

Smad2 mediates Erk1/2 activation by TGF- β 1 in suspended, but not in adherent, gastric carcinoma cells

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Abstract. Integrin-mediated cell adhesion enables cells to respond to extracellular stimuli for diverse cellular functions including proliferation, leading to differential biological activities from cells in suspension. Integrins can transduce signals (directly) to intracellular molecules and also collaborate with other membrane receptor-mediated signal pathways, including TGF- β 1 pathway. TGF- β 1 induces growth inhibition in epithelial cells and is known to transduce intracellular signaling in Smad-dependent or -independent manner. Currently effects of cell adhesion status on the TGF- β 1-mediated Erk1/2 regulation and on its Smad-(in)dependency are not known. In this study, we examined effects of cell adhesion status on the TGF- β 1-mediated Erk1/2 regulation, and roles of Smad proteins on the cell adhesion-mediated effects, using a gastric carcinoma cell variant. First, we found that cell adhesion-dependent Erk1/2 activation responded differentially to TGF- β 1, depending on cell adhesion status; TGF- β 1 treatment resulted in activation of Erk1/2 in suspended cells, whereas a decrease was noted in adherent cells. This activation of Erk1/2 by TGF- β 1 in suspension was more enhanced by an overexpression of Smad2, but not of other Smads 2, 4, and 7, but abolished by a Smad2 reduction via an introduction of its siRNA. In contrast, PKB/Akt regulation by TGF- β 1 was not different in suspension or in

adhesion, and Smad7, but not the other Smads, activated PKB/Akt phosphorylation on TGF- β 1 treatment, indicating a specificity of Smad2-mediated and cell adhesion status-dependent activation of Erk1/2 activity.

Introduction

Cell adhesion to extracellular matrix (ECM) proteins occurs via cell adhesion receptor including integrins and would allow for cells to efficiently respond to extracellular stimuli for spreading, proliferation, migration, survival, and gene transcription (1-4). Whereas diverse *in vitro* cell systems including normal and established cells in suspension show inert signal activities, certain malignantly cancer cells may show abnormally activated signaling activities even in suspension, leading to anchorage-independency. Not only structural anchorage of cells to the proper place where appropriate binding partners of ECM proteins exist, but also bi-directional signal transduction between extracellular and intracellular spaces are accomplished via this integrin-mediated cell adhesion (1-4).

A group of cell adhesion receptors, integrins are heterodimeric cell surface receptors consisting of an α and a β subunit. So far, 18 α and 8 β subunits are known to assemble for about two dozen combinations (1-4). Integrin-mediated signaling can be divided into two categories: direct signaling transduced by integrins engaging directly with ECM proteins leading to regulation of downstream intracellular signaling molecules and collaborative (indirect) signaling where integrins co-signal with other receptor-mediated signal pathways, including growth factor receptors, G-protein coupled receptors (GPCRs) (3,5-8), or TGF- β 1 signaling pathway (9).

Transforming growth factor- (TGF-) β 1 is a multi-functional cytokine that inhibits epithelial cell growth and also stimulates growth of fibroblasts by binding to a heterodimeric receptor consisting of both type 1 (TBR1) and type 2 (TBR2) serine/threonine kinase receptors (10,11). Activation of the receptor complex propagates intracellular signal transduction involving Smad proteins to regulate numerous developmental and homeostatic processes via regulations in gene induction (12). Recently, TGF- β 1-mediated, but Smad-

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Abbreviations: ECM, extracellular matrix; Fn, fibronectin; SNU16 α 5Ad, an adherent variant cell line from an anchorage-independent SNU α 516 gastric carcinoma cell line overexpressing exogenously human integrin α 5 subunit; siRNA, short-interfering RNA

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independent signaling pathways have also been evidenced in diverse cell culture systems (13-18). Accumulating evidence shows that TGF- β 1 can activate diverse intracellular signaling molecules including MAPKs consisting of Erk1/2, JNKs, and p38 MAPK (19-21).

TGF- β 1-mediated Erk1/2 activation involves Ras (22,23). Depending on its activation kinetics, slow activation of Erk1/2 may result from Smad-dependent transcription responses, whereas rapid activation (within 30 min) may occur in a Smad-independent manner (22). In addition, activation of other MAPK signaling including JNKs and p38 MAPK by TGF- β 1 could be shown even in the systems deficient of Smad4 or with dominant negative Smads (20,21). TGF- β 1-mediated activation of Erk1/2 can also enhance Smad phosphorylation and regulates Smad activation, leading to TGF- β 1 expression and Smad-dependent transcriptions (reviewed in ref. 18), indicating a convergence between Smads and MAPK pathways, depending on cellular contexts.

In this study, we tested whether Smad-(in)dependency of TGF- β 1-mediated Erk1/2 activation might be differential depending on cell adhesion status (i.e., suspension versus adhesion). Interestingly, we found that TGF- β 1 treatment of a gastric carcinoma variant cell line in suspension resulted in an increase in Erk1/2 activity, whereas in adhesion TGF- β 1 mediated a decrease in Erk1/2 activity. Further, Smad2, but not other Smads, mediated much more activation of Erk1/2 in suspension, but not in adhesion. This suggests a cell adhesion-dependent Smad2 dependency of Erk1/2 activation by TGF- β 1 in the gastric carcinoma cells.

Materials and methods

Cells. A Korean derived gastric cancer cell line (SNU16) was purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). SNU16 cells were stably transfected to overexpress human integrin α 5 subunit by Lipofectamine Plus-mediated methods, and selected with G418 drug selection and by immunobead (Dynabeads M-450, Dynal A.S., Oslo, Norway) selection with the use of monoclonal anti-human α 5 (P1D6) antibody (SNU16 α 5). Subclones enriched with adherent cells, SNU16 α 5Ad, were obtained from subsequent cultures by collecting adherent cells among mostly anchorage-independent cells of SNU16 α 5. SNU16 α 5Ad cells were grown at 37°C and 5% CO₂ in RPMI-1640 culture media containing 10% fetal bovine serum and 0.2 mg/ml of G418.

Cell lysate preparation and Western blot analysis. SNU16 α 5Ad cells trypsinized were incubated in suspension within the serum-free RPMI-1640 media plus 1% BSA for 1 h, before replating on fibronectin-precoated dishes (15 μ g/ml). TGF- β 1 (1 or 5 ng/ml, Chemicon, Temecula, CA) was added directly to the replating media and the treatment lasted for 6 or 20 h. In case that adenovirus for either β -galactosidase (Lac Z), FLAG-tagged Smad2, or Smad7 was separately infected before cell manipulation, SNU16 α 5Ad cells in 150 mm culture dishes were infected with proper virus. Twenty hours later, cells were maintained in suspension or replated onto fibronectin-precoated dishes in the absence or presence of TGF- β 1 treatment for further 20 h. In case that Smad2 knock-out via introduction of SiRNA was performed

prior to keeping in suspension, siRNA against Smad2 (Cellogenetics Inc., Rockville, MD) was transfected using Lipofectamine 2000[®] reagent according to the manufacturer's protocols. Twenty-four hours after the transfection, cells were maintained in suspension in the absence or presence of TGF- β 1 treatment for 20 h. Cell lysates were prepared as described in previous studies (24,25). The lysates were used in Western blots using anti-Erk1/2, phospho-Erk1/2, phospho-Y⁴¹⁶Src, phospho-S⁴⁷³PKB/Akt, and PKB/Akt (Cell Signaling Technology, Beverly, MA), FLAG (Sigma), c-Src (New England Biolabs, Beverly, MA), Ras, GST (BD Transduction Laboratories, San Jose, CA), and Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA). In the cases where the same PVDF membrane was reprobed with another primary antibody, the membrane was stripped by incubation in a stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β -mercaptoethanol) at 65°C for 30 min, washed for 1 h (3 times x 20 min) with Tris-based saline with 0.05% Tween-20 (TBST), reblocked with TBST containing 1% BSA plus 2% non-fat milk proteins, and then reprobed with another primary antibody.

Ras-GTP determination. The GST-RBD pull-down assay to determine Ras-GTP level using GST-RBD (Ras binding domain of Raf-1;1-140) and cell lysates were performed as described previously (26).

Results

We have an interest in the roles of cross-talk between integrins and TGF- β 1 signal pathways on regulation of cellular functions, such as growth, adhesion, spreading, and migration, using a gastric carcinoma cell variant cell line. The gastric SNU16 α 5Ad cells used in this study required a long time to be adherent upon replating to fibronectin-precoated dishes; for a significant cell population enough for biochemical assays to be adherent before cell harvests, incubation following replating should be more than 6 h. As shown in Fig. 1A (left), replating onto fibronectin for 6 h did not show a significant increase in Erk1/2 activation on cell adhesion. However, we have observed cell adhesion-dependent activation of Erk1/2 (Fig. 1A), when cells were replated for 20 h. Further, we have tested how TGF- β 1 treatment affected the cell adhesion-dependent Erk1/2 activity regulation. At the same time when cells were either maintained in suspension or replated onto fibronectin, TGF- β 1 at 1 or 5 ng/ml was directly added to the cell incubation media. Basically the data by 1 ng/ml TGF- β 1 treatment were very similar to data from the experiments where TGF- β 1 was treated at 5 ng/ml (data not shown). Interestingly, TGF- β 1 treatment of cells in suspension resulted in Erk1/2 activation, whereas cells in adhesion showed instead a decrease in Erk1/2 activity on TGF- β 1 treatment (Fig. 1B). This differential effect by TGF- β 1 on Erk1/2 activity in a cell adhesion-dependent manner appears specific, because another intracellular signaling molecule, PKB/Akt, showed decreases in activities in both suspension and adhesion on TGF- β 1 treatment (Fig. 1C).

It is known that TGF- β 1-mediated Erk1/2 activation is through the Ras protein (22,23). Thus, we next tested whether

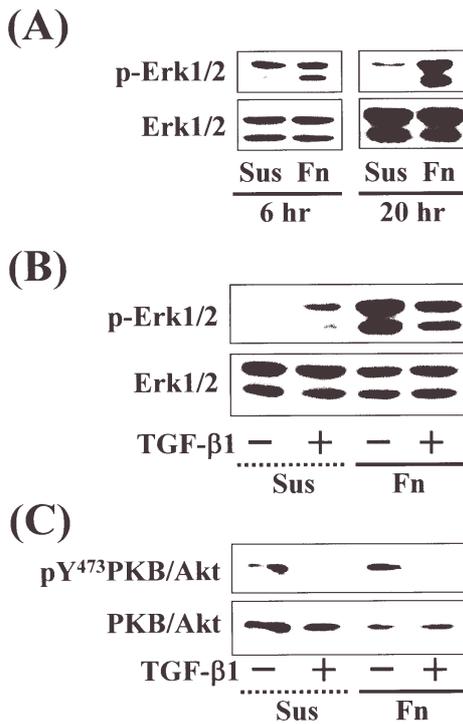


Figure 1. Cell adhesion-dependent regulation of TGF- β 1-mediated Erk1/2, but not PKB/Akt, activities. Adherent gastric cells (SNU16 α 5Ad) were trypsinized, collected, washed with RPMI-1640 containing BSA 1%, kept in suspension with rolling over for 1 h at 37°C, and then either incubated in suspension or replated onto fibronectin-precoated (15 μ g/ml) dishes for 20 h except for the indicated period at 37°C before preparation of whole cell lysate. Data are representative of several independent experiments. (A), Cell adhesion via a longer replating onto fibronectin results in Erk1/2 activation. Cell lysates from cells either kept in suspension (Sus) or replated onto fibronectin (Fn) for 6 or 20 h were used for immunoblotting with primary antibodies against the indicated molecules. (B), TGF- β 1 treatment results in an increase in Erk1/2 activity in suspended cells, but a decrease in adherent cells. Keeping cells in suspension (Sus) or replating cells onto fibronectin (Fn) were done as indicated above. Treatment of TGF- β 1 was done by a direct addition of TGF- β 1 (5 ng/ml) to replating media (RPMI-1640 plus BSA 1%) at the time when cells were replated. (C), TGF- β 1-mediated decrease in PKB/Akt activity was cell adhesion-independent. Lysates prepared as in (B) were immunoblotted for the indicated molecules.

the differential regulation of Erk1/2 activities by TGF- β 1 in a cell adhesion-dependent manner is also mediated by Ras in our system. Cell lysates prepared from cells either in suspension or adhesion in the absence or presence of TGF- β 1 treatment, were analyzed for determination of GTP-loading to Ras by a GST-RBD (Ras Binding Domain of Raf1) pulling down assay. It was shown that TGF- β 1 treatment to cells in suspension resulted in an increase in Ras-GTP levels (i.e., activation of Ras) and cells in adhesion showed a decrease in the Ras-GTP level (Fig. 2), being consistent with the TGF- β 1 mediated regulation of Erk1/2 activities. Therefore, this is well consistent with the previous reports that TGF- β 1-mediated Erk1/2 activation is through Ras (22,23). Further, this showed that TGF- β 1 effects activation of Ras and thus Erk1/2 are differential, depending on cell adhesion status.

Generally it is thought that TGF- β 1-mediated regulation of Erk1/2 activities is Smad-dependent or -independent,

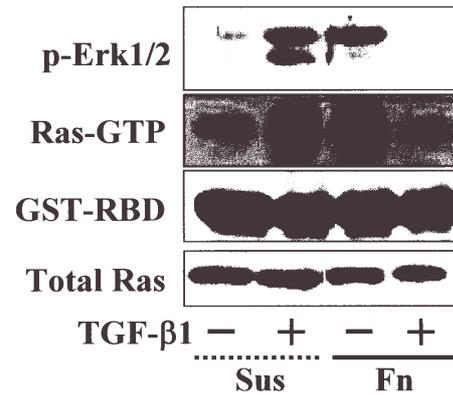


Figure 2. TGF- β 1-mediated Erk1/2 regulation is dependent on cell adhesion status via the Ras protein. Manipulation of cells and preparation of cell lysates were done as indicated in Fig. 1. GTP loading to Ras was analyzed by a pull-down assay using GST-RBD (Ras binding domain of Raf-1;1-140) immobilized to beads and SNU16 α 5Ad cell lysates prepared from the indicated conditions. Data shown are representative from two independent experiments.

depending on cellular environment and nature of TGF- β 1 stimulation (18,27). Further, its Smad-(in)dependency has not been studied with regards to cell adhesion status so far. To see whether TGF- β 1-mediated Erk1/2 activity regulation either in suspension or adhesion is (in)dependent of Smad2, we infected cells with adenovirus with a cDNA either for β -galactosidase (Lac Z, as a negative control) or FLAG-tagged Smad2, before incubation of cells in suspension or adhesion (by replating) in the absence or presence of TGF- β 1 treatment and then lysate preparation. Infection efficiency was examined either by staining for β -galactosidase-positive cells or FLAG-positive cells, resulting in infection of $\geq 90\%$ cells similar to each other (data not shown). In TGF- β 1-treated cell suspensions, Smad2-overexpressing cells showed a significantly higher Erk1/2 activity compared to TGF- β 1-treated cells expressing β -galactosidase (Lac Z) (Fig. 3A). However, cells already in adhesion showed a minimal Erk1/2 activity by TGF- β 1 treatment alone and thus further Smad2 expression could not show a significant influence on the Erk1/2 activity (Fig. 3A). TGF- β 1 signal pathway is inhibited by Smad7 (27). Therefore, it was tested to answer whether overexpression of Smad7 via a viral infection may antagonize the TGF- β 1 effects on Erk1/2 activity. Cells were infected to overexpress either β -galactosidase (Lac Z) or FLAG-tagged Smad7 before cell manipulations. In contrast to Smad2, Smad7 overexpression in suspended cells resulted in a decrease in TGF- β 1-mediated Erk1/2 activity, compared to cells expressing β -galactosidase treated with TGF- β 1 (Fig. 3B). Next, we tested whether other Smad proteins, such as Smad3 and 4, might have effects similar to Smad2 on TGF- β 1-mediated Erk1/2 activity in suspended cells. Overexpression of each Smad protein or β -galactosidase via separated viral infections was performed and the infected cells were manipulated to be either in suspension or adhesion in the absence or the presence of TGF- β 1 treatment. As shown in Fig. 4, Smad2-mediated increase and Smad7-

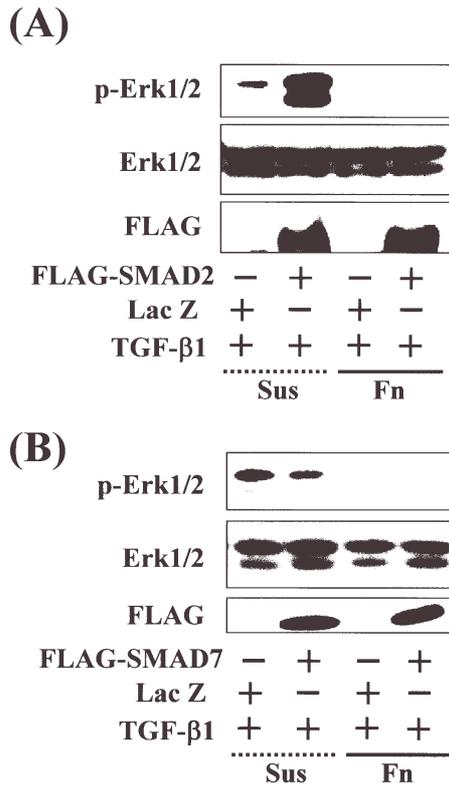


Figure 3. Smad2-mediated increase and Smad7-mediated decrease in Erk1/2 activities by TGF-β1 in suspended cells. Cells were first infected with adenovirus with cDNA either for β-galactosidase (Lac Z), FLAG-tagged Smad2, or FLAG-tagged Smad7. Twenty hours later, infected cells were manipulated to be incubated in suspension or replated onto fibronectin. At the same time of maintenance in suspension or replating onto fibronectin, TGF-β1 treatment was done as indicated in Fig. 1. Lysates were used for Western blotting using primary antibodies against the indicated molecules. Data are representative of two independent experiments.

mediated decrease in Erk1/2 activities by TGF-β1 treatment to suspended cells, but not to adherent cells, were predominant. However, Smad3 or 4 did not show any significant effects on the Erk1/2 activities either in suspension or adhesion, except for a hardly-detectable increase by Smad3 in adherent cells (Fig. 4). Meanwhile, PKB/Akt activity was similarly regulated by TGF-β1 treatment both in suspension and adhesion; TGF-β1 treatment caused decrease, Smad2 and 3 caused further decreases, Smad4 did not cause any change, but Smad7 resulted in an increase, cell adhesion-independently (Fig. 4). Further, the preferential Smad2-mediated activation of Erk1/2 by TGF-β1 only in suspended cells was confirmed by another approach of using an siRNA against Smad2 (siRNA-Smad2). When cells were first transfected with siRNA-Smad2 and then kept in suspension, TGF-β1-mediated activation of Erk1/2 was abolished (Fig. 5). In this observation, suppression of Smad2 was effective, but the level of Smad3 appeared to be up-regulated by TGF-β1 treatment (Fig. 5). This observation of Smad3 levels again suggests that Erk1/2 activation by TGF-β1 is not mediated by Smad3. Therefore, it is likely that gastric carcinoma cells may have a Smad2-mediated activation of Erk1/2 activity by TGF-β1

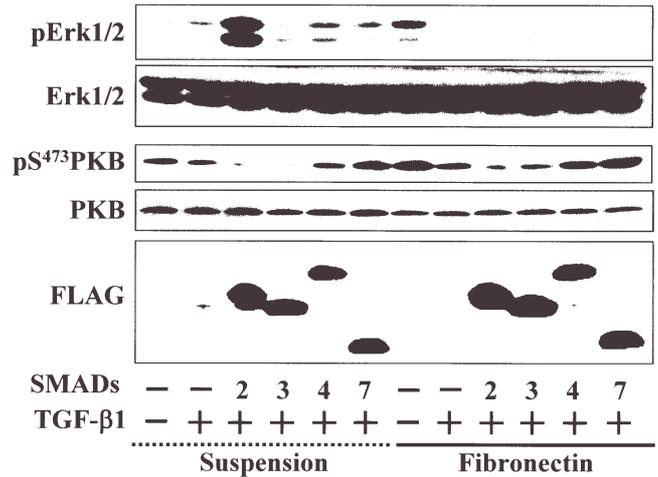


Figure 4. Differential regulation of Erk1/2, but not PKB/Akt, activity via Smads in a cell adhesion-dependent manner. Cells were infected with adenovirus with cDNA either for β-galactosidase (Lac Z) or each Smad form, before further manipulation and TGF-β1 treatment and then lysates were harvested, as indicated in Fig. 3. Lysates were used for immunoblotting for the indicated molecules. Data shown are representative of two isolated experiments.

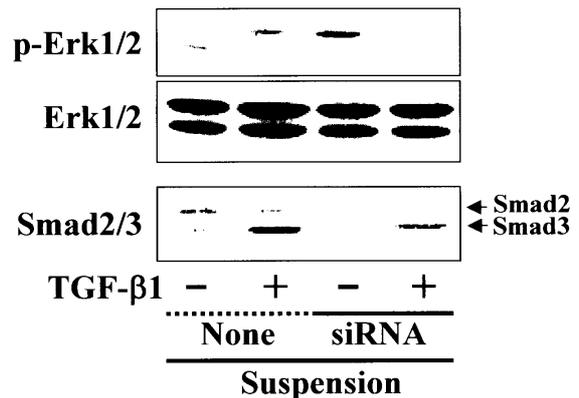


Figure 5. TGF-β1-mediated enhancement of Erk1/2 activity in suspension is abolished by suppression of Smad2 protein. Cells were first transfected with siRNA to down-regulate Smad2. Twenty-four hours after transfection, cells were manipulated to be in suspension in the absence or presence of TGF-β1 treatment for 20 h. Then lysates were prepared and used for Western blotting for indicated molecules. Data shown are representative of two independent experiments.

treatment, which may be counteracted by Smad7, in a cell adhesion status-dependent manner.

Discussion

In this study, we observed that Smad2 mediates TGF-β1-induced activation of Erk1/2 activity in suspended gastric cells. In addition, this Smad2-mediated Erk1/2 activation in suspended cells was abolished by suppression of Smad2 using its siRNA. However, adherent cells did show a decrease in Erk1/2 activity on TGF-β1 treatment for a long period (20 h), and did not significantly respond to any Smad

overexpression. This suggests that, at least in suspension of gastric carcinoma cells, Erk1/2 is activated by TGF- β 1 in a Smad2-mediated manner.

TGF- β 1 transduces intracellular signaling activities in Smad-dependent or -independent manner. Generally Smad-dependent TGF- β 1 signal transduction results in complex regulation of transcription responses, leading to growth arrest, inhibition and apoptosis of epithelial cells. In addition, in cases of Smad-independent pathways, TGF- β 1 still transduces signals to diverse intracellular signaling molecules including MAPKs (Erk1/2, JNKs, and p38 SAPK), RhoA GTPases (Rac1, CDC42, and RhoA), c-Src, and so on (18,27). Through this signal transduction independent of Smads, TGF- β 1 signal pathway can also regulate diverse cellular functions such as epithelial-mesenchymal transdifferentiation (EMT) (9).

Erk1/2 is a well-known mitogenic signaling molecule activated by diverse growth-stimulating stimuli and enhances cyclin D1 accumulation leading to G1/S cell cycle progression (28). However, it is also regulated by a cytokine growth-inhibitory to epithelial cells, TGF- β 1. In many systems, TGF- β 1 treatment has resulted in activation of Erk1/2 (29,30) and it is through Ras protein (19,22,23). In addition to the previous studies, in this study, SNU16 α 5Ad showed an activation of Ras/Erks cascade by TGF- β 1 only when the cells were in suspension. Unlike the previous study in which TGF- β 1 was treated for a short time, \leq 90 min (19), the current study showed clearly that TGF- β 1 treatment for 20 h (or at least 6 h is enough for adhesion of a significant cell population) resulted in an increase in Erk1/2 activity only in suspended cells, but a decrease in adherent cells. The longer treatment was performed because we had interests in a cell-adhesion-dependent regulation of TGF- β 1-mediated Erk1/2 activity and this cell line showed a minimally significant cell adhesion 6 h after subculture or replating. Usually normal and established cells show no activation by growth factors and hormones when cells are in suspension or not spread (i.e., plated on polylysine without spreading). So, SNU16 α 5Ad seems somehow to have an ability to respond to extracellular stimuli even in suspension, by virtue of a Smad2-mediated pathway. This may be due to the fact that this cell line is a subclone of a malignant cancer and anchorage-independent gastric carcinoma cell line SNU16, and may suggest that the selection of adherent cells did not result in a complete re-conversion to anchorage-dependent normal cells. However, in this cell system, Erk1/2 seems not always activated by TGF- β 1. Even though Erk1/2 was activated by TGF- β 1 in suspension, TGF- β 1 treatment to adherent SNU16 α 5Ad cells resulted in an opposite response, a decrease in Erk1/2 activity, indicating a cell adhesion-dependent regulation of Erk1/2 activity by TGF- β 1 in this cell system. This suggests that differential regulation mechanisms underlie the TGF- β 1-mediated regulation of Erk1/2 activity, depending on cell adhesion status. Further, this differential regulation of Erk1/2 activities by TGF- β 1 appears not to involve differential level of Smad2 in suspension, because similar expression level of Smad2 was observed either in the absence or the presence of TGF- β 1 treatment.

Like this study (as shown in an adherent condition), inhibitory effects of TGF- β 1 on Erk1/2 activity have also been reported. Previously it was shown that Erk2 activation by basic fibroblast growth factor (bFGF) was inhibited by TGF- β 1 treatment in smooth muscle cells (31). In the latter study, there seem presumably different mechanistic regulations of Erk1/2 by TGF- β 1, depending on nature of TGF- β 1 treatment; interestingly, a shorter TGF- β 1 treatment (40 min) reduced threonine-phosphorylation of Erk2, indicating an action of a threonine phosphatase, whereas a longer treatment (4 h) reduced both threonine- and tyrosine-phosphorylation levels, indicating a blocking of the Erk cascade or directly Erk itself (31). Therefore, in the gastric carcinoma cells, TGF- β 1 may differentially regulate Erk1/2 activity, depending on TGF- β 1 treatment period and cellular environment such as cell adhesion status, as suggested previously (27).

In this study, Smad2, but not the other Smads 3 or 4, mediated a dramatic activation of Erk1/2 only in suspended cells, indicating a Smad2-mediated regulation of Erk1/2 activity by TGF- β 1 treatment depending on cellular environment. Similar to this study, it was also reported that Smad4 could mediate activation of Erk1/2 in pancreatic acinar cells with a maximal peak at 4 h after TGF- β 1 treatment, in a concentration-dependent and protein synthesis-dependent manner (32). Also TGF- β 1-mediated Erk1/2 activation through Smads and Ras led to TGF- β 1 production (22,33). In addition to the previous studies, this current study further indicates that TGF- β 1-mediated regulation of Erk1/2 and its Smad2-dependency may be dependent on cell adhesion status.

Many previous studies have also shown that TGF- β 1-mediated regulation of Erk1/2 can result in Smad phosphorylation and thus affects Smad signaling (18,20). In addition to studies of TGF- β 1-mediated regulation of Erk1/2 or MAPKs explained above, there is evidence that Erk1/2 or other MAPKs (JNK and p38) could regulate Smad signaling, suggesting a bidirectional cross-talk between Smads and MAPKs including Erk1/2. Ras/Erks cascade or oncogenic Ras leads to Erks-mediated phosphorylation of Smad2 and 3 in the linker region between MAD homology domain 1 (MH1) and MH2, with accumulation of phosphorylated Smads in nucleus (34). Erk1/2 activated by TGF- β 1 can also lead to TGF- β 1 expression via Smad-dependent transcription (22,33). Therefore, the bi-directional cross-talk or convergence between Smads and MAPKs including Erk1/2 is suggested to diversify or define cellular response to TGF- β 1 (18). Therefore, Smad2-mediated activation of Erk1/2 on TGF- β 1 treatment only in suspended gastric carcinoma cells may presumably result in transcriptions of genes in the absence of cell adhesion signaling. Adherent cells may restrict TGF- β 1-mediated transcription processes via a reduction of Erk1/2 activity, which may result from antagonistic cross-talks of TGF- β 1 signaling with integrin-mediated cell adhesion signaling.

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