the sense Gadd45b transcript. G6pd, glucose-6-phosphate dehydrogenase. C, Phosphorylation of endogenous p38 MAPK in total cell lysates prepared from C10 cells stably transfected with either an empty vector (Control) or an antisense Gadd45b expression vector (AS-G-45b) and untreated or treated with TGF- β 1 for 24- and 48 hours, was analyzed by immunoblotting. Phosphorylated (P) and non-phosphorylated forms of p38 MAPK are shown in the upper and lower panels, respectively. D, C10 cells stably transfected with either an empty vector (Control), a Gadd45b expression vector (G-45b) or an antisense Gadd45b expression vector (AS-G-45b) were treated with vehicle (open bars) or TGF- β 1 for 24- (gray bars) and 48 hours (dark bars). DNA fragmentation was detected by ELISA assay. *, $p < 0.01$ versus control.

To confirm the effect of GADD45b on activation of p38 MAPK, C10 cells were stably transfected with antisense Gadd45b cDNA (AS-G-45b) to block expression of the endogenous Gadd45b gene. Antisense Gadd45b suppressed endogenous Gadd45b mRNA expression induced by TGF- β 1 (Fig 4B), and p38 MAPK activity was substantially reduced 48 hours after treatment with TGF- β 1 in AS-G-45b cells compared to control cells (Figure 4C). These results suggest that endogenous GADD45b has a role in p38 MAPK activation. Next, to test whether GADD45b plays a role as a mediator of apoptosis induced by TGF- β 1, we examined the ability of these cells to undergo apoptosis with or without TGF- β 1. Apoptosis increased by ~50% in G-45b cells compared to control cells in the absence of TGF- β 1, and overexpression of GADD45b increased the appearance of apoptosis in C10 cells by 24 hours (Figure 4D). However, apoptosis induced by TGF- β 1 decreased in AS-G-45b cells compared to control cells 48 hours after TGF- β 1 addition (Figure 4D). This result suggests that expression of endogenous GADD45b is necessary for TGF- β -mediated p38 MAPK activation and apoptosis

3.5 TGF- β 1 increases GADD153 mRNA

Because TGF- β 1-mediated apoptosis in C10 cells involves increased expression of Gadd45b mRNA only after 0.5- and 1 hour, it is possible that additional proteins may be involved in TGF- β 1-mediated apoptosis in these cells. Besides GADD45b, GADD153/CAATT enhancer binding protein homologous protein (CHOP), another member of the GADD family, has been implicated in processes that initiate apoptosis (Corazzari et al., 2003; Maytin et al., 2001; Murphy et al., 2001). We investigated whether GADD153 has a role in TGF- β -mediated apoptosis in C10 cells. Semi-quantitative and real-time RT-PCR amplification showed that Gadd153 mRNA had a gradual, but sustained, increase after TGF- β 1 addition (Figure 5A), and by 3 hours, there was a 1.5-fold increase in expression of Gadd153 mRNA that increased significantly to 11- and 9-fold by 24- and 48 hours, respectively (Figure 5B).

We next used actinomycin D and cyclohexamide to investigate whether TGF- β 1-induced Gadd153 mRNA expression is transcriptionally regulated and requires prior de novo protein synthesis or not. The addition of actinomycin D, an inhibitor of transcription, inhibited TGF- β 1-induced Gadd153 mRNA expression (Figure 5C), indicating that up-regulation of Gadd153 expression is transcriptionally dependent. To address whether the induction of Gadd153 mRNA expression by TGF- β 1 requires de novo protein synthesis, the effect of the protein synthesis inhibitor cyclohexamide was examined. Addition of cyclohexamide by itself caused an increase in Gadd153 mRNA levels (Figure 5C). The level of Gadd153 mRNA expression was not significantly changed by addition of cyclohexamide and TGF- β 1 together compared to cyclohexamide alone. This suggests that although Gadd153 mRNA is regulated by TGF- β 1 in C10 cells, the induction of GADD153 by TGF- β 1 depends on prior de novo protein synthesis events. The increase in Gadd153 mRNA level in the presence of cyclohexamide could be explained by an increase in mRNA stability or loss of transcriptional repressors by cyclohexamide.

3.6 GADD153 expression increases with overexpression of GADD45b

Since expression of Gadd45b mRNA is induced early by TGF- β 1 in C10 cells, while induction of Gadd153 mRNA occurs more gradually and maximizes at a later time compared to Gadd45b mRNA, we examined whether GADD45b could affect the expression of GADD153.

To test this, we used C10 cells that we had previously stably transfected with a (HA)-tagged sense or antisense Gadd45b expression plasmid. The basal level of GADD153 protein in GADD45b-expressing cells (G-45b) increased in the absence of TGF- β 1, and treatment of these cells with TGF- β 1 showed further increased expression of GADD153 protein after 24 hours (Figure 6A). However, antisense Gadd45b (AS-G-45b) significantly suppressed endogenous GADD153 protein expression in the absence of TGF- β 1 in AS-G-45b cells.

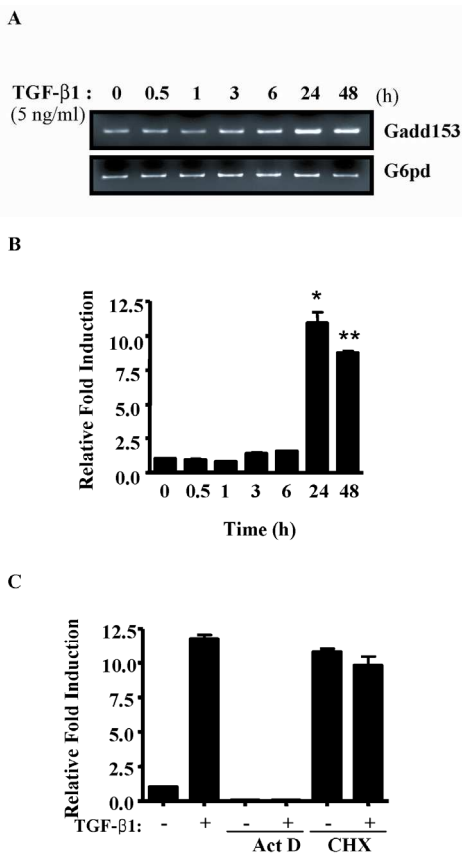


Fig. 5. TGF- β 1 increases expression of Gadd153 mRNA in C10 cells. Time course of expression of Gadd153 mRNA transcripts after stimulation by TGF- β 1 using A, semi-quantitative and B, real-time RT-PCR amplification. Total RNA was prepared, reverse-transcribed and equal amounts of the first-strand cDNA were subjected to PCR amplification with specific primers for Gadd153 or glucose-6-phosphate dehydrogenase (G6pd). The results shown are the means S.E. of two independent experiments performed in duplicate. *, $p < 0.005$, **, $p < 0.001$ versus control. C, C10 cells were treated with actinomycin (Act D; 1 μ g/ml) or cycloheximide (CHX; 5 μ g/ml) 1 hour before TGF- β 1 addition, and incubated for 24 hours with or without TGF- β 1. Total RNA was prepared and subjected to real-time RT-PCR using a primer specific for the Gadd153 transcript. The results shown are the means S.E. of three independent experiments performed in duplicate.

Expression of GADD153 protein was induced by TGF- β 1 in C10 cells stably transfected with antisense Gadd45b cDNA, but at levels that were markedly reduced compared to control C10 cells. These results suggest that GADD45b is capable of inducing Gadd153 expression in response to TGF- β 1 in C10 cells through a direct or indirect route. The ability of TGF- β 1 to stimulate GADD153 expression in AS-G-45b cells could be explained by a residual level of Gadd45b that may be sufficient to stimulate a level of GADD153 expression, or alternatively, both Gadd45b-dependent and Gadd45b-independent mechanisms may exist for stimulating GADD153 expression in C10 cells.

To confirm that endogenous GADD45b has an effect on TGF- β 1-induced expression of GADD153, we used a hamster Gadd153 promoter Luciferase reporter construct. Cotransfection of Gadd45b plasmid showed that increasing amounts of Gadd45b using 0.1-, 0.5- and 1- μ g of Gadd45b cDNA, augmented transcriptional activity of Gadd153-Luc, with maximal induction occurring using 0.5- μ g (Figure 6B). As a control, cotransfection of Gadd45b plasmid had no effect on the transcriptional activity of the TGF- β 1-responsive p3TP-Lux reporter. These results suggest that GADD45b is involved in transactivation of the Gadd153 promoter.

Having shown that GADD45b increases the activity of the Gadd153 promoter, we next set out to determine the minimum region of the Gadd153 promoter that was required for induction of Gadd45b. To accomplish this, we utilized a hamster Gadd153 promoter construct (pGadd153-778+21-Luc) and a series of deletion constructs (pGadd153-225+21-Luc, pGadd153-165+21-Luc, pGadd153-105+21-Luc and pGadd153-36+21-Luc). Addition of 0.5- μ g of Gadd45b cDNA augmented transcriptional activity of pGadd153-778+21-Luc 3-fold (Figure 6C). Deletion of the Gadd153 promoter up to position -225 still conferred induction by GADD45b. However, deletion of additional nucleotides from -165 to -36 of the Gadd153 promoter resulted in reduced ability of GADD45b to induce the Gadd153 promoter. In addition, cotransfection of Gadd45b with p3TP-Lux showed a minimal effect similar to that of pGadd153-36+21-Luc, indicating specificity of the effect of GADD45b on GADD153. This suggests the existence of a sequence that is responsive to the effects of GADD45b in region -778 to -225 of the Gadd153 promoter.

3.7 Overexpression of GADD153 activates p38 MAPK and induces apoptosis in the absence of TGF- β 1

To address the role of GADD153 in TGF- β 1-mediated apoptosis in lung cells, we next investigated whether overexpression of GADD153 could activate p38 MAPK and lead to apoptosis in the absence of TGF- β 1 in C10 cells. To test this, C10 cells were stably transfected with a Gadd153 expression vector or empty vector. Figure 7A shows increased expression of GADD153 protein in C10 cells stably transfected with Gadd153 expression plasmid (G-153-1 and G-153-2), but not in control cells. While TGF- β 1 treatment induced p38 MAPK activation in control cells, p38 MAPK in GADD153-expressing cells (G-153-1, G-153-2) was activated even in the absence of TGF- β 1 (Figure 7B). In GADD153-expressing cells, apoptosis in the absence of TGF- β 1 exceeded that in control cells that had been treated with TGF- β 1, and sensitivity to TGF- β 1-induced apoptosis was increased (Figure 7C and D). To further explore the involvement of p38 MAPK activation in GADD153-expressing cells, we used the potent inhibitor of p38 MAPK, SB203580. Treatment of GADD153-expressing cells with SB203580 strongly inhibited TGF- β 1-induced apoptosis at 48 hours (Figure 7D). Our results suggest that the induction of GADD153 by TGF- β 1 is part of the TGF- β 1-mediated apoptotic pathway, and is upstream of p38 MAPK in this pathway.

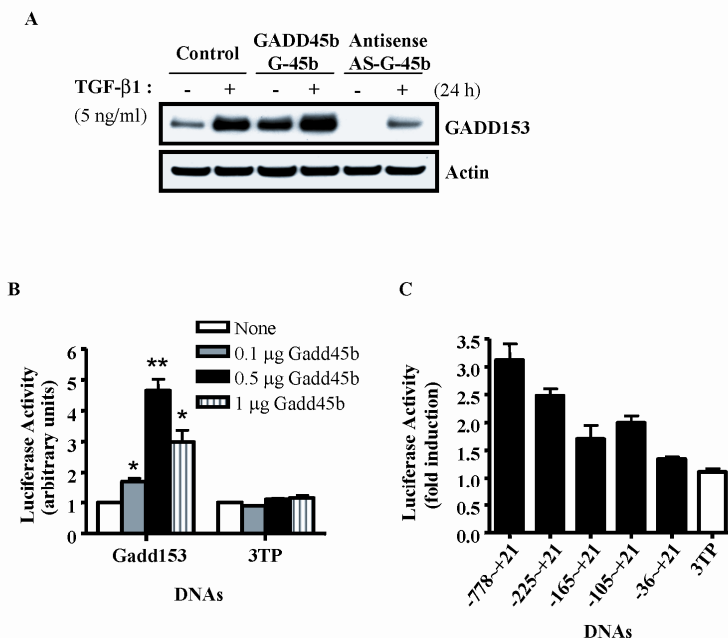


Fig. 6. Effect of GADD45b on transactivation of GADD153.

A, C10 cells stably transfected with either an empty vector (Control), a sense Gadd45b expression vector (G-45b) or an antisense Gadd45b expression vector (AS-G-45b) were treated with TGF- β 1 or vehicle for 24 hours, and total cell lysates were analyzed by immunoblotting using GADD153-specific antibodies. Immunoblotting with actin antibodies is shown in the lower panel. B, C10 cells were transiently cotransfected with 0.5- μ g Gadd153 (-778-+21)-Luc of the hamster Gadd153 promoter or 3TP-Luc plasmid as a negative control with increasing amounts of the Gadd45b expression plasmid. Cells were harvested 30 hours after transfection and assessed for Luciferase activity. The results shown are the means S.E. of three independent experiments performed. *, $p < 0.05$; **, $p < 0.01$ versus control. C, C10 cells were transiently cotransfected with 0.5- μ g of Gadd45b expression plasmid and 0.5- μ g of deletion mutants of the hamster Gadd153 promoter, including pGadd153 (-778-+21)-Luc, pGadd153 (-225-+21)-Luc, pGadd153 (-165-+21)-Luc, pGadd153 (-105-+21)-Luc and pGadd153 (-36-+21)-Luc (filled bars) along with p3TP-Luc (open bar). Luciferase activity was measured. The results shown are the means S.E. of two independent experiments performed in triplicate.

3.8 GADD153 expression and TGF- β 1-mediated apoptosis is blocked by Smad7

It has previously been reported that GADD45b is activated by TGF- β 1 in a Smad-dependent manner (Takekawa et al., 2002; Yoo et al., 2003). Our results showed that GADD153 induction occurs through GADD45b, which in turn, activates p38 MAPK, and leads to apoptosis in the presence of TGF- β 1 in C10 cells. These findings evoked the possibility that TGF- β 1-mediated apoptosis in C10 cells may be mediated by the Smad pathway. To determine whether the Smad pathway was required for TGF- β -mediated signaling of apoptosis, we asked whether overexpression of Smad7, an inhibitory Smad, would inhibit

this effect in C10 cells. In the Smad7-overexpressing cells detected by Smad7 antibody, expression of GADD153 protein was below the level of detection, and TGF- β 1 addition did not result in induction of GADD153 protein as it did in control cells (Figure 8A). The basal level of apoptosis and the sensitivity to TGF- β 1-mediated apoptosis was significantly reduced in C10 cells that overexpressed Smad7 (Figure 8B). The morphological changes described earlier did not occur in Smad7-overexpressing C10 cells treated with TGF- β 1 (Figure 8C). This indicates that TGF- β 1-mediated apoptosis is Smad-dependent and requires induction of GADD153. To confirm that induction of GADD153 is important in determining sensitivity of C10 cells to TGF- β 1-mediated apoptosis, we tested the ability of GADD153 to affect apoptosis in C10 cells that overexpressed Smad7. Transient restoration of GADD153 in C10 cells expressing Smad7 significantly augmented apoptosis in the absence of TGF- β 1 and the level of apoptosis was not significantly affected by TGF- β 1 treatment (Figure 8D). This suggests that the induction of GADD153 expression is a necessary and sufficient step for the induction of apoptosis in C10 cells that overexpress Smad7.

Sensitivity of C10 cells to TGF- β 1-mediated apoptosis, we tested the ability of GADD153 to affect apoptosis in C10 cells that overexpressed Smad7. Transient restoration of GADD153 in C10 cells expressing Smad7 significantly augmented apoptosis in the absence of TGF- β 1 and the level of apoptosis was not significantly affected by TGF- β 1 treatment (Figure 8D). This suggests that the induction of GADD153 expression is a necessary and sufficient step for the induction of apoptosis in C10 cells that overexpress Smad7.

3.9 TGF- β 1 regulates IL-6 production through a Smad2-dependent pathway

Since overexpression of Smad7 not only blocked the basal apoptosis, but also resulted in a significant decrease in TGF- β 1-mediated apoptosis compared to vector transfected control cells, it may be that TGF- β 1 inhibits cell survival signaling pathways as well as activates apoptosis signaling pathways in C10 cells in a Smad-dependent manner. Among the cytokines that have been shown to participate in cellular survival, IL-6 can activate the phosphatidylinositol-3 kinase (PI3K)/AKT survival pathway (Sierra, 2005). In addition, IL-6 modulates TGF- β 1-induced apoptosis via the PI3K/AKT pathway (Chen et al., 1999). To investigate possible crosstalk between TGF- β 1 and IL-6, we examined IL-6 production by C10 cells that stably overexpress Smad7. Basal production of IL-6 was found to be 5-fold higher in Smad7-overexpressing C10 cells compared to control cells (Figure 9A), and IL-6 production increased by accumulation in Smad7-overexpressing C10 cells after addition of TGF- β 1. In contrast, no significant change in IL-6 production occurred in control C10 cells after treatment with TGF- β 1. This result suggests that inhibition of the Smad signaling pathway by Smad7 leads to increased production of IL-6 in C10 cells.

To investigate whether serine/threonine kinase AKT, a downstream target of PI3K, is involved, we also examined activation of AKT in C10 cells overexpressing Smad7. Figure 9B shows phosphorylation, and thus activation, of AKT in Smad7-overexpressing C10 cells. Activation of AKT was maintained at the basal level for up to 24 hours after treatment with TGF- β 1 in C10 cells that overexpress Smad7, while activation of AKT was not detected in control C10 cells. To address the effect of IL-6 on TGF- β 1-induced apoptosis, C10 cells were treated with either TGF- β 1 or increasing amounts of IL-6. TGF- β 1 induced apoptosis, while addition of IL-6 increased the ability of C10 cells to survive without TGF- β 1 (Figure 9C). The ability of IL-6 to promote cell survival in C10 cells was detected using an IL-6 concentration as low as 1-ng/ml, maximized these effects using 10-ng/ml IL-6 and sustained these effects

using 50- and 100-ng/ml IL-6. Next, to investigate whether IL-6 affects apoptosis induced by TGF- β 1, C10 cells were treated with TGF- β 1 and IL-6 in combination using concentrations of 10-, 50- or 100-ng/ml of IL-6. In the presence of TGF- β 1, no treatments with IL-6 protected C10 cells from apoptosis induced by TGF- β 1 (Figure 9D). These findings suggest that IL-6 promotes cell survival in the absence of TGF- β 1 in C10 cells, but does not inhibit apoptosis induced by TGF- β 1, and also suggest that the apoptotic pathway may be predominant over IL-6-mediated survival in these cells.

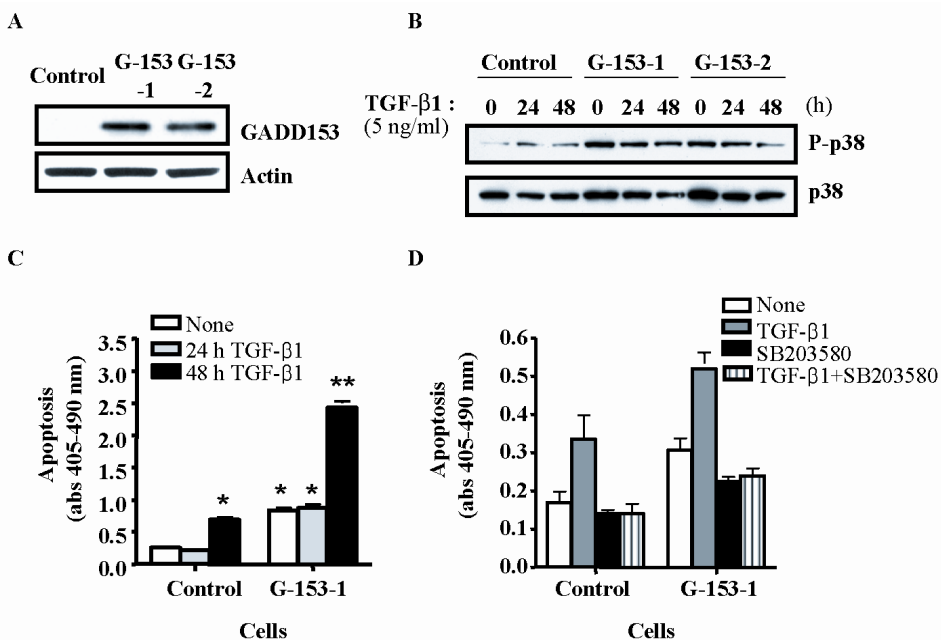


Fig. 7. Overexpression of GADD153 activates p38 MAPK and increases TGF- β 1-mediated apoptosis in C10 cells.

A, C10 cells were stably transfected with either an empty vector (Control) or a Gadd153 expression vector (G-153-1 and G-153-2). Total cell lysates were isolated and immunoblotting was performed using GADD153-specific antibodies. Immunoblotting with actin antibodies is shown in the lower panel. B, Phosphorylation of endogenous p38 MAPK in total cell lysates prepared from C10 cells stably transfected with either an empty vector (Control) or a Gadd153 expression vector (G-153-1 and G-153-2) and treated with TGF- β 1 or vehicle for 24- and 48 hours was analyzed by immunoblotting. Phosphorylated (P) and non-phosphorylated forms of p38 MAPK are indicated in the upper and lower panels, respectively. C, C10 cells stably transfected with either an empty vector (Control) or a Gadd153 expression vector (G-153-1) were treated with vehicle (open bars) or with TGF- β 1 for 24- (gray bars) and 48 hours (dark bars). DNA fragmentation was detected by ELISA assay. *, $p < 0.01$; **, $p < 0.001$ versus control. D, C10 cells stably transfected with either an empty vector (Control) or a Gadd153 expression vector (G-153-1) were treated with vehicle (open bars), TGF- β 1 (gray bars), 10 μ M SB203580 (dark bars) or TGF- β 1 and SB203580 in combination (lined bars) for 48 hours. DNA fragmentation was detected by ELISA assay.

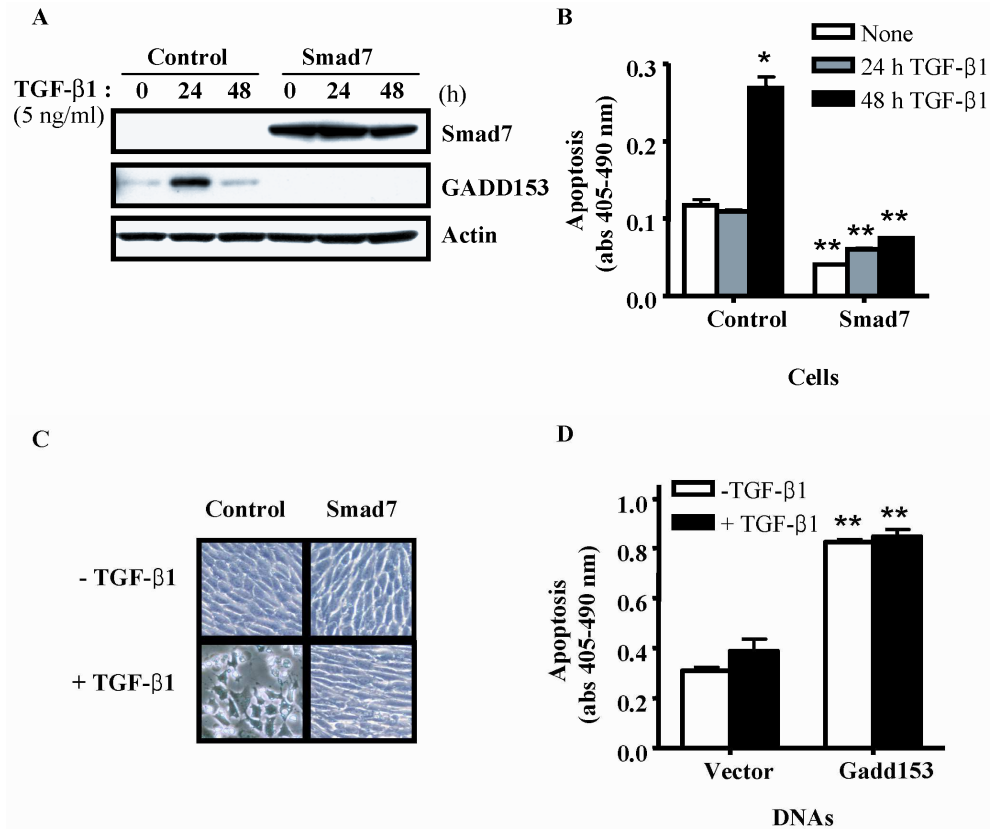


Fig. 8. Overexpression of Smad7 blocks GADD153 expression and TGF- β 1-mediated apoptosis.

A, C10 cells were stably transfected with either an empty vector (Control) or a Flag-tagged Smad7 expression vector (Smad7) and treated with TGF- β 1 for 24- and 48 hours. Total cell lysates were isolated and immunoblotting was performed using Smad7 and GADD153 antibodies. Immunoblotting with actin antibodies is shown in the lower panel. B, C10 cells stably transfected with either an empty vector (Control) or a Smad7 expression vector (Smad7) were treated with vehicle (open bars) or with TGF- β 1 for 24- (gray bars) and 48 hours (dark bars). DNA fragmentation was detected by ELISA assay. *, $p < 0.05$, **, $p < 0.01$ versus control. C, Morphology of C10 cells stably transfected with either an empty vector (Control) or a Smad7 expression vector (Smad7) by phase contrast microscopy at 48 hours after treatment with or without TGF- β 1. D, C10 cells stably transfected with a Smad7 expression vector were transiently transfected with either an empty vector (Vector) or Gadd153 cDNA expression vector (Gadd153) and cultured with vehicle (open bars) or TGF- β 1 (filled bars) for 48 hours. DNA fragmentation was detected by ELISA assay.

** $p < 0.01$ versus control.

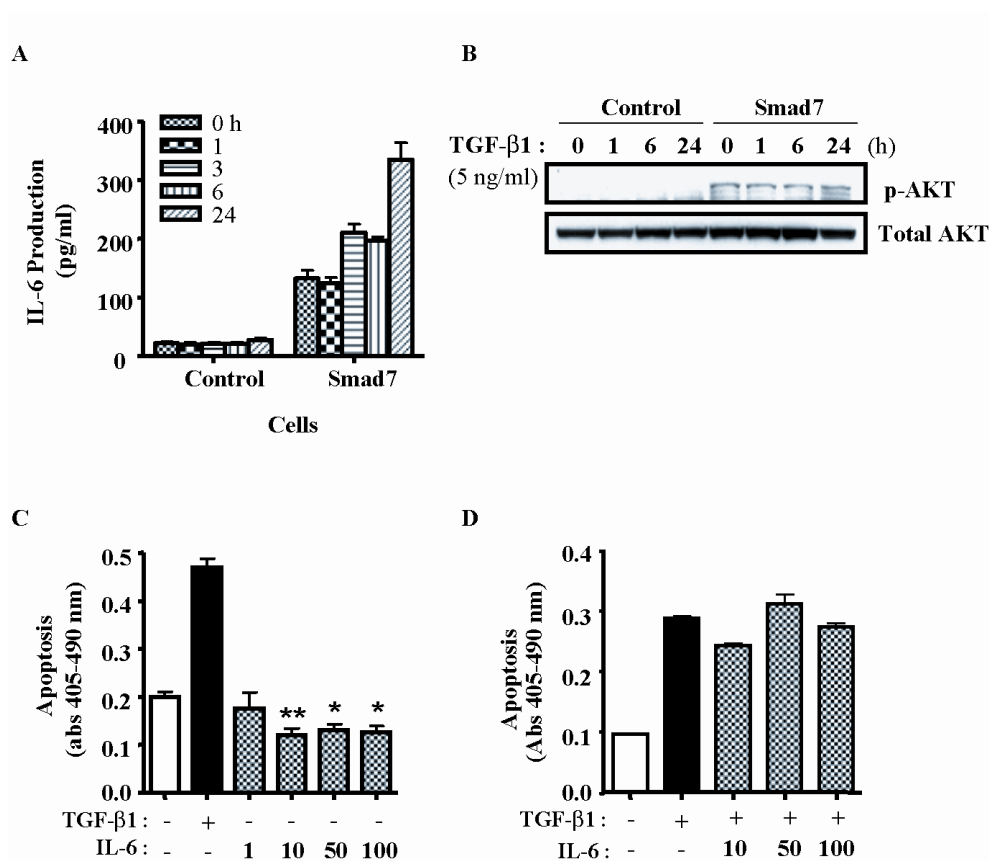


Fig. 9. Overexpression of Smad7 increases IL-6 production in the presence or absence of TGF- β 1.

C10 cells were stably transfected with either an empty vector (Control) or a Flag-tagged Smad7 expression vector (Smad7). A, The cells were treated with TGF- β 1 for the indicated times. Conditioned media were isolated and the level of IL-6 protein production was detected using a Quantikine assay as described in Materials and Methods. The results shown are the means S.E. of two independent experiments performed in triplicate. B, Time course of phosphorylation of endogenous AKT after stimulation by TGF- β 1. Total cell lysates were prepared from untreated or TGF- β 1-treated cells and used for immunoblotting. Phosphorylated (P) and non-phosphorylated total forms of AKT are indicated in the upper and lower panels, respectively. C,D, C10 cells were treated with vehicle (open bars) or TGF- β 1 (filled bars) or IL-6 (1-, 10-, 50- or 100-ng/ml) (stippled bars) in C, or a combination of TGF- β 1 and IL-6 (10-, 50- or 100-ng/ml) (stippled bars) in D, for 48 hours as indicated. DNA fragmentation was detected by ELISA assay at 48 hours after treatments.

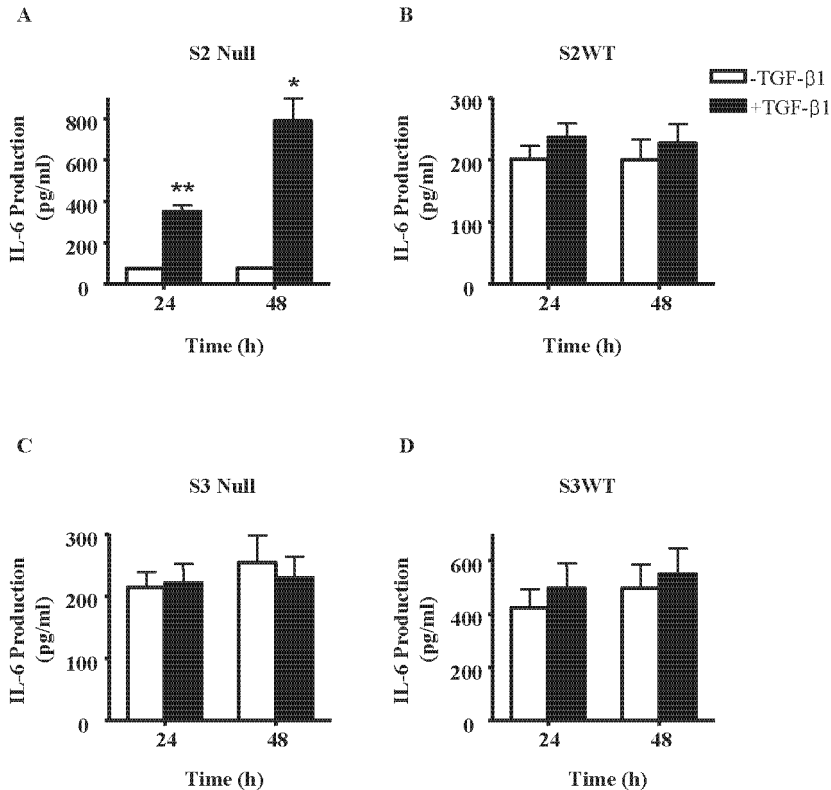


Fig. 10. Production of IL-6 in embryo fibroblasts derived from Smad2- and Smad3-deficient mice and their wildtype littermates.

Embryo fibroblasts from A, Smad2 null, B, Smad2 wildtype, C, Smad3 null and D, Smad3 wildtype mice were cultured with vehicle (open bars) or TGF-β1 (filled bars) for 24- and 48 hours. Conditioned media were isolated and the level of secreted IL-6 protein in pg/ml was detected using a Quantikine assay as described in Materials and Methods. The results shown are the means S.E. of two independent experiments performed in duplicate.

*, $p < 0.01$, **, $p < 0.001$ versus control.

Finally, to determine which Smad affects expression of IL-6, we examined the secretion of IL-6 in embryo fibroblasts from Smad2- and Smad3-deficient mice and their wildtype littermates. Treatment of Smad2 null mouse embryo fibroblasts (MEFs) with TGF-β1 demonstrated that production of IL-6 was enhanced by TGF-β1 7-fold by 24 hours that increased to 15-fold by 48 hours, while no change was detected in the amount of IL-6 that was produced from wildtype MEFs (Figure 10B). In contrast, no change in IL-6 production was detected in Smad3 null and wildtype MEFs after treatment with TGF-β1 for up to 48 hours. This suggests that TGF-β1 regulates IL-6 production via Smad2, but not via Smad3, in C10 cells.

4. Discussion

The present study was undertaken to investigate the potential effect of TGF- β 1 on apoptosis in immortalized normal mouse lung C10 cells. We reasoned that it would be easier to understand the mechanism of TGF- β 1-mediated apoptosis in normal epithelial cells in which the TGF- β 1 signaling pathway was functional than in tumor cells in which the TGF- β 1 pathway may not be functional. Addition of TGF- β 1 to C10 cells activated the p38 MAPK pathway, and the p38 MAPK inhibitor SB203580 significantly reduced the TGF- β 1-mediated apoptotic response. These findings suggest that activation of p38 MAPK is involved in TGF- β 1-mediated apoptosis in C10 cells. It has been reported that stress-activated protein kinases like p38 MAPK can mediate pro-apoptotic signals from TGF- β Receptors in multiple cell types, including lung cells (Undevia et al., 2004). Interestingly, although ERK1/ERK2 and JNK activation is associated with TGF- β Receptor-mediated apoptosis in some cell types, addition of TGF- β 1 to C10 cells had only a marginal effect on activation of ERK and no effect on JNK. Thus, ERK and JNK do not appear to play essential roles in TGF- β 1-mediated apoptosis in these lung cells.

TGF- β 1-mediated apoptosis in C10 cells has revealed an unexpected degree of complexity. TGF- β 1-mediated apoptosis in C10 cells is dependent on signaling through Smads and p38 MAPK. The delayed kinetics of p38 MAPK activation in TGF- β 1-mediated apoptosis in C10 cells suggest that one or more additional components may be involved in this process. Earlier reports have provided strong evidence that GADD45b is a critical upstream component in the apoptotic pathway of TGF- β 1 in human pancreatic carcinoma cells and mouse hepatocytes (Takekawa et al., 2004; Yoo et al., 2003). A recent report outlined a role for Smad3 and Smad4 in activating Gadd45b through its third intron to facilitate G2 progression following addition of TGF- β 1 (Major & Jones, 2004). We examined the expression of Gadd45b mRNA in response to TGF- β 1 in C10 cells. As in earlier reports, TGF- β 1 induces expression of GADD45b in C10 cells. Overexpression of GADD45b accelerates the appearance of apoptosis in C10 cells by at least 24 hours, and down-regulation of GADD45b expression with an antisense construct inhibits TGF- β -mediated apoptosis. These results suggest that GADD45b participates in TGF- β 1-mediated apoptosis in C10 cells.

The participation of GADD45b in TGF- β 1-mediated apoptosis in C10 cells does not rule out the possible involvement of other factors that may cooperate with GADD45b in TGF- β 1-mediated apoptosis. The time interval between the initial induction of Gadd45b mRNA expression by TGF- β 1 stimulation and the appearance of apoptosis suggest that other factors may be involved. We explored the possible involvement of other GADD family members, including GADD5a and GADD45g. However, transcripts for Gadd45a or Gadd45g in the absence or presence of TGF- β 1 were detected only at very low levels in C10 cells. Earlier reports showed that the stress-inducible transcription factor GADD153/CHOP induced apoptosis in mammalian cells through p38 MAPK-dependent and -independent mechanisms (Corazzari et al., 2003; Maytin et al., 2001; Murphy et al., 2001). GADD153 has been implicated in apoptosis, and cells isolated from Gadd153 null mice have been shown to be resistant to apoptosis-inducing regimes (Zinszner et al., 1998). In our study, Gadd153 mRNA had a gradual and sustained increase in Gadd153 mRNA after treatment with TGF- β 1 that reached a maximum at 24- to 48 hours. The timing of Gadd153 mRNA induction is consistent with the appearance of TGF- β 1-mediated apoptosis in C10 cells. Addition of actinomycin D and cyclohexamide showed that up-regulation of GADD153 expression is dependent on transcription and requires de novo protein synthesis. This suggests that

although Gadd153 mRNA is regulated by TGF- β 1 in C10 cells, the induction of GADD153 by TGF- β 1 depends on prior protein synthesis events.

Our results demonstrate that expression of GADD153 protein is induced in C10 cells stably transfected with Gadd45b cDNA, while it is markedly reduced in antisense Gadd45b-expressing cells compared to control C10 cells untreated or treated with TGF- β 1. Cotransfection of Gadd45b plasmid with Gadd153-Luc showed that increasing amounts of Gadd45b cDNA augmented transcriptional activity of Gadd153-Luc. These results suggest that GADD45b has a role in transactivation of GADD153 expression in response to TGF- β 1 in C10 cells in a direct or indirect manner. This is the first report of regulation of GADD153 by GADD45b. Our findings also show that the induction of GADD153 is sufficient to activate p38 MAPK and to trigger apoptosis in the absence of TGF- β 1 in C10 cells.

In this study, we also demonstrated that GADD153 induction by TGF- β 1 and TGF- β 1-mediated apoptosis was completely inhibited in C10 cells when Smad signaling was blocked by Smad7. Transient restoration of GADD153 in C10 cells overexpressing Smad7 significantly augmented apoptosis in the absence of TGF- β 1, and the level of apoptosis was not affected by TGF- β 1 treatment. Our results suggest that GADD153 is a determining factor in TGF- β 1-mediated apoptosis in C10 cells and induction of GADD153 may be useful to potentiate apoptosis in cells in which the TGF- β 1 signaling pathway is not functional or only minimally functional, which is often the case in tumor cells.

In addition to the p38 MAPK regulated apoptosis pathway, we also identified crosstalk between TGF- β 1 and IL-6 in C10 cells. In C10 cells that overexpress Smad7, the basal level of IL-6 production was higher than in control cells and activation of AKT occurred without TGF- β 1. Addition of IL-6 alone resulted in increased survival, and IL-6 in combination with TGF- β 1 failed to block apoptosis induced by TGF- β 1. The slow kinetics of delayed onset of TGF- β 1-induced apoptosis may be attributed to the activation of the PI3K/AKT cell survival pathway during the early stage of TGF- β treatment (Yu et al., 2002). Activation of the AKT pathway by TGF- β 1 has been shown to be mediated by a p38 MAPK-mediated mechanism (Horowitz et al., 2004). Activation of AKT in response to TGF- β 1 addition does not appear to play an important role in normal C10 cells, indicating that this pathway probably does not participate in delaying the onset of apoptosis. However, if events occur which lead to the overexpression of Smad7, and thus decreased Smad-dependent TGF- β 1 signaling, the activation of AKT may play a more prominent role. It has been reported that overexpression of Smad7 induced tumorigenicity in human colon carcinoma cells by blocking TGF- β -induced growth inhibition and apoptosis (Halder et al., 2005). It appears that the TGF- β 1/p38 MAPK-mediated apoptosis pathway in which GADD45b and GADD153 participate as intermediates, plays a more prominent role than the IL-6/AKT pathway does in C10 cells as outlined in Figure 11. Although our scenario depicts direct activation of GADD153 by GADD45b, this may or may not occur. Other factors may be involved. In addition, the survival pathway may be a less frequently used pathway in normal C10 cells compared to the apoptosis pathway. The predominance of these pathways may also change in tumor cells. Future studies will be needed to examine the role of these alternative pathways and crosstalk signaling that may be involved or abrogated in tumor cells.

TGF- β 1 induces GADD45b expression in a Smad-dependent manner. GADD45b then activates GADD153, which, in turn, activates p38 MAPK. Activated p38 MAPK results in apoptosis. This may be the predominant pathway in C10 cells. Alternatively, TGF- β may down-regulate IL-6 production through Smad2-dependent signaling. Elevated IL-6 may be able to induce activation of AKT to block or reduce apoptosis and increase cell survival. If

Smad signaling is blocked by Smad7 or reduced by another agent or phenomenon, it may be sufficient to confer a pro-survival/apoptosis-resistant phenotype to C10 cells.

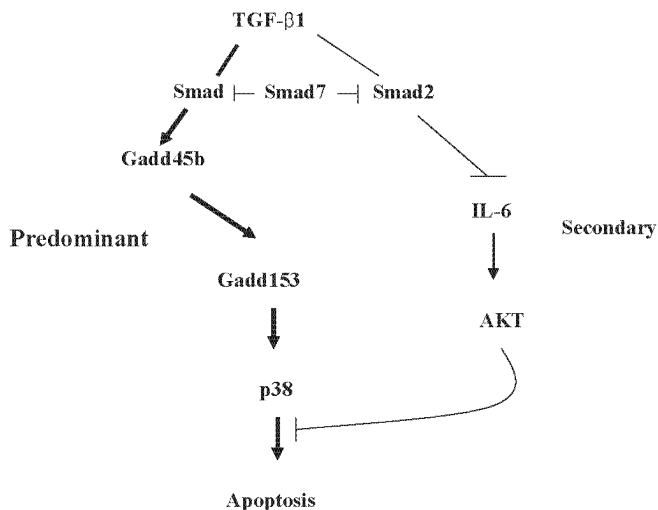


Fig. 11. A possible mechanism of TGF- β 1-mediated apoptosis in mouse lung epithelial C10 cells.

5. Conclusion

Resistance to apoptosis has been shown to be one of the characteristics of cancer cells during progressive tumorigenesis (Hanahan & Weinberg, 2000). Thus, it would be promising for the treatment of cancer, to activate TGF- β 1-mediated apoptosis by modulating the function of TGF- β 1 in specific tumor cell types. Our study has shown that in lung C10 cells that overexpress GADD153, both the basal level and sensitivity to apoptosis are increased, suggesting that the induction of GADD153 is an important step in the sensitivity of these lung cells to apoptosis. Future studies will need to be performed to evaluate whether increased levels of GADD153 can be stimulated effectively to increase TGF- β -mediated apoptosis in lung tumor cells in which this does not occur or occurs less frequently, and thus, bring about their destruction, and an effective therapy that can be used in the treatment of lung cancer.

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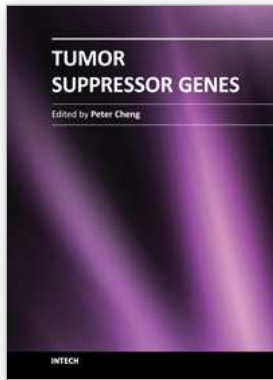
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Functional evidence obtained from somatic cell fusion studies indicated that a group of genes from normal cells might replace or correct a defective function of cancer cells. Tumorigenesis that could be initiated by two mutations was established by the analysis of hereditary retinoblastoma, which led to the eventual cloning of RB1 gene. The two-hit hypothesis helped isolate many tumor suppressor genes (TSG) since then. More recently, the roles of haploinsufficiency, epigenetic control, and gene dosage effects in some TSGs, such as P53, P16 and PTEN, have been studied extensively. It is now widely recognized that deregulation of growth control is one of the major hallmarks of cancer biological capabilities, and TSGs play critical roles in many cellular activities through signaling transduction networks. This book is an excellent review of current understanding of TSGs, and indicates that the accumulated TSG knowledge has opened a new frontier for cancer therapies.

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Phone: +86-21-62489820
Fax: +86-21-62489821

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