

Modulation of the transforming growth factor- β 1-induced Smad phosphorylation by the extracellular matrix receptor β 1-integrin

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Abstract. Integrins, heterodimeric receptors for the extracellular matrix, are known to modulate transforming growth factor- β 1 (TGF- β 1)-mediated cell behavior. However, the interplay between β 1-integrin and Smad signaling, regulated by TGF- β 1, is not clearly understood. This study focuses on the alterations of the regulatory Smads (R-Smads) by TGF- β 1 in β 1-integrin-transfected HepG2 cells. The phosphorylation at the C-terminal site of R-Smads by TGF- β 1 was impaired in the β 1-integrin-transfected cells. However, the R-Smads were constitutively phosphorylated at the linker region in a MAP kinase-dependent manner. Furthermore, the expression of a mutant Smad3, that lacks the phosphorylation sites in the linker region, restored the TGF- β 1-induced Smad transcriptional activity. These results suggest that β 1-integrin impairs the TGF- β 1-mediated signals through the altered phosphorylation of the R-Smads.

Introduction

Hepatocellular carcinoma (HCC) is ranked as the fifth most common malignant neoplasm in the world and it is closely associated with hepatitis B and/or C viral infections (1,2). The chronic infection that is related to the hepatitis viruses causes persistent inflammation of the liver which results in cirrhosis, a condition characterized by an excessive accumulation of the extracellular matrices. Liver cirrhosis is associated with the development of HCC in both clinical observations and in experimental animal models (1,3,4).

Transforming growth factor- β 1 (TGF- β 1) is a potent stimulator of the extracellular matrix (ECM) production in the liver and it also participates in the development of liver cirrhosis (5). TGF- β 1 plays a dual role in carcinogenesis. TGF- β 1 inhibits the proliferation of the epithelial, the endothelial and the hematopoietic cells and therefore acts as a tumor suppressor (6). TGF- β 1 induces an epithelial-to-mesenchymal transition (EMT) in cells which increases tumor invasiveness resulting in the promotion of tumor metastasis (7). TGF- β transduces the signals from its receptor to the nucleus through the Smad proteins. The phosphorylation of the receptors and the intracellular signaling molecules play an important role in most of the signal transduction pathways. The activated TGF- β type I receptor (T β RI) phosphorylates the C-terminal SSXS motif of the receptor-regulated Smads (R-Smads), such as Smad2 and Smad3. The phosphorylated R-Smads can oligomerize with Smad4 to form a functional complex which is translocated to the nucleus, where it induces transcriptional induction or repression of the target genes (8,9). The linker region of the Smad proteins can be phosphorylated by several kinases, including the mitogen-activated protein (MAP) kinases, such as the extracellular signal-regulated kinase (Erk), the c-Jun terminal kinase (JNK) and the p38 or the cyclin-dependent kinase (10-13). Several studies have demonstrated that the phosphorylation of the linker region of the R-Smads can cause an alteration of the TGF- β 1 signaling, thus resulting in the loss of the tumor suppressive function.

β 1-integrin is a major cell surface adhesion receptor that transmits signals from the ECM and that plays critical roles in growth, differentiation and carcinogenesis (14). β 1-integrin can block the apoptosis that is induced by several reagents and can therefore function as a survival factor (15). The over-expression of β 1-integrin in HCC cells inhibits the growth suppressive signals and the apoptosis induced by TGF- β 1 (16). However, the mechanisms by which β 1-integrin modulates the TGF- β 1-induced Smad signaling is not known. Therefore, this study investigated the changes of the TGF- β 1-induced R-Smads phosphorylation by β 1-integrin in order to elucidate the mechanisms involved in the β 1-integrin-initiated alteration of the TGF- β signaling.

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Materials and methods

Cells and reagents. The HepG2 cells were obtained from the Japanese Cancer Research Resources (Osaka, Japan). The HepG2 cells overexpressing β 1-integrin, generated by stable transformation of a human β 1A-integrin-enhanced green fluorescent protein (GFP) fusion gene, were described previously (17,18). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) containing 10% fetal calf serum (Gibco-BRL). The human recombinant TGF- β 1 was purchased from R&D Systems (Minneapolis, MN), The PD98059 was purchased from Promega (Madison, WI), the SB203580 was purchased from Sigma (St. Louis, MO) and the SP600125 was purchased from Calbiochem (San Diego, CA).

Plasmids, transfection and the luciferase assay. The TGF- β -inducible luciferase-reporter plasmid containing the four Smad-binding elements (SBE4-Luc) (19) and the mammalian expression plasmids containing the mutations in the linker region of Smad2 and Smad3 (Smad2/EPsm and Smad3/EPsm, respectively) (10) were obtained from Addgene (Cambridge, MA). The human β 1A-integrin expression plasmid was described previously (17,18). The Smad-dependent transcriptions were detected by a luciferase assay according to the method described by the supplier (Dual-Luciferase Reporter Assay System; Promega). The luciferase expression plasmid, pRL-SV40 (Promega), was co-transfected to normalize any variations in the transfection efficiency. The HCC cells were seeded onto 6-well plates at a density of 1×10^5 cells/well without antibiotics and were then incubated at 37°C until 80% confluence was attained. The confluent cells were washed twice with OPTI-MEM I Reduced Medium (Life Technologies, Rockville, MD) prior to the addition of 2 ml of OPTI-MEM I Reduced Medium containing 1 μ g of the target-gene reporter plasmid, 0.5 μ g of the pRL-SV40 luciferase plasmid and 15 μ l of the Lipofectamine 2000 reagent (Life Technologies). After 6 h of incubation, the medium was changed and the transfected cells were treated with the indicated doses of TGF- β 1. After 24 h of treatment, the cell were washed twice with PBS and carefully scraped into a 1X passive lysis buffer (Promega). The cell extracts were assayed immediately for luciferase activity using a Berthold Luminometer (MLR-100 Micro Lumino Reader; Corona Electric, Ibaragi, Japan).

Antibodies. The rabbit polyclonal anti-Smad2, anti-Smad3, and anti-Histone H1 antibodies were purchased from Zymed Laboratories (San Francisco, CA). The antibodies against the phosphorylated form of the C-terminal region of Smad2 (Ser465/467) and Smad3 (Ser423/425), as well as the antibody against the phosphorylated form of the linker region of Smad2 (Ser245/250/255), were purchased from Cell Signaling (Boston, MA). The antibody against the phosphorylated form of the linker region of Smad3 was described previously (11,12). The rabbit polyclonal anti-human β -actin antibody was purchased from Biomedical Technologies (Stoughton, MA).

Western blotting. The cultured HepG2 cells were treated with 2 ng/ml of TGF- β 1 and recovered at the indicated times. The cells were lysed with an extraction buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4 and 1 mM NaF. The lysate was sonicated for 5 min at 4°C and was then clarified by centrifugation at 15,000 rpm for 10 min, after which the supernatant was collected. After the quantification of the protein with a protein assay kit (Bio-Rad, Hercules, CA), 40 μ g of the protein was mixed with a NuPAGE sample buffer (Novex, San Diego, CA). The mixture was separated by SDS polyacrylamide gel electrophoresis and transferred to a polyvinylamide difluoride (PVDF) membrane (Bio-Rad). The membrane was then blocked for 1-2 h at room temperature by incubating with 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20. This was followed by incubation with a primary antibody for 30 min at room temperature. The specific bands were visualized by a further incubation with an HRP (horseradish peroxidase)-conjugated secondary antibody, followed by a chemiluminescence reaction using the ECL system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The rabbit polyclonal anti- β -actin antibody was used as a control and an anti-Histone H1 antibody was used as a control for the nuclear fraction.

The subcellular fractionation of cells. The cytoplasmic and the nuclear fractions were obtained by using a NE-PER kit (Pierce Biotechnology Inc, Rockford, IL) according to the manufacturer's protocol. The nuclear fraction was further extracted with 0.6 M NaCl containing 10 mM Hepes, 5 mM MgCl_2 , 0.5% NP40, 1% Triton X-100, 1 mM PMSF, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin.

Statistical analysis. Differences were analyzed using Student's t-test and $P < 0.05$ was considered significant. All experiments were performed at least three times. The data are presented as the mean \pm standard deviation (SD).

Results

The suppression of TGF- β 1-induced Smad-dependent transcription by β 1-integrin. Since our previous study showed that the overexpression of β 1-integrin inhibited the TGF- β 1-induced growth suppression of HCC cells (16), we first examined whether or not β 1-integrin can alter the TGF- β 1-induced Smad-dependent transcription. As shown in Fig. 1, TGF- β 1 increased the Smad-dependent transcription in HepG2 cells, but failed to increase the luciferase activity in the β 1-integrin-transfected cells, thus indicating that β 1-integrin mediated the inhibition of the Smad-dependent transcription.

The alteration of the phosphorylation status of the Smad proteins in the β 1-integrin transfectants. The phosphorylation status of Smad2 and Smad3 in both the C-terminal and the linker regions was examined in wild-type, mock- and β 1-integrin-transfected HepG2 cells under unstimulated conditions. Fig. 2 shows that the C-terminal region of Smad2 (pSmad2C) and Smad3 (pSmad3C) remained unphosphorylated in the β 1-integrin transfectants, in the wild-type cells and in the mock-transfected cells. However, the linker

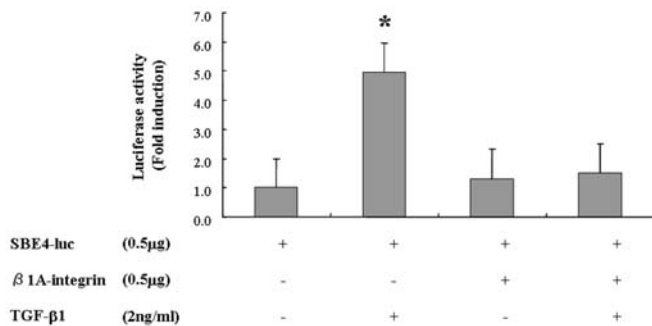


Figure 1. The inhibition of TGF-β1-induced Smad-dependent transcriptional activity by β1-integrin. The HepG2 cells were transfected with the SBE4-luciferase reporter gene plasmid with or without the β1-integrin expression plasmid and were treated with 2 ng/ml of TGF-β1 for 24 h. All experiments were performed in triplicates and data are shown as the mean fold induction of control with SD. *P<0.05 compared with control cells without TGF-β1.

region of Smad2 (pSmad2L) and Smad3 (pSmad3L) in the β1-integrin transfectants were phosphorylated, but those in the wild-type and the mock-transfected cells remained unphosphorylated.

The modulation of the TGF-β1-induced phosphorylation of Smad proteins in the β1-integrin-transfected cells. The phosphorylation changes that occur after the TGF-β1 treatment were investigated (Fig. 3). In the wild-type and in the mock-transfected cells, 2 ng/ml of TGF-β1 transiently phosphorylated the C-terminal region of Smad2 (pSmad2C) and Smad3 (pSmad3C), but did not induce phosphorylation in the linker region of Smad2 (pSmad2L) and Smad3 (pSmad3L). However, TGF-β1 could not induce the phosphorylation of the C-terminal region of Smad2 and Smad3 in the β1-integrin transfectants, even though it induced the phosphorylation in the linker region of Smad2. The phosphorylation in the linker region of Smad3 was not affected by TGF-β1 in the wild-type, in the mock- and β1-integrin-transfected cells.

The altered nuclear translocation of the phosphorylated form of Smad proteins in the β1-integrin-transfected cells. The

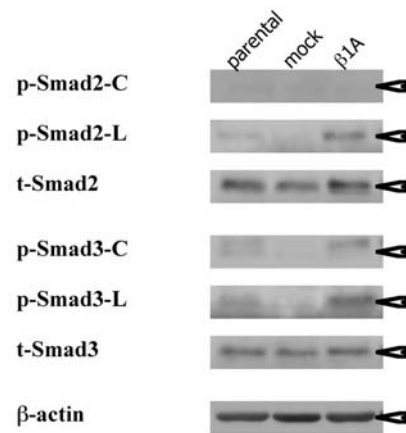


Figure 2. The expression and the phosphorylation status of Smad2 and Smad3 in the parental, the mock- and β1A-integrin-transfected HepG2 cells. The cells without TGF-β1 stimulation were analyzed by Western blotting with anti-Smad2 and anti-Smad3 antibodies. pSmad2C, Smad2 phosphorylated at C-terminal site; pSmad2L, Smad2 phosphorylated at the linker region; tSmad2, total Smad2; pSmad3C, Smad3 phosphorylated at the C-terminal site; pSmad3L, Smad3 phosphorylated at the linker region; tSmad3, total Smad3.

ability of the β1-integrin to modulate the nuclear translocation of Smad proteins after the TGF-β1 treatment was investigated (Fig. 4). The cells were separated into the cytoplasmic (Cy) and nuclear (Nu) fractions with or without TGF-β1. TGF-β1 induced the phosphorylation of the C-terminal region of Smad2 and Smad3 and stimulated the nuclear accumulation of the C-terminal phosphorylated form of Smad2 and Smad3 in the wild-type and in the mock-transfected HepG2 cells. The linker region of Smad2 and Smad3 was not phosphorylated by TGF-β1 and the accumulation of the linker-phosphorylated forms of Smad2 and Smad3 were not observed. In contrast, TGF-β1 could not induce C-terminal phosphorylation of Smad2 and Smad3 and did not stimulate the accumulation of the C-terminal phosphorylated form of Smad2 and Smad3 in the β1-integrin-transfected cells. Although the phosphorylation of the linker region of Smad2 and Smad3 was not observed in the wild-type and in the mock-transfected cells, the linker

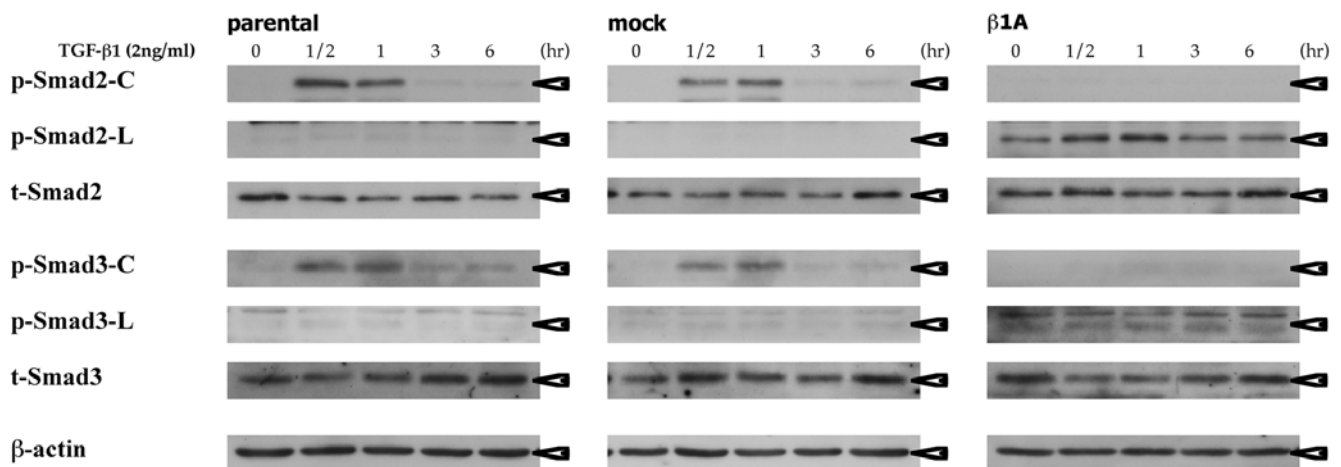


Figure 3. The modulation of the TGF-β1-induced phosphorylation of the Smad proteins in the parental, the mock- and β1A-integrin-transfected HepG2 cells. The cells were treated with 2 ng/ml of TGF-β1, were harvested at the indicated times and were analyzed by Western blotting using each antibody. pSmad2C, Smad2 phosphorylated at C-terminal site; pSmad2L, Smad2 phosphorylated at the linker region; tSmad2, total Smad2; pSmad3C, Smad3 phosphorylated at the C-terminal site; pSmad3L, Smad3 phosphorylated at the linker region; tSmad3, total Smad3.

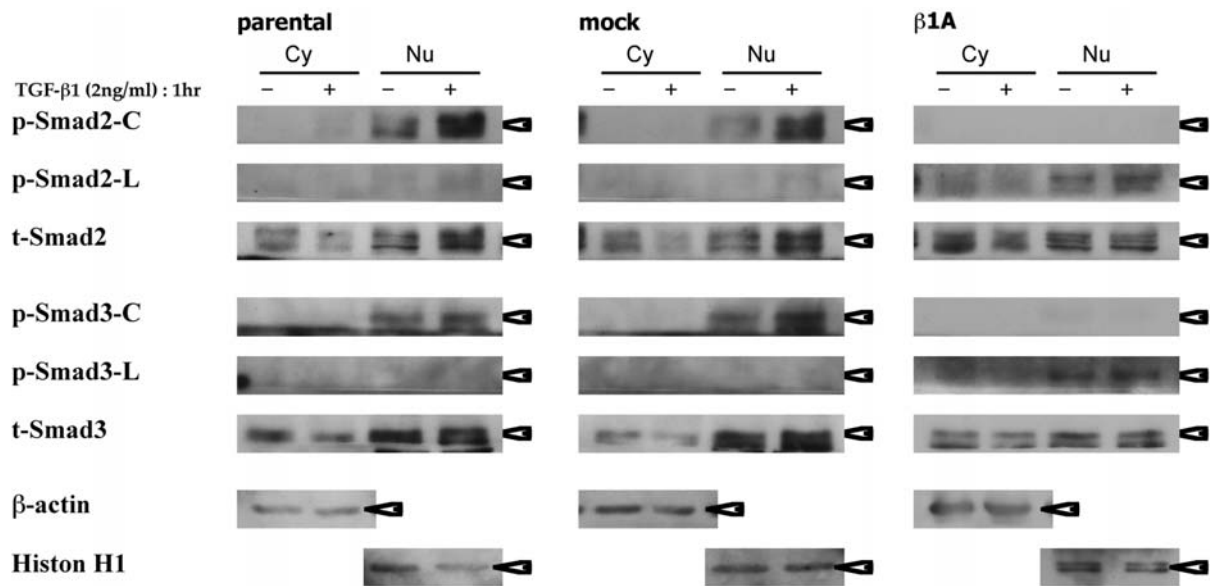


Figure 4. The altered nuclear translocation of the phosphorylated Smad proteins after TGF- β 1 treatment in β 1A-integrin-transfected HepG2 cells. The cells were separated into the cytoplasmic (Cy) and nuclear (Nu) fractions with or without 2 ng/ml of TGF- β 1 for 1 h and were analyzed by Western blotting. Histone H1 was detected as the control for the nuclear proteins. pSmad2C, Smad2 phosphorylated at C-terminal site; pSmad2L, Smad2 phosphorylated at linker region; tSmad2, total Smad2; pSmad3C, Smad3 phosphorylated at the C-terminal site; pSmad3L, Smad3 phosphorylated at the linker region; tSmad3, total Smad3.

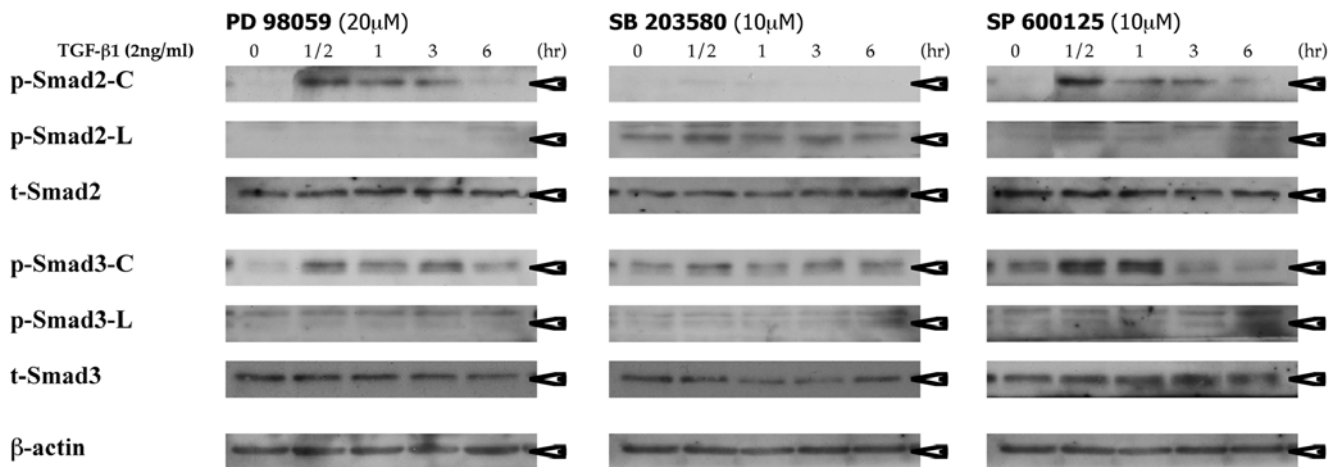


Figure 5. The involvement of the MAP kinase pathway in the alteration of the Smad phosphorylation in the β 1A-integrin-transfected cells. The HepG2 cells overexpressing β 1A-integrin were treated with PD98509 (ERK inhibitor; left), SB203580 (p38 inhibitor; center) and SP600125 (JNK inhibitor; right) for 6 h prior to the 1-h treatment with 2 ng/ml of TGF- β 1. pSmad2C, Smad2 phosphorylated at the C-terminal site; pSmad2L, Smad2 phosphorylated at the linker region; tSmad2, total Smad2; pSmad3C, Smad3 phosphorylated at the C-terminal site; pSmad3L, Smad3 phosphorylated at the linker region; tSmad3, total Smad3.

region of Smad2 and Smad3 was already phosphorylated in the β 1-integrin-transfected cells at the basal level and the linker-phosphorylated form of Smad2 and Smad3 was present in the nucleus irrespective of the TGF- β 1 stimulation.

The involvement of the MAP kinase pathway in the alterations of the TGF- β 1-regulated Smad phosphorylation by β 1-integrin. Since the ERK, the p38 and JNK pathways are involved in the alterations of the TGF- β 1-induced growth suppression in the β 1-integrin-transfected HCC cells (16), further experiments were conducted to determine whether or not the MAP kinase pathway is involved in the changes of the TGF- β 1-regulated Smad phosphorylation in β 1-integrin-transfected cells. The HepG2 cells, overexpressing β 1-integrin, were pretreated with

PD98509 (ERK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor), respectively. The pretreated cells were then treated with 2 ng/ml of TGF- β 1 and were analyzed by Western blotting for changes in the phosphorylation state of the Smad proteins (Fig. 5). The treatment with the ERK and the JNK inhibitors restored the TGF- β 1-induced C-terminal phosphorylation of Smad2 that was impaired in the β 1-integrin transfectants. However, the p38 inhibitor did not restore the TGF- β 1-induced phosphorylation of the C-terminal region of Smad2. Phosphorylation of the linker region of Smad2 was not affected by the ERK and the JNK inhibitors. In comparison, the p38 inhibitor induced the phosphorylation of the linker region of Smad2, indicating that the p38 pathway contributes to the suppression of the phosphorylation of the linker region

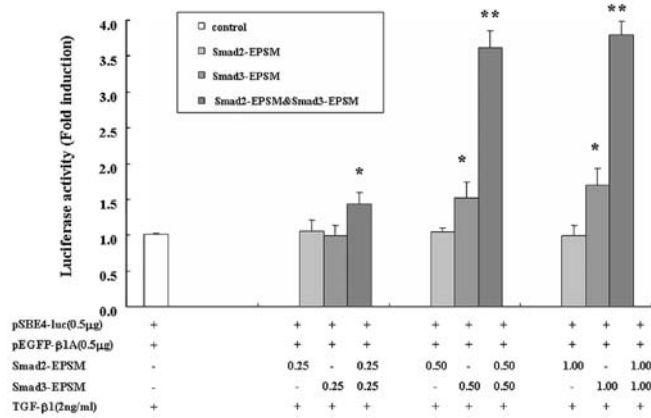


Figure 6. The restoration of the TGF- β 1-induced Smad-mediated transcription by mutant Smads lacking phosphorylation sites in the linker region. The HepG2 cells were transfected with the SBE4 reporter plasmid and the β 1-integrin expression plasmid together with mutant Smads lacking phosphorylation sites in the linker region (Smad2-ESPM and Smad3-ESPM) were then treated with 2 ng/ml of TGF- β 1 for 24 h. The data are shown as the mean \pm SD from three independent experiments. *P<0.05, **P<0.01 compared with control without Smad2-ESPM and Smad3-ESPM.

Table I. Effects of the ERK, p38 and JNK pathways on the phosphorylation of Smad proteins in the linker and C-terminal regions.

Phosphorylation site of Smads	ERK	p38	JNK
pSmad2-C	↓	→	↓
pSmad2-L	↑	↓	↑
pSmad3-C	↓	↓	↓
pSmad3-L	↑	↑	↑

pSmad2C, Smad2 phosphorylated at C-terminal site; pSmad2L, Smad2 phosphorylated at linker region; pSmad3C, Smad3 phosphorylated at the C-terminal site; pSmad3L, Smad3 phosphorylated at the linker region; ↑, stimulation; ↓, inhibition; →, equivocal.

of Smad2. The phosphorylation of the C-terminal site of Smad3 by TGF- β 1 was restored by all three inhibitors, but the phosphorylation of the linker region of Smad3 was suppressed by these inhibitors, suggesting that the ERK, the p38 and the JNK pathways all participated in the linker phosphorylation of Smad3 by β 1-integrin.

The restoration of the TGF- β 1-induced Smad-dependent transcription by Smads mutated in the linker-region phosphorylation sites. Because the phosphorylation of the linker region of Smad2/3 appears to play important roles in the alteration of Smad signaling, we transfected the Smad2/3 plasmids (bearing mutations in the linker region and lacking phosphorylation sites) and tested whether or not the TGF- β 1-induced Smad-dependent transcription was restored. As shown in Fig. 6, the transfection of the Smad3/ESPM partially restored the TGF- β 1-induced Smad transcription. Although the Smad2/ESPM alone did not restore the TGF- β 1-induced Smad transcription, the co-transfection of the Smad2/ESPM and the

Smad3/ESPM further increased the luciferase activity induced by TGF- β 1.

Discussion

TGF- β is highly expressed in many malignant tumors, including HCC, with varying sensitivity of the tumor cells to TGF- β . The loss of the growth inhibitory effects of TGF- β is regarded as an important pathway to carcinogenesis. The resistance to TGF- β results from the repression of T β RI, T β RII, Smad2 and/or Smad4 (20). Previous studies showed that the linker region of the R-Smads can be phosphorylated by several kinases, and this linker-phosphorylated form responds to the TGF- β 1-induced growth inhibitory signal that is mediated by the phosphorylation of the C-terminal SSXS motif. The loss of the C-terminal phosphorylation of the R-Smads, which results in the repression of the anti-proliferative effects of TGF- β , is caused by the phosphorylation of the linker region of Smad2/3. The phosphorylation of the R-Smads are caused by an oncogenic Ras-mediated ERK MAP kinase activation (10) and/or by the cyclin-dependent kinases, CDK4 and CDK2 (14). The TGF- β signaling is modulated by the growth factors and/or the inflammatory cytokines through JNK and/or p38 (11,12). Chronic inflammation is closely linked to the development of many types of cancers, including HCC (21). Matsuzaki *et al* (22) recently reported that the phosphorylation at the linker region of the R-Smads, by the inflammatory cytokines acting through the JNK pathway, correlated with the loss of the phosphorylation at the C-terminal site of the R-Smads, thus leading to the promotion of hepatocarcinogenesis.

Experimental and clinical observations have suggested that liver fibrosis is a prerequisite for hepatocarcinogenesis (1-4). β 1-integrin, a major subunit of the heterodimeric receptor for ECM, plays important roles in carcinogenesis (23). Integrin serves as a survival factor by protecting the cells from various apoptosis-inducing factors, including TGF- β (15-17). β 1-integrin-induced activation of MAP kinase plays a critical role in protecting the HCC cells from the growth inhibition and apoptosis induced by TGF- β 1 (16). However, the alterations of the Smad signaling by β 1-integrin in the HCC cells remain to be elucidated. This study demonstrated that the TGF- β 1-induced phosphorylation status of the R-Smads was altered in the β 1-integrin-overexpressing HCC cells, and that the MAP kinases differentially contributed to the phosphorylation of the linker and to the C-terminal region of R-Smads (Table I). A specific feature of β 1-integrin-transfected cells is the loss of the phosphorylation of the C-terminal site in both Smad2 and Smad3 after TGF- β 1 treatment. However, the linker region of Smad2 and Smad3 is constitutively phosphorylated in the β 1-integrin-transfected cells. In accordance with these results, Matsuzaki *et al* (22) observed an increased phosphorylation at the linker region of Smad3 in the hepatocytes adjacent to the fibrotic areas in liver that were chronically infected with the hepatitis C virus.

In this study, the differential phosphorylation of Smad2 and Smad3 was observed when the cells were treated with a p38 inhibitor. Although the p38 inhibitor increased the phosphorylation of the Smad2 linker region in the β 1-integrin-transfected HCC cells, the phosphorylation state of the Smad3

linker region remained unchanged. Smad3 plays an essential role in the TGF- β -mediated signal transduction that leads to the suppression of cell growth and the reduction in HCC development (24). The activation of p38 MAP kinase was required for the Smad3-mediated suppression of HCC development. However, it is not clear if Smad2, Smad3 or both proteins are responsible for the TGF- β -induced EMT, cell motility and invasion. Ju *et al* showed that Smad2-deleted hepatocytes were sensitive to TGF- β -induced growth arrest, apoptosis and EMT, but that the Smad3-deleted hepatocytes were not (25). In addition, the Smad2-deleted hepatocytes spontaneously acquired the characteristic features of the EMT, thus suggesting that Smad2 suppressed the hepatocyte growth and the EMT, independent of the TGF- β signaling. Therefore, elucidating the mechanism by which Smad2 and Smad3 regulate the TGF- β signaling pathway is important for the development of a better strategy for cancer therapy.

Because the alterations in the TGF- β signaling pathway play an important role in promoting carcinogenesis, the TGF- β signaling appears to be an attractive target for cancer prevention and therapy. However, TGF- β plays a tumor-promoting and tumor-suppressive role, therefore, shutting down the TGF- β signaling may be unfavorable because it may lead to the suppression of TGF- β -mediated growth inhibition. Hence, caution should be taken when the TGF- β signal is targeted for inhibition because blockage of TGF- β does not always lead to the inhibition of cancer development and progression.

With respect to the tumor-promoting action of the linker-phosphorylated form of R-Smad and the tumor-suppressing action of the C-terminal phosphorylated form, the selective phosphorylation of the C-terminal site and/or the selective inhibition of the linker region of R-Smads might be ideal processes for the restoration of TGF- β -mediated tumor-suppressive action. Recently, protein phosphatase 1A, a magnesium-dependent α -protein phosphatase 2C α (PPM1A/PP2Ca), was reported to dephosphorylate the C-terminal region of Smad2/3 and to act as a negative regulator of the TGF- β -induced signals (26). This suggests that PPM1A inhibition might restore the growth-inhibitory action of TGF- β .

The phosphorylation of the linker region in the R-Smads, especially in Smad3, seems to play an important role in shifting the TGF- β signaling from a tumor-suppressive to an oncogenic state by increasing the invasion and the metastatic potential through the EMT (27). Since the MAP kinase pathway and the CDKs are involved in the phosphorylation of the linker region in the R-Smads, the inhibitors of these kinases might be useful for the recovery of the TGF- β -mediated tumor-suppressive signaling. Recently, the dephosphorylation and/or the blockade of phosphorylation in the linker region of Smad2 and/or Smad3 were shown to restore the altered TGF- β signaling (28-30). Therefore, the specific inhibition of the phosphorylation of the linker-region might provide a more selective and effective approach for the prevention and the treatment of cancers.

References

- Okuda K: Hepatocellular carcinoma. *J Hepatol* 32: 225-237, 2000.
- Llovet JM, Burroughs A and Bruix J: Hepatocellular carcinoma. *Lancet* 362: 1907-1917, 2003.
- Sakaida I, Hironaka K, Uchida K, Suzuki C, Kayano K and Okita K: Fibrosis accelerates the development of enzyme-altered lesions in the rat liver. *Hepatology* 28: 1247-1252, 1998.
- Omata M and Shiratori Y: Long-term effects of interferon therapy on histology and development of hepatocellular carcinoma in hepatitis C. *J Gastroenterol Hepatol (Suppl 15)*: E134-E140, 2000.
- Friedman SL: The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med* 328: 1828-1835, 1993.
- Moses HL, Yang EY and Pietenpol JA: TGF- β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* 63: 245-247, 1990.
- Zavadil J and Bottinger EP: TGF- β and epithelial-to-mesenchymal transitions. *Oncogene* 24: 5764-5774, 2005.
- Heldin CH, Miyazono K and ten Dijke P: TGF- β signaling from cell membrane to nucleus through SMAD proteins. *Nature* 390: 465-471, 1997.
- Massague J, Seonae J and Wotton D: Smad transcription factors. *Genes Dev* 19: 2783-2810, 2005.
- Kretzschmar M, Doody J, Timokhina I and Massague J: A mechanism of repression of TGF β /Smad signaling by oncogenic Ras. *Genes Dev* 13: 804-816, 1999.
- Mori S, Matsuzaki K, Yoshida K, Furukawa F, Tahashi Y, Yamagata H, Sekimoto G, Seki T, Matsui H, Nishizawa M, Fujisawa J and Okazaki K: TGF-beta and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at linker regions. *Oncogene* 23: 7416-7429, 2004.
- Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sungano Y, Yamagata H, Matsushita M, Seki T, Inagaki Y, Nishizawa M, Fujisawa J and Inoue K: p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. *Hepatology* 38: 879-889, 2003.
- Matsuura I, Denissova NG, Wang G, He D, Long J and Liu F: Cyclin-dependent kinases regulated the antiproliferative function of Smads. *Nature* 430: 226-231, 2004.
- Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69: 11-25, 1992.
- Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C, Chilvers ER, Dransfield I, Donnelly SC, Strieter R and Haslett C: Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. *Nat Med* 5: 662-668, 1999.
- Zhang H, Ozaki I, Mizuta T, Yoshimura T, Matsuhashi S, Eguchi Y, Yasutake T, Hisatomi A, Sakai T and Yamamoto K: Transforming growth factor- β 1-induced apoptosis is blocked by β 1-integrin-mediated mitogen-activated protein kinase activation in human hepatoma cells. *Cancer Sci* 95: 878-886, 2004.
- Zhang H, Ozaki I, Mizuta T, Matsuhashi S, Yoshimura T, Hisatomi A, Tadano J, Sakai T and Yamamoto K: Beta1-integrin protects hepatoma cells from chemotherapy-induced apoptosis via a mitogen-activated protein-kinase-dependent pathway. *Cancer* 95: 896-906, 2002.
- Zhang H, Ozaki I, Mizuta T, Yoshimura T, Matsuhashi S, Hisatomi A, Tadano J, Sakai T and Yamamoto K: Mechanism of β 1-integrin-mediated hepatoma cell growth involves p27 and S-phase kinase-associated protein 2. *Hepatology* 38: 305-313, 2003.
- Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzer KW, Vogelstein B and Kern SE: Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* 1: 611-617, 1998.
- Levy L and Hill CS: Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* 17: 41-58, 2006.
- Karin M, Lawrence T and Nizet V: Innate immunity gone awry linking microbial infections to chronic inflammation and cancer. *Cell* 124: 823-835, 2006.
- Matsuzaki K, Murata M, Yoshida K, Sekimoto G, Uemura Y, Sakaida N, Kaibori M, Kamiyama Y, Nishizawa M, Fujisawa J, Okazaki K and Seki T: Chronic inflammation associated with hepatitis C virus infection perturbs hepatic transforming growth factor β signaling, promoting cirrhosis and hepatocellular carcinoma. *Hepatology* 46: 48-57, 2007.
- Hanahan D and Weinberg RA: The hallmarks of cancer. *Cell* 100: 57-70, 2000.
- Yang Y, Zhang G, Feigenbaum L and Zhang YE: Smad3 reduces susceptibility to hepatocellular carcinoma by sensitizing hepatocytes to apoptosis through downregulation of Bcl-2. *Cancer Cell* 9: 445-447, 2006.

25. Ju W, Ogawa A, Heyer J, Nierhof D, Yu L, Kucherlapati R, Shafritz DA and Bottinger EP: Deletion of Smad2 in mouse liver reveals novel functions in hepatocytes growth and differentiation. *Mol Cell Biol* 26: 654-667, 2006.
26. Lin X, Duan X, Liang Y, Su Y, Wrighton KH, Long J, Hu M, Davis CM, Wang J, Brunicardi FC, Shi Y, Chen Y, Meng A and Feng X: PPM1A functions as a Smad phosphatases to terminate TGF- β signaling. *Cell* 125: 915-928, 2006.
27. Matsuzaki K: Smad3 phosphoisoform mediated signaling during sporadic human colorectal carcinogenesis. *Histol Histopathol* 21: 645-662, 2006.
28. Wrighton KH, Willis D, Long J, Liu F, Lin X and Feng X: Small C-terminal phosphatases dephosphorylate the regulatory linker region of Smad2 and Smad3 to enhance transforming growth factor- β signaling. *J Biol Chem* 281: 38365-38375, 2006.
29. Sapkota G, Knockarert M, Alarcon C, Montalvo E, Brivanlou AH and Massague J: Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor- β pathways. *J Biol Chem* 281: 40412-40419, 2006.
30. Sekimoto G, Matsuzaki K, Yoshida K, Mori S, Murata M, Seki T, Matsui H, Fujisawa J and Okazaki K: Reversible Smad-dependent signaling between tumor suppression and oncogenesis. *Cancer Res* 67: 5090-5096, 2007.