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SB-431542 and Gleevec Inhibit Transforming Growth Factor-β-Induced Proliferation of Human Osteosarcoma Cells

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ABSTRACT

Transforming growth factor-β (TGF-β) has growth-stimulating effects on mesenchymal cells and several tumor cell lines. The signaling pathway for this effect is, however, not well understood. We examined how TGF-β stimulates proliferation of MG63 human osteosarcoma cells. Two distinct type I receptors for TGF-β, ALK-1 and ALK-5, were expressed and functional in MG63 cells. Of these two receptors, ALK-5 appears to be responsible for the growth stimulation because expression of constitutively active ALK-5, but not ALK-1, stimulated proliferation of MG63 cells. SB-431542 (0.3 μM), a novel inhibitor of ALK4/5/7 kinase, suppressed TGF-β-induced growth stimulation. DNA microarray analysis as well as quantitative real-time PCR analysis of RNAs from TGF-β-treated cells demonstrated that several growth factors, including platelet-derived growth factor AA, were induced in response to TGF-β in MG63 cells. Gleevec (1 μM) as well as AG1296 (5 μM) inhibited TGF-β-induced growth stimulation of MG63 cells, suggesting that platelet-derived growth factor AA was mainly responsible for the growth-stimulatory effect of TGF-β. We also examined the mechanisms of perturbation of growth-suppressing signaling in MG63 cells. We found that expression of c-Myc, which is down-regulated by TGF-β in many other cells, was up-regulated in MG63 cells, suggesting that up-regulation of c-Myc expression may be the mechanism canceling growth-suppressing signaling of TGF-β in MG63 cells.

INTRODUCTION

TGF[6]-β is a prototype of the cytokines of the TGF-β superfamily, which includes TGF-βs, activins/inhibins, and BMPs (1). Members of the TGF-β superfamily have pleiotropic functions in a wide variety of target cells in physiological as well as pathological processes. One outstanding feature of TGF-β is its growth-inhibitory effect (2, 3). TGF-β acts as a potent growth inhibitor for divergent cell types, including epithelial, endothelial, and hematopoietic cells. Loss of TGF-β-mediated growth inhibition is thought to play a role in tumorigenesis (2). TGF-β also acts as a growth stimulator for some cells of mesenchymal origin such as fibroblasts, chondrocytes, and osteoblasts, as well as several tumor cell lines (4).

The signal transduction pathways leading to the growth inhibition have been studied well at the molecular level (2). TGF-β binds to two different types of serine/threonine kinase receptors, termed type I and type II. Type I receptor is activated by type II receptor upon ligand binding and transduces signals into cytoplasm through phosphorylation of receptor-regulated Smads. ALK-5 is a ubiquitously expressed type I receptor for TGF-β, transmitting signals via Smad2/3. ALK-1 is also a type I receptor for TGF-β; it is predominantly expressed in vascular endothelial cells and transmits signals via Smad1/5 (5). Phosphorylated receptor-regulated Smads interact with Co-Smad (Smad4), translocate to the nucleus, and regulate transcription of target genes in cooperation with transcriptional activators/repressors as well as with coactivators/corepressors (6).

In most types of cells, TGF-β arrests cell cycle progression in the G_1 phase by down-regulating expression of c-Myc and cdc25A and up-regulating that of CDKIs, $p21^{WAF1/CIP1}$ and/or $p15^{Ink4B}$ (2). A Smad-responsive element was identified in the c-myc promoter (7, 8), and the activated Smad complex (Smad2/3-Smad4) has been found to suppress transcription of c-myc in cooperation with p107 and E2F4/5 (8). Down-regulation of c-Myc thus leads to induction of $p21^{WAF1/CIP1}$ and $p15^{Ink4B}$, because c-Myc physically interacts with Smad2/3 and suppresses the function of Sp1-Smad complex in the transcriptional activation of $p21^{WAF1/CIP1}$ and $p15^{Ink4B}$ (9, 10). In contrast, pathways leading to the growth stimulation by TGF-β have not been well elucidated. Thus far, several mechanisms for this growth stimulation have been proposed. TGF-β-induced up-regulation of growth factors including PDGF and FGF has been reported in fibroblasts, Ito cells, and prostate cancer cells (11–18). Down-regulation of CDKIs by TGF-β has been reported in rat osteoblasts ($p57^{Kip2}$; Refs. 19, 20) and H-ras-transfected colon carcinoma cells ($p21^{WAF1/CIP1}$; Refs. 21). Recently, Goumans *et al.* (22) reported that ALK-1 but not ALK-5 acts as a signaling receptor for TGF-β in the transduction of cell proliferation signals in endothelial cells.

Osteosarcoma is one of the most common nonhematological primary malignant tumors of bone (23). Bone is an organ that produces and stores large amounts of TGF-β (24). Recent findings suggest that TGF-β is involved in the progression of osteosarcoma; TGF-β stimulates the growth of several osteosarcoma cell lines in culture (25–27), and overexpression of TGF-β is observed in osteosarcoma tissue by immunohistochemistry, mRNA *in situ* hybridization, and RT-PCR (28, 29). It thus appears important to elucidate how TGF-β acts on osteosarcoma cells.

In the present study, we examined the signal transduction mechanism for the growth-stimulating effect of TGF-β on MG63 human osteosarcoma cells. We found that SB-431542, a novel TGF-β type I kinase inhibitor, as well as Gleevec, inhibited TGF-β-stimulated proliferation of MG63 cells.

MATERIALS AND METHODS

Chemicals, Antibodies, and Recombinant Adenoviruses. SB-431542 was synthesized as described (30) and stored as a solution in DMSO. This solution was used after diluting with medium for each assay. Gleevec (STI571) capsules were purchased from Novartis Pharma (Basel, Switzerland). The contents of one capsule were dissolved in 17 ml of distilled water, centrifuged, filtered, and used as 10 mM stock solution. AG1296 was from Calbiochem (San Diego, CA). TGF-β1, TGF-β3, and BMP-6 were from R & D systems (Minneapolis, MN). Activin-A was a generous gift from Dr. Eto (Ajinomoto Co. Ltd., Tokyo, Japan). FGF-2, HB-EGF, PDGF-AA, and PDGF-BB were

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[6] The abbreviations used are: TGF, transforming growth factor; BMP, bone morphogenetic protein; ALK, activin receptor-like kinase; CDKI, cyclin-dependent kinase inhibitor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; RT-PCR, reverse transcription-PCR; HB-EGF, heparin-binding EGF-like growth factor; PAI-1, plasminogen activator inhibitor 1; PlGF, placenta growth factor.

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from PeproTech (Rocky Hill, NJ). Anti-phospho-Smad1/5 antibody was from Cell Signaling Technology (Beverly, MA). Anti-phospho-Smad2 antibody was from United Biomedical, Inc. (Hauppauge, NY). Anti-Smad1 antibody and anti-Smad2 antibody were from Transduction Laboratories (Lexington, KY). Anti-c-Myc antibody and anti-p21 antibody were from Oncogene Research Products (San Diego, CA). Anti-lamin A/C antibody was from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-α-tubulin antibody (T9026) was from Sigma-Aldrich (St. Louis, MO). Antisera against type I receptors were described previously (31). Recombinant adenoviruses carrying LacZ, a constitutively active form of ALK-1 (ALK-1QD), or ALK-5 (ALK-5TD) were described previously (32, 33).

Cell Culture. MG63 human osteosarcoma cells were maintained in Minimum Essential Medium (Life Technologies, Inc., Carlsbad, CA) containing 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin. NIH3T3 cells were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin.

Cell Proliferation Assay. To explore the effects of ligands, cells were seeded at a density of 8×10^4 cells/well in 6-well plates and starved (0.1% FCS for MG63 cells and 0.5% FCS for NIH3T3 cells) for 24 h before ligand stimulation. Media containing various ligands were exchanged at 48-h intervals. Cells were trypsinized and counted by a Coulter counter on days 2, 4, and 6 after ligand stimulation. The experiments were performed in triplicate. To explore the effects of constitutively active receptors, cells were seeded at a density of 2×10^5 cells/well in 6-well plates. The next day, cells were infected with adenoviruses carrying various cDNAs at a multiplicity of infection of 100. Cells were trypsinized and counted on day 3.

Thymidine Incorporation Assay. Cells were seeded at a density of 2×10^4 cells/well in 24-well plates and cultured overnight. Then serum concentration in the medium was decreased to 0.1%, and the cells were incubated for another 24 h, followed by stimulation with various ligands. Forty-eight h after stimulation, the cells were labeled with [^{3}H]thymidine for 2 h. Thymidine incorporation into the TCA-insoluble fraction was analyzed as described previously (34).

Immunoblotting, Affinity Cross-Linking, and Immunoprecipitation. Immunoblotting was performed as described previously (34). Nuclear extract was prepared using NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL). For affinity cross-linking, recombinant TGF-β1 was iodinated using the Chloramine-T method. MG63 cells were affinity-labeled with [^{125}I]-labeled TGF-β1 using 0.27 mM disuccinimidyl suberate (Pierce Biotechnology), followed by immunoprecipitation using specific antisera against ALK-1 or ALK-5. Immune complexes were analyzed as described previously (34).

RNA Extraction and RT-PCR Analysis. Total RNA was extracted from MG63 cells using ISOGEN (Nippon Gene, Toyama, Japan). For RT-PCR analysis, first-strand DNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamer primers. Expression of various signaling components was examined by semiquantitative RT-PCR analysis. PCR products were separated by electrophoresis in agarose gels (1%) and visualized with ethidium bromide. The primer sequences, PCR programs, and expected sizes of PCR products were described previously (35).

Oligonucleotide Microarray Analysis. Total RNAs were extracted from MG63 or HaCaT cells at 0, 1, and 4 h after stimulation by TGF-β3 (1 ng/ml). We used the total RNAs to prepare cRNA and conducted oligonucleotide microarray analysis using GeneChip Human Genome U95A (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions.

Quantitative Real-Time PCR Analysis. Total RNAs were extracted from MG63 cells after various treatments, and first-strand cDNAs were synthesized using the Superscript First-Strand Synthesis System (Invitrogen) with random hexamer primers. Quantitative real-time RT-PCR analysis was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) as described (33). The primer sequences are available upon request.

RESULTS

TGF-β Stimulates Proliferation of MG63 Cells. MG63 cells were cultured in the presence of 0.1% FBS, and cell numbers were counted at 48-h intervals (Fig. 1*A*). Cell numbers were significantly increased (1.5-fold at day 4) by TGF-β treatment (1 ng/ml), although this effect was rather late in onset and first appeared at day 4. We also examined the effect of TGF-β on DNA synthesis by MG63 cells (Fig. 1*B*). TGF-β caused a dose-dependent increase in [^{3}H]thymidine incorporation. Maximal response was observed above the TGF-β concentration of 100 pg/ml. The response was maintained even at higher concentrations up to 5 ng/ml. These results indicate that TGF-β positively regulates proliferation of MG63 cells.

ALK-1 Is Expressed and Functional in MG63 Cells. To characterize the signaling pathways of the TGF-β superfamily in MG63 cells, we first examined the expression profiles of signaling components of TGF-β superfamily, *i.e.*, the receptors and Smad proteins, by RT-PCR analysis. Most of the signaling components except for ALK-6, ActR-IIB, and Smad6 were detected in MG63 cells (data not shown). Smad6 was not detected without TGF-β stimulation but was detected after stimulation with TGF-β. Notably, ALK-1 was expressed in MG63 cells. ALK-1 is a type I receptor for TGF-β predominantly expressed in vascular endothelial cells. In vascular endothelial cells, ALK-1 is reported to be involved in TGF-β-induced cell growth and cell motility (22). Because it appeared possible that the growth-stimulatory effect of TGF-β on MG63 cells could be attributed to the presence of ALK-1, we examined whether ALK-1 is functional in MG63 cells.

MG63 cells were affinity-labeled with [^{125}I]-labeled TGF-β followed by cross-linking and immunoprecipitation by antisera against ALK-1 or ALK-5 (Fig. 2*A*). Cross-linked bands were observed when anti-ALK-1 or anti-ALK-5 was used but not when preimmune serum was used. This result indicated that ALK-1 as well as ALK-5 is expressed on the MG63 cell surface and functional in binding to TGF-β.

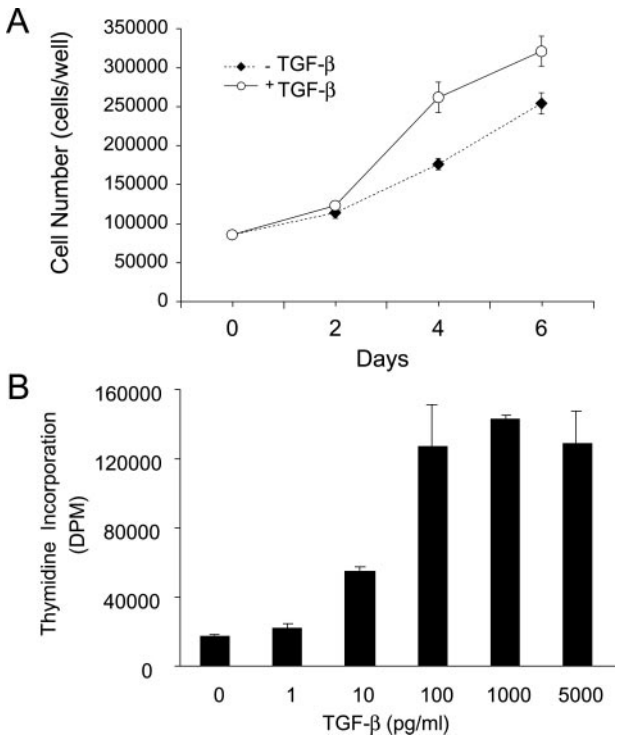

Fig. 1. TGF-β positively regulates proliferation of MG63 cells. *A*, cell proliferation assay. MG63 cells were cultured in the presence or absence of TGF-β3 (1 ng/ml), and cell numbers were counted on days 2, 4, and 6 after treatment. Each value represents the mean of triplicate determinations; *bars*, SD. *B*, [^{3}H]thymidine incorporation assay. MG63 cells were treated with various concentrations of TGF-β3 (0–5 ng/ml) and labeled with [^{3}H]thymidine for 2 h. Each value represents the mean of triplicate determinations; *bars*, SD.

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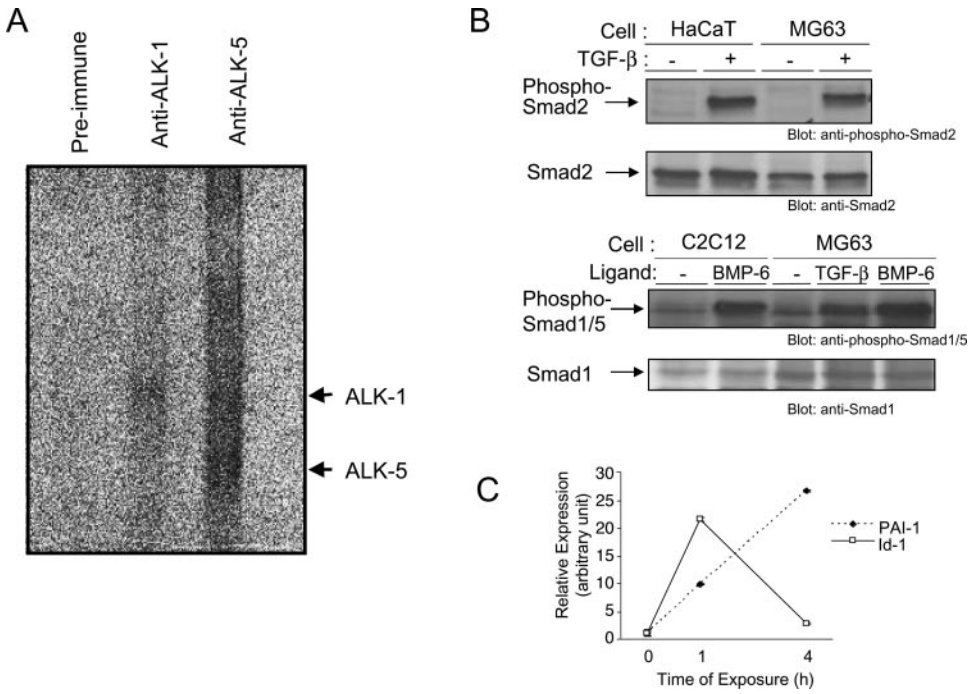

Fig. 2. ALK-1 is expressed and functional in MG63 cells. *A*, affinity cross-linking and immunoprecipitation analysis. MG63 cells were affinity-labeled with [^{125}I]-labeled TGF-β1, followed by immunoprecipitation using specific antisera against ALK-1 or ALK-5. *B*, immunoblotting analysis of phosphorylated Smads. MG63 cells were starved for 24 h and treated with TGF-β3 (1 ng/ml) for 1 h (for Smad2 phosphorylation) or 2 h (for Smad1/5 phosphorylation). Total cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were blotted with anti-phospho-Smad2 antibody and anti-Smad2 antibody (*upper panel*) or anti-phospho-Smad1/5 antibody and anti-Smad1 antibody (*lower panel*). Positive controls were as follows: HaCaT cells treated with TGF-β3 (1 ng/ml) for 1 h (for Smad2), and C2C12 cells treated with BMP-6 (50 ng/ml) for 1 h (for Smad1/5). *C*, relative mRNA expression of target genes for ALK-5 (PAI-1) and ALK-1 signal (Id-1) was measured by quantitative real-time PCR. Total RNAs were extracted from MG63 cells before and after TGF-β treatment. Fold-induction by TGF-β treatment is indicated. Each value represents the mean of triplicate determinations; *bars*, SD.

We next examined whether ALK-1 in MG63 cells can transmit signals. It has been reported that TGF-β stimulation of endothelial cells induces phosphorylation of Smad1/5 as well as Smad2/3; the former is ALK-1-dependent, and the latter is ALK-5-dependent (22). We observed phosphorylation of Smad1/5 as well as Smad2/3 in MG63 cells in response to TGF-β stimulation (Fig. 2*B*). We also quantified the expression level of mRNA for PAI-1 (as an ALK-5 target) as well as Id1 (as an ALK-1 target; Ref. 22; also Fig. 4*A*) by quantitative real-time PCR analysis (Fig. 2*C*). In response to TGF-β, expression of PAI-1 as well as Id1 was induced, suggesting that both ALK-5 and ALK-1 signals were transmitted in MG63 cells.

SB-431542 Inhibited Cell Proliferation Induced by TGF-β. The next question we addressed was which of the type I receptors is responsible for the growth stimulation of MG63 cells. We used SB-431542, which is reported to be a specific inhibitor of ALK-4, ALK-5, and ALK-7, the type I receptors for activin, TGF-β, and nodal (36). Cell proliferation assay was performed in the presence of 0.3 μM SB-431542. As shown in Fig. 3*A*, SB-431542 inhibited cell proliferation induced by TGF-β. In contrast, it did not inhibit PDGF-BB-induced cell proliferation, thus excluding the possibility of a nonspecific effect.

The specificity of inhibition of type I receptors was examined by Smad phosphorylation and quantitative real-time PCR analysis. Unexpectedly, SB-431542 inhibited the phosphorylation of both Smad2/3 and Smad1/5 induced by TGF-β (Fig. 3*B*), although it did not inhibit the phosphorylation of Smad1/5 induced by BMP-6. Furthermore, SB-431542 inhibited TGF-β-induced Id-1 expression as well as PAI-1 (data not shown). These results indicated that SB-431542 affected both ALK-1- and ALK-5-mediated signaling induced

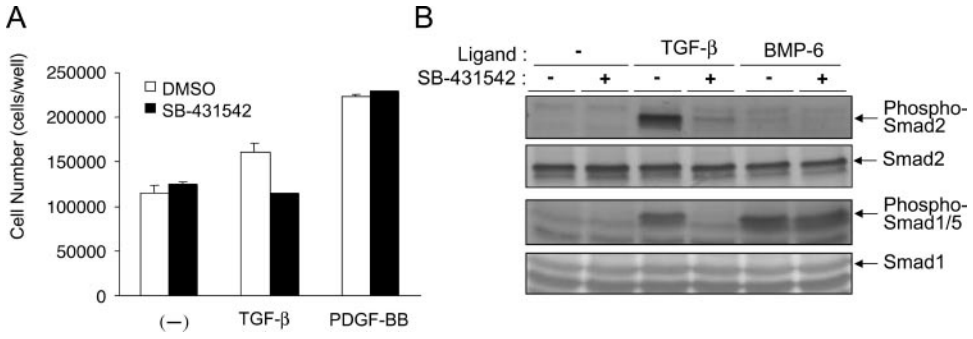

Fig. 3. Effect of SB-431542 on TGF-β-stimulated responses in MG63 cells. *A*, effect of SB-431542 on TGF-β-stimulated cell proliferation. MG63 cells were treated with TGF-β3 (1 ng/ml) or PDGF-BB (10 ng/ml), and cell numbers were counted on day 4. SB-431542 (0.3 μM) or DMSO (vehicle) was added 30 min before ligand stimulation. Each value represents the mean of triplicate determinations; *bars*, SD. *B*, effect of SB-431542 on Smad phosphorylation. MG63 cells were treated with TGF-β3 (1 ng/ml) or BMP-6 (50 ng/ml) for 1 h in the presence or absence of SB-431542 (0.3 μM), which was added 30 min before ligand stimulation. Phosphorylation of Smad proteins was examined by immunoblotting using anti-phospho-Smad2 and anti-Smad2 (*upper two panels*) or anti-phosho-Smad1/5 antibody and anti-Smad1 (*lower two panels*).

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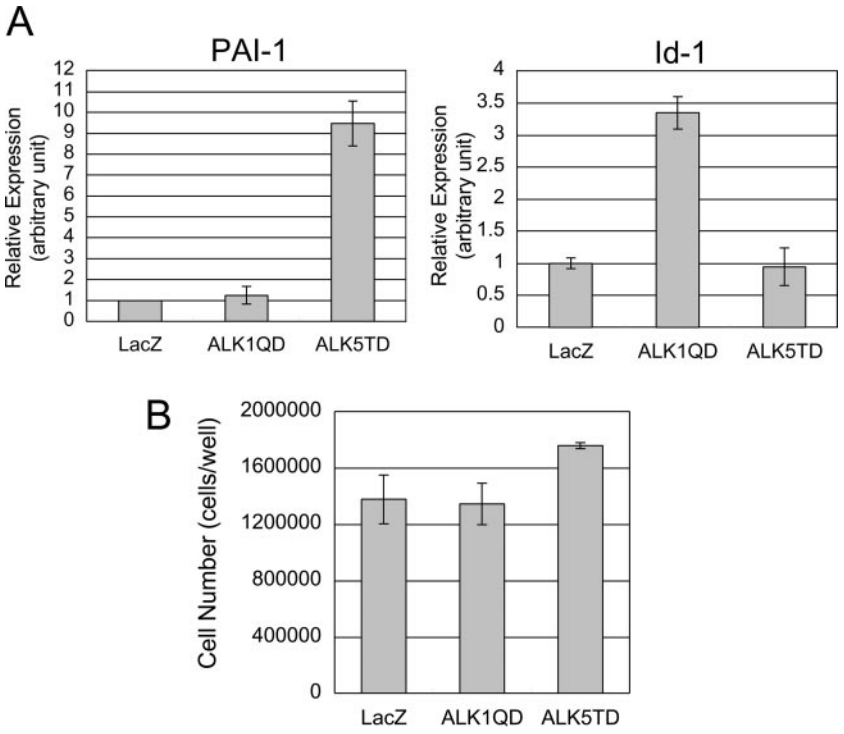

Fig. 4. Effects of constitutively active forms of ALK-1 (ALK-1QD) and ALK-5 (ALK-5TD) on MG63 cells. MG63 cells infected with recombinant adenoviruses carrying LacZ, ALK-1QD, or ALK-5TD at a multiplicity of infection of 100 plaque-forming units/cell were harvested 72 h after infection. *A*, induction of target genes for ALK-5 (PAI-1) and ALK-1 (Id-1) was examined by quantitative real-time PCR analysis. *B*, cell numbers were counted. Each value represents the mean of triplicate determinations; *bars*, SD.

by TGF-β ligand. The type I receptor responsible for growth stimulation could not be identified with the use of SB-431542.

Constitutively Active ALK-5 but not ALK-1 Induced Proliferation of MG63 Cells. We next examined effects of constitutively active forms of ALK-1 (ALK-1QD) and ALK-5 (ALK-5TD) on proliferation of MG63 cells. MG63 cells were infected with adenoviruses carrying LacZ, ALK-1QD, or ALK-5TD. Expression of ALK-1QD caused induction of Id-1, whereas expression of ALK-5TD caused induction of PAI-1 (Fig. 4*A*), indicating that both receptors transmitted signals in MG63 cells. Results of cell proliferation assay, however, demonstrated that ALK-5 but not ALK-1 induced cell proliferation in MG63 cells (Fig. 4*B*). We thus concluded that ALK-5 is mainly responsible for growth stimulation by TGF-β in MG63 cells.

Activin-A but not BMP-6 Stimulated Proliferation of MG63 Cells. We also examined the effects of other ligands in the TGF-β superfamily, *i.e.*, activin-A and BMP-6, on proliferation of MG63 cells. In most cell types, activin-A transmits signals via ALK-4 and Smad2/3, whereas BMP-6 transmits Smad1/5 signals. The selectivity of signal transduction in MG63 cells was confirmed on the basis of induction of target genes. Activin-A induced PAI-1, although the intensity of induction was weaker than that by TGF-β, whereas it did not induce Id1. In contrast, BMP-6 induced Id1, but induced PAI-1 only weakly (Fig. 5*A*).

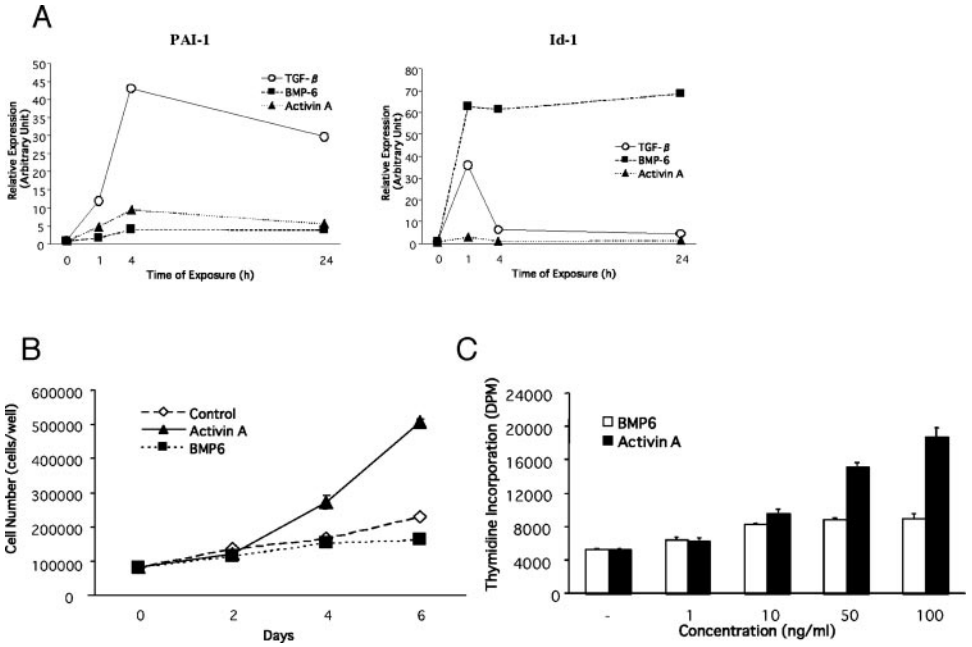

Fig. 5. Effects of activin-A and BMP-6 on MG63 cells. *A*, induction of target genes (PAI-1 and Id-1) was examined in cells treated with TGF-β3 (1 ng/ml), BMP-6 (50 ng/ml), or activin-A (100 ng/ml) for 0, 1, 4, or 24 h by quantitative real-time PCR. Each value represents the mean of triplicate determinations; *bars*, SD. *B*, cell proliferation assay was performed in cells treated with activin-A (100 ng/ml) or BMP-6 (50 ng/ml). Cells were counted on days 2, 4, and 6. Each value represents the mean of triplicate determinations; *bars*, SD. *C*, [^{3}H]thymidine incorporation assay was performed in the presence of various concentrations of activin-A or BMP-6 (0–100 ng/ml). Each value represents the mean of triplicate determinations; *bars*, SD.

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Table 1 *Genes regulated by TGF-β in MG63 cells and HaCaT cells*

Total RNAs were extracted from MG63 or HaCaT cells at 0, 1, and 4 hours after stimulation by TGF-β3 (1 ng/ml) and used to prepare cRNA. Oligonucleotide microarray analysis was conducted using GeneChip Human Genome U95A (Affymetrix). Intensity <50 means that the level of expression is as low as background.

		MG63					HaCaT				
		Control	TGF-β 1 h		TGF-β 4 h		Control	TGF-β 1 h		TGF-β 4 h	
Accession number	Description	Intensity	Intensity	Fold change	Intensity	Fold change	Intensity	Intensity	Fold change	Intensity	Fold change
Representative TGF-β target genes											
M14083	*PAI-1*	105.2	1021.1	9.4	3173.3	29.7	50.7	259.3	5.1	695.9	12.9
X51345	*jun B*	388.5	1429.9	3.7	466.9	1.6	291.1	596.6	2.8	474.9	2.1
AF010193	*Smad7*	88.1	412.2	4.7	228.6	2.6	46.4	173.3	4.7	117.9	3.2
U76702	*follistatin-like 3*	8.4	59.8	7.1	385.3	35.8	80.9	121.2	1.5	482.7	4.7
U03106	*p21*	133	341.8	2.8	239.4	1.1	252.7	470.5	1.9	470.5	2.6
V00568	*c-Myc*	166	500.8	3	263.4	1.6	295.3	198.1	−1.5	137.4	−2.1
Representative BMP target genes											
X77956	*Id-1*	106.3	2291.3	21.6	295.6	2.8	1740.9	2477.6	1.4	914.4	−1.9
D13891	*Id-2*	78.2	555.1	7.1	125.6	1.6	37.8	101.2	2.8	25.6	−1.7
AL021154	*Id-3*	171.8	1037.1	6	544.1	3.2	748.1	963.5	1.3	879.6	1.2
AF035528	*Smad6*	53	100.9	1.9	119.1	2.2	6.2	17.6	1.4	20.4	>2.2
Growth factors and receptors											
X06374	*PDGF-A*	46.7	46.7	2.3	240.6	7.5	127.3	100.5	−1.3	146	1.1
X63966	*PDGF-B*	−59.3	−56.6	>1.5	−34.8	<−1.9	−10.9	−14.7	<−1.7	−20	<−2.3
M21574	*PDGFRα*	398.4	375.4	−1.1	228.2	−2	9.1	9.9	1.1	8	−1.1
J03278	*PDGFRβ*	231.6	243.6	1.1	553.3	2.5	40.4	16.1	−1.9	23.4	−1.2
M27968	*FGF-2*	99.3	105	1.1	327.1	3.6	13.5	13.5	1.3	17.4	1.3
X54936	*PIGF*	−3.6	8.7	>3.1	117.1	>18.3	11	6.1	−1.8	−1.4	<−2.8
M60278	*HBEGF*	0.9	158.7	>26.7	260	>34.4	−12.1	31.5	>9.4	0.6	>2.8

Effects of these ligands on proliferation of MG63 cells were then examined. Activin-A stimulated proliferation and thymidine incorporation in MG63 cells at concentrations around 50–100 ng/ml, whereas BMP-6 was only weakly effective (Fig. 5, *B* and *C*). These findings further confirmed that the Smad2/3 pathway but not the Smad1/5 pathway plays a major role in transmitting proliferation signals in MG63 cells.

DNA Microarray Analysis of TGF-β-regulated Genes in MG63 Cells. To elucidate the signaling pathway involved in the cell proliferation induced by TGF-β, we analyzed TGF-β-regulated genes using DNA microarray. We used HaCaT cells as a reference, because their growth is inhibited by TGF-β. Several representative target genes of TGF-β signaling were induced in MG63 cells (Table 1), indicating that the signaling pathway of TGF-β was not greatly altered in the cells. However, we found four unique features:

(*a*) Several growth factors were induced in MG63 cells in response to TGF-β stimulation. They were PDGF-A, FGF-2, HB-EGF, and PlGF. In HaCaT cells, significant induction of these growth factors was not observed. It is thus possible that growth stimulation of MG63 cells by TGF-β is mediated through the induction of these growth factors.

(*b*) Expression of c-Myc was up-regulated in MG63 cells. In most cells including HaCaT cells, expression of c-Myc is down-regulated after TGF-β stimulation.

(*c*) Expression of $p21^{WAF1/CIP1}$ was up-regulated in both MG63 cells and HaCaT cells, only transiently in MG63 cells but in rather sustained fashion in HaCaT cells.

(*d*) As expected from the observation that MG63 cells express ALK-1, Id proteins were rapidly induced in MG63 cells. As shown in Fig. 4, the possibility of involvement of ALK-1 in the growth-stimulating signaling had already been excluded. We thus further examined the first three features.

TGF-β-induced Growth Factors in MG63 Cells: Their Effects on Proliferation of MG63 Cells. Induction of PDGF-A, FGF-2, HB-EGF, and PlGF by TGF-β was confirmed by real-time PCR analysis (Fig. 6 and data not shown). These results are consistent with those of DNA microarray analysis. We also observed that induction of these growth factors was completely inhibited by SB-431542 (data not shown). Among the induced growth factors, however, PlGF seemed unlikely to be involved in the growth stimulation of MG63 cells, because the receptor for PlGF (Flt1) is not expressed in the cells.

We next examined effects of growth factors that were up-regulated by TGF-β in MG63 cells. As shown in Fig. 7*A*, PDGF-AA and FGF-2 were effective in stimulating thymidine incorporation in MG63 cells, whereas HB-EGF was only weakly effective.

To determine which of the three growth factors, PDGF-AA, FGF-2, and HB-EGF, is mainly involved in the growth stimulation by TGF-β, we used a tyrosine kinase inhibitor, Gleevec (STI571), which inhibits PDGF receptor kinase (Ref. 37; Fig. 7*B*). MG63 cells were cultured in the presence of the inhibitor and treated with TGF-β, PDGF-AA, FGF-2, or HB-EGF. Gleevec (1 μM) inhibited the growth stimulation induced by TGF-β as well as that by PDGF-AA but did not inhibit the stimulatory effects of FGF-2 and HB-EGF. A similar result was obtained when AG1296, another inhibitor of the PDGF receptor

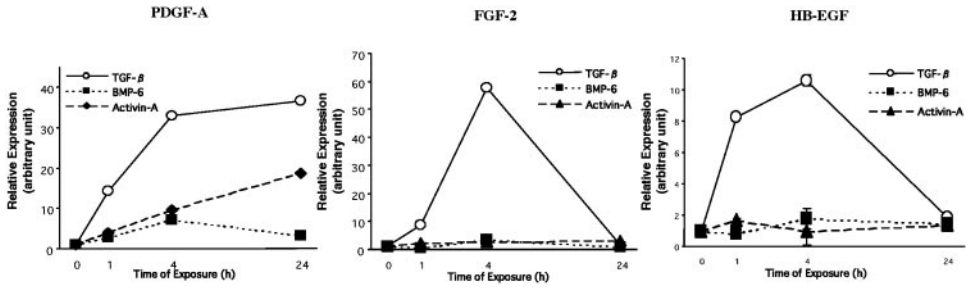

Fig. 6. Up-regulation of growth factors by signaling from the TGF-β superfamily. MG63 cells were starved for 24 h and treated with TGF-β3 (1 ng/ml), BMP-6 (50 ng/ml), or activin-A (100 ng/ml). Total RNAs were extracted from the cells at 0, 1, 4, and 24 h after treatment and analyzed by quantitative real-time PCR using specific primers for PDGF-A, FGF-2, or HB-EGF. Each value represents the mean of triplicate determinations; *bars*, SD.

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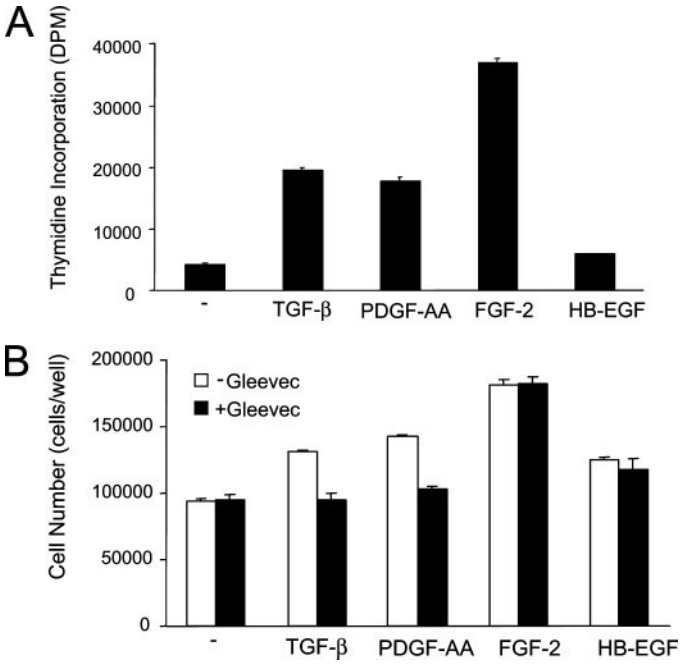

Fig. 7. Effect of up-regulated growth factors on proliferation of MG63 cells. *A*, [^{3}H]thymidine incorporation assay was performed in the presence of TGF-β3 (1 ng/ml), PDGF-AA (10 ng/ml), FGF-2 (5 ng/ml), or HB-EGF (5 ng/ml). Each value represents the mean of triplicate determinations; *bars*, SD. *B*, cell proliferation assay was performed in the presence of various ligands (concentration of each ligand is described above). The effect of Gleevec (1 μM) was also examined. Gleevec was added 30 min before ligand stimulation. Cells were counted on day 4. Each value represents the mean of triplicate determinations; *bars*, SD.

kinase (38), was used (5 μM; data not shown). These results suggested that the effect of TGF-β was mediated principally through PDGF signaling, although the involvement of FGF signaling cannot be excluded.

We also examined the induction of growth factors by activin-A and BMP-6 (Fig. 6). Activin-A, which stimulated growth of MG63 cells, induced PDGF-A but neither FGF-2 nor HB-EGF. BMP-6, which did not stimulate the growth of MG63 cells, induced none of the three growth factors. These results further supported a major role of PDGF signaling in the growth stimulation of MG63 cells by ligands of the TGF-β superfamily.

Control of Cell Cycle Regulators by TGF-β in MG63 Cells. It is also important to elucidate why growth-inhibitory signaling by TGF-β is ineffective in MG63 cells. As described above, expression of both c-Myc and p21$^{WAF1/CIP1}$ was up-regulated on DNA microarray analysis. We confirmed the induction of c-Myc and p21$^{WAF1/CIP1}$ using quantitative real-time PCR analysis and found that it was inhibited by SB-431542 (data not shown). p15 mRNA was not detected in MG63 cells.

We further examined the protein levels of c-Myc and p21$^{WAF1/CIP1}$ after TGF-β treatment (Fig. 8*A*). Expression of c-Myc protein was up-regulated from 12 h after TGF-β treatment, and this up-regulation was maintained even at 24 h after the treatment. Expression of p21$^{WAF1/CIP1}$ protein was also up-regulated, but maximal expression was observed at 12 h, with decrease to basal level by 24 h. We also confirmed that nuclear p21$^{WAF1/CIP1}$, which has cell cycle inhibitory activity, was actually up-regulated by cell-fractionation experiment (Fig. 8*B*). These results suggest that, among the growth-inhibitory signals by TGF- β, the pathway leading to c-Myc down-regulation was abrogated, and that a signal up-regulating c-Myc expression may be transmitted in MG63 cells.

Effects of SB-431542 and Gleevec on TGF-β-induced Proliferation of NIH3T3 Cells. Finally, we examined the effects of SB-431542 and Gleevec on TGF-β-induced proliferation of cell lines other than MG63. Among several cell lines tested, NIH3T3 cells exhibited TGF-β-induced growth stimulation, which is comparable to that of MG63 cells. The growth stimulation was inhibited by SB-431542 but not by Gleevec (Fig. 9). These results suggested that TGF-β-induced growth stimulation is mediated through various mechanisms, which appear to depend on cell types.

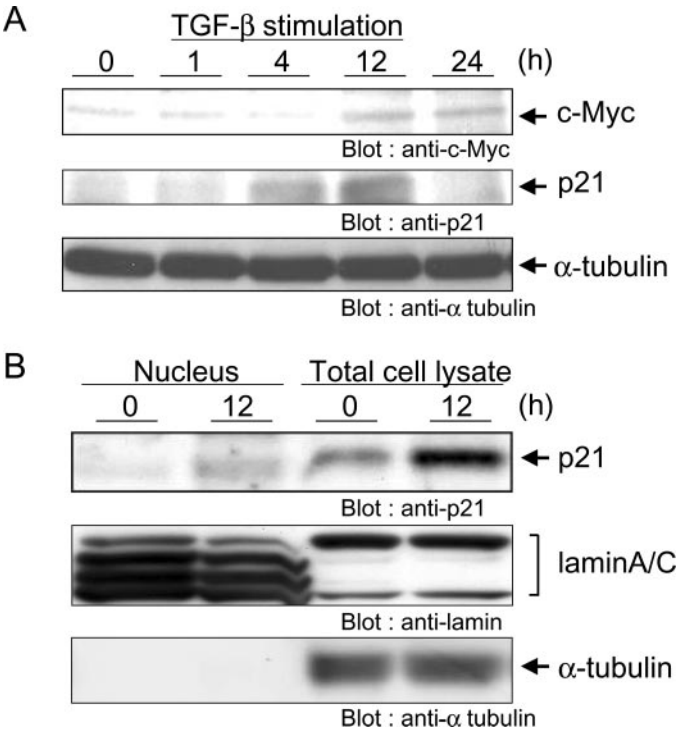

Fig. 8. Up-regulation of c-Myc and p21$^{WAF1/CIP1}$ by TGF-β in MG63 cells. *A*, MG63 cells were starved for 24 h and then treated with TGF-β3 (1 ng/ml). At various times after treatment (0, 1, 4, 12, and 24 h), cell lysates were prepared. Protein levels of c-Myc (*upper panel*) and p21$^{WAF1/CIP1}$ (*middle panel*) and α-tubulin (*bottom panel*) were examined by immunoblotting of the total lysates with anti-c-Myc, anti-p21, or anti-α-tubulin antibody, respectively. *B*, total cell lysates and nuclear extracts were prepared from MG63 cells at 0 and 12 h after TGF-β treatment (1 ng/ml). Lamin A/C and α-tubulin were used to confirm equal loading of samples as well as the integrity of fractionation.

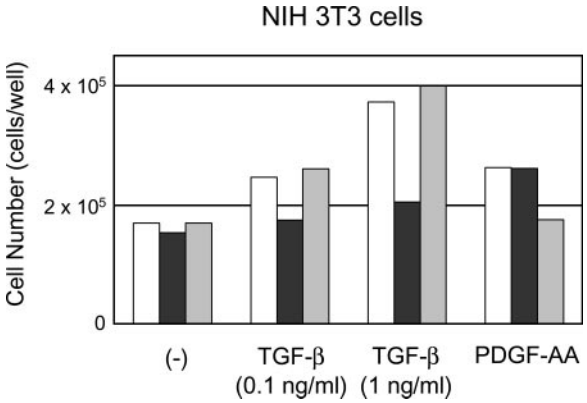

Fig. 9. Effects of SB-431542 and Gleevec on TGF-β-induced proliferation of NIH3T3 cells. NIH3T3 cells were treated with TGF-β (0.1 and 1 ng/ml) or PDGF-AA (10 ng/ml), and cell numbers were counted on day 2 after ligand stimulation. Inhibitors were added 30 min before ligand stimulation. □, control samples; ■, SB-431542 (0.3 μM)-treated samples; ▨, Gleevec (1 μM)-treated samples.

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DISCUSSION

In the present study, we examined the pathway of signal transduction by TGF-β leading to proliferation of MG63 osteosarcoma cells. Although the effects of TGF-β on proliferation of MG63 cells are controversial (39–42), we observed significant stimulation of proliferation after TGF-β treatment in the presence of 0.1% FBS, based on an increase in cell number as well as of thymidine incorporation. We found that growth-stimulatory signal was transmitted via the ALK-5 pathway, and that PDGF-A is induced by TGF-β. We also demonstrated that SB-431542, a novel ALK-5 kinase inhibitor, as well as Gleevec (STI571), an inhibitor of PDGF receptor kinase, inhibited the growth stimulation by TGF-β.

Interestingly, ALK-1, a TGF-β type I receptor preferentially expressed in endothelial cells, was found to be expressed on the MG63 cell surface and to bind TGF-β. We further confirmed that ALK-1 was functionally active based on the phosphorylation of Smad1/5 as well as gene expression profiles of targets of the Smad1/5 pathway. The up-regulated genes include those for Id proteins (Id1, Id2, and Id3). Id proteins were originally identified as "inhibitor of differentiation," and their positive roles in cell cycle progression have been reported recently (43). Id proteins negatively regulate CDK inhibitors. In addition, Id2 physically interacts with hypophosphorylated pRb and antagonizes its antiproliferative effect. It thus seemed possible that induction of Id by the ALK-1 pathway is involved in the growth-stimulating effect of TGF-β in MG63 cells. In the present study, however, we observed that ALK-1 signaling is not sufficient for the growth stimulation of MG63 cells. The function of ALK-5 is sufficient and indispensable for the growth stimulation, although the possibility cannot be excluded that ALK1 cooperates with ALK5 and is involved in the growth stimulation.

Analyses of the TGF-β-regulated genes in MG63 cells revealed up-regulation of four growth factors; among them, only PDGF-AA and FGF-2 were effective in stimulating proliferation of MG63 cells when exogenously added. Using specific inhibitors of PDGF receptor kinases, Gleevec and AG1296, we found that PDGF-AA mainly contributed to the growth-promoting action of TGF-β in MG63 cells. This may be because expression of FGF-2 was only transiently up-regulated, whereas that of PDGF-AA was up-regulated in a sustained fashion.

We further examined the expression of cell cycle regulators. Miyazaki *et al.* (44) reported that TGF-β stimulates or down-regulates cell growth through down- or up-regulation of $p21^{WAF1/CIP1}$. $p21^{WAF1/CIP1}$ is one of the direct targets of Smad proteins (10, 45). In MG63 cells, $p21^{WAF1/CIP1}$ was up-regulated by TGF-β, but cell proliferation was still stimulated. This result suggested that certain signaling pathways of TGF-β leading to growth inhibition are still active, but that some mechanism canceling the growth inhibition by $p21^{WAF1/CIP1}$ may become predominant. In this respect, it should be noted that the induction of $p21^{WAF1/CIP1}$ by TGF-β in MG63 cells was only transient. We also observed that c-Myc was up-regulated in a sustained fashion in response to TGF-β stimulation in MG63 cells. Induction of c-Myc by TGF-β has already been reported by Leof *et al.* (11) in AKR-2B cells, which are also growth stimulated by TGF-β. One of the functions of c-Myc in cell cycle progression is to down-regulate expression of CDKIs including $p21^{WAF1/CIP1}$. Thus, up-regulation of c-Myc in MG63 cells may cause the transient induction of $p21^{WAF1/CIP1}$, which may lead, at least in part, to abrogation of growth-inhibitory signaling by TGF-β.

In the present study, we used SB-431542 to selectively inhibit ALK-5 signaling but observed an inhibitory effect of SB-431542 on ligand-induced ALK-1 signaling in MG63 cells. Inman *et al.* (36) reported that this inhibitor was not effective on the constitutively active form of ALK-1 in which Gln-201 was mutated to Asp (QD mutant). There appears to be two possible explanations for this observation. One is that SB-431542 has differential effects on ligand-activated ALK-1 kinase and mutationally activated ALK-1 kinase. Alternatively, ALK-1 requires ALK-5 kinase activity for its activation by ligands. The mechanism of inhibition of ALK-1 signaling by SB-431542 remains to be elucidated.

Many tumor cells are not responsive to TGF-β and sometimes secrete TGF-β. TGF-β may induce growth factor secretion from stromal cells, and the secreted growth factors may in turn enhance proliferation of cancer cells. This is thought to be one mechanism by which TGF-β activity enhances malignancy of cancer (46). Thus, inhibition of growth factor induction may lead to suppression of tumor growth. Moreover, PDGF was reported to cause high interstitial fluid pressure in tumor tissues. High interstitial fluid pressure acts as a barrier to tumor transvascular transport, decreasing uptake of antitumor reagents by tumors. Recently, Pietras *et al.* (47) reported that inhibition of PDGF activity by Gleevec enhanced the efficacy of chemotherapeutic treatment by decreasing the tumor interstitial fluid pressure. In the present study, we found that SB-431542, a novel ALK-4/5/7 inhibitor, can block induction of growth factors including PDGF. Thus, synthetic kinase inhibitors of ALK-5 may have additional effects *in vivo*, mediated through inhibition of PDGF induction, if used together with chemotherapeutic drugs. Development of clinically available ALK-5 kinase inhibitors is thus highly desired.

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REFERENCES

1. Miyazono, K., Kusanagi, K., and Inoue, H. Divergence and convergence of TGF-β/BMP signaling. J. Cell. Physiol., *187:* 265–276, 2001.
2. Massagué, J., Blain, S. W., and Lo, R. S. TGFβ signaling in growth control, cancer, and heritable disorders. Cell, *103:* 295–309, 2000.
3. ten Dijke, P., Goumans, M-J., Itoh, F., and Itoh, S. Regulation of cell proliferation by Smad proteins. J. Cell. Physiol., *191:* 1–16, 2002.
4. Moses, H. L., Yang, E. Y., and Pietenpol, J. A. TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. Cell, *63:* 245–247, 1990.
5. Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., Li, L., Miyazono, K., ten Dijke, P., Kim, S., and Li, E. Activin receptor like kinase-1 modulates transforming growth factor-β1 signaling in the regulation of angiogenesis. Proc. Natl. Acad. Sci. USA, *97:* 2626–2631, 2000.
6. Miyazono, K., ten Dijke, P., and Heldin, C-H. TGF-β signaling by Smad proteins. Adv. Immunol., *75:* 115–157, 2000.
7. Yagi, K., Furuhashi, M., Aoki, H., Goto, D., Kuwano, H., Sugamura, K., Miyazono, K., and, Kato, M. c-myc is a downstream target of the Smad pathway. J. Biol. Chem., *277:* 854–861, 2002.
8. Chen, C-R., Kang, Y., Siegel, P. M., and Massagué, J. E2F4/5 and p107 as Smad cofactors linking the TGFβ receptor to c-*myc* repression. Cell, *110:* 19–32, 2002.
9. Feng, X-H., Liang, Y-Y., Liang, M., Zhai, W., and Lin, X. Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-β-mediated induction of the CDK inhibitor $p15^{Ink4B}$. Mol. Cell, *9:* 135–143, 2002.
10. Pardali, K., Kurisaki, A., Morén, A., ten Dijke, P., Kardassis, D., and Moustakas, A. Role of Smad proteins and transcription factor Sp1 in $p21^{WAF1/CIP1}$ regulation by transforming growth factor-β. J. Biol. Chem., *275:* 29224–29256, 2000.
11. Leof, E. B., Proper, J. A., Goustin, A. S., Shipley, G. D., DiCorleto, P. E., and Moses, H. L. Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor β: a proposed model for indirect mitogenesis involving autocrine activity. Proc. Natl. Acad. Sci. USA, *83:* 2453–2457, 1986.
12. Battegay, E. J., Raines, E. W., Seifert, R. A., Bowen-Pope, D. F., and Ross, R. TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell, *63:* 515–524, 1990.
13. Win, K. M., Charlotte, F., Mallat, A., Cherqui, D., Martin, N., Mavier, P., Preaux, A. M., Dhumeaux, D., and Rosenbaum, J. Mitogenic effect of transforming growth factor- β1 on human Ito cells in culture: evidence for mediation by endogenous platelet-derived growth factor. Hepatology, *18:* 137–145, 1993.
14. Seifert, R. A., Coats, S. A., Raines, E. W., Ross, R., and Bowen-Pope, D. F. Platelet-derived growth factor (PDGF) receptor α-subunit mutant and reconstituted cell lines demonstrate that transforming growth factor-β can be mitogenic through PDGF A-chain-dependent and -independent pathways. J. Biol. Chem., *269:* 13951–13955, 1994.

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15. Sintich, S. M., Lamm, M. L. G., Sensibar, J. A., and Lee, C. Transforming growth factor-β1-induced proliferation of the prostate cancer cell line, TSU-Pr1: the role of platelet-derived growth factor. Endocrinology, *140:* 3411–3415, 1999.
16. Kay, E. P., Lee, H. K., Park, K. S., and Lee, S. C. Indirect mitogenic effect of transforming growth factor-β on cell proliferation of subconjunctival fibroblasts. Invest. Ophthalmol. Vis. Sci., *39:* 481–486, 1998.
17. Kay, E. P., Lee, M. S., Seong, G. J., and Lee, Y. G. TGF-βs stimulate cell proliferation via an autocrine production of FGF-2 in corneal stromal fibroblasts. Curr. Eye Res., *17:* 286–293, 1998.
18. Strutz, F., Zeisberg, M., Renziehausen, A., Raschke, B., Becker, V., van Kooten, C., and Müller, G. A. TGF-β1 induces proliferation in human renal fibroblasts via induction of basic fibroblast growth factor (FGF-2). Kidney Int., *59:* 579–592, 2001.
19. Urano, T., Yashiroda, H., Muraoka, M., Tanaka, K., Hosoi, T., Inoue, S., Ouchi, Y., Tanaka, K., and Toyoshima, H. $p57^{Kip2}$ is degraded through the proteasome in osteoblasts stimulated to proliferation by transforming growth factor β1. J. Biol. Chem., *274:* 12197–12200, 1999.
20. Nishimori, S., Tanaka, Y., Chiba, T., Fujii, M., Imamura, T., Miyazono, K., Ogasawara, T., Kawaguchi, H. Igarashi, T., Fujita, T., Tanaka, K., and Toyoshima, H. Smad-mediated transcription is required for transforming growth factor-β1-induced $p57^{Kip2}$ proteolysis in osteoblastic cells. J. Biol. Chem., *276:* 10700–10705, 2001.
21. Yan, Z., Kim, G-Y., Deng, X., and Friedman, E. Transforming growth factor β1 induces proliferation in colon carcinoma cells by Ras-dependent, smad-independent down-regulation of p21cip1. J. Biol. Chem., *277:* 9870–9879, 2002.
22. Goumans, M-J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., and ten Dijke, P. Balancing the activation state of the endothelium via two distinct TGF-β type I receptors. EMBO J., *21:* 1743–1753, 2002.
23. Ragland, B. D., Bell, W. C., Lopez, R. R., and Siegal, G. P. Cytogenetics and molecular biology of osteosarcoma. Lab. Invest., *82:* 365–373, 2002.
24. Seyedin, S. M., Thompson, A. Y., Bentz, H., Rosen, D. M., McPherson, J. M., Conti, A., Siegel, N. R., Galluppi, G. R., and Piez, K. A. Cartilage-inducing factor-A: apparent identity to transforming growth factor-β. J. Biol. Chem., *261:* 5693–5695, 1986.
25. Pfeilschifter, J., D'Souza, S. M., and Mundy, G. R. Effects of transforming growth factor-β on osteoblastic osteosarcoma cells. Endocrinology, *121:* 212–218, 1987.
26. Kloen, P., Jennings, C. L., Gebhardt, M. C., Springfield, D. S., and Mankin, H. J. Expression of transforming growth factor-β (TGF-β) receptors, TGF-β1 and TGF-β2 production and autocrine growth control in osteosarcoma cells. Int. J. Cancer, *58:* 440–445, 1994.
27. Reed, B. Y., Zerwekh, J. E., Antich, P. P., and Pak, C. Y. Fluoride-stimulated [^{3}H]thymidine uptake in a human osteoblastic osteosarcoma cell line is dependent on transforming growth factor β. J. Bone Miner. Res., *8:* 19–25, 1993.
28. Franchi, A., Arganini, L., Baroni, G., Calzolari, A., Capanna, R., Campanacci, D., Caldora, P., Masi, L., Brandi, M. L., and Zampi, G. Expression of transforming growth factor β isoforms in osteosarcoma variants: association of TGF β1 with high-grade osteosarcomas. J. Pathol., *185:* 284–289, 1998.
29. Kloen, P., Gebhardt, M. C., Perez-Atayde, A., Rosenberg, A. E., Springfield, D. S., Gold, L. I., and Mankin, H. J. Expression of transforming growth factor-β (TGF-β) isoforms in osteosarcomas: TGF-β3 is related to disease progression. Cancer (Phila.), *80:* 2230–2239, 1997.
30. Callahan, J. F., Burgess, J. L., Fornbald, J. A., Gaster, L. M., Harling, J. D., Harrington, F. P., Heer, J., Kwon, C., Lehr, R., Mathur, A., Olson, B. A., Weinstock, J., and Laping, N. J. Identification of novel inhibitors of the transforming growth factor-β1 (TGF-β1) type 1 receptor (ALK5). J. Med. Chem., *45:* 999–1001, 2002.
31. ten Dijke, P., Yamashita, H., Ichijo, H., Franzén, P., Laiho, M., Miyazono, K., and Heldin, C-H. Characterization of type I receptors for transforming growth factor-β and activin. Science (Wash. DC), *264:* 101–104, 1994.
32. Fujii, M., Takeda, K., Imamura, T., Aoki, H., Sampath, T. K., Enomoto, S., Kawabata, M., Kato, M., Ichijo, H., and Miyazono, K. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol. Biol. Cell, *10:* 3801–3813, 1999.
33. Ota, T., Fujii, M., Sugizaki, T., Ishii, M., Miyazawa, K., Aburatani, H., and Miyazono, K. Targets of transcriptional regulation by two distinct type l receptors for transforming growth factor-β in human umbilical vein endothelial cells. J. Cell. Physiol., *193:* 299–318, 2002.
34. Ebisawa, T., Tada, K., Kitajima, I., Tojo, K., Sampath, T. K., Kawabata, M., Miyazono, K., and Imamura, T. Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J. Cell Sci., *112:* 3519–3527, 1999.
35. Nishihara, A., Watabe, T., Imamura, T., and Miyazono, K. Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension. Mol. Biol. Cell, *13:* 3055–3063, 2002.
36. Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., and Hill, C. S. SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol., *63:* 65–74, 2002.
37. Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Muller, M., Druker, B. J., and Lydon, N. B. Inhibition of the Abl protein-tyrosine kinase *in vitro* and *in vivo* by a 2-phenylaminopyrimidine derivative. Cancer Res., *56:* 100–104, 1996.
38. Kovalenko, M., Gazit, A., Böhmer, A., Rorsman, C., Rönnstrand, L., Heldin, C-H., Waltenberger, J., Böhmer, F. D., and Levitzki, A. Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. Cancer Res., *54:* 6106–6114, 1994.
39. Subramaniam, M., Oursler, M. J., Rasmussen, K., Riggs, B. L., and Spelsberg, T. C. TGF-β regulation of nuclear proto-oncogenes and *TGF-β* gene expression in normal human osteoblast-like cells. J. Cell. Biochem., *57:* 52–61, 1995.
40. Saito, T., Kinoshita, A., Yoshiura, K., Makita, Y., Wakui, K., Honke, K., Niikawa, N., and Taniguchi, N. Domain-specific mutations of a transforming growth factor (TGF)-β1 latency-associated peptide cause Camurati-Engelmann disease because of the formation of a constitutively active form of TGF-β1. J. Biol. Chem., *276:* 11469–11472, 2001.
41. Tokuyama, H., and Tokuyama, Y. Bovine colostric transforming growth factor-β-like peptide that induces growth inhibition and changes in morphology of human osteogenic sarcoma cells (MG-63). Cell Biol. Int. Rep., *13:* 251–258, 1989.
42. Pirskanen, A., Jaaskelainen, T., and Maenpaa, P. H. Effects of transforming growth factor β1 on the regulation of osteocalcin synthesis in human MG-63 osteosarcoma cells. J. Bone Miner. Res., *9:* 1635–1642, 1994.
43. Yokota, Y., and Mori, S. Role of ld family proteins in growth control. J. Cell. Physiol., *190:* 21–28, 2002.
44. Miyazaki, M., Ohashi, R., Tsuji, T., Mihara, K., Gohda, E., and Namba, M. Transforming growth factor-β1 stimulates or inhibits cell growth via down- or up-regulation of p21/Waf1. Biochem. Biophys. Res. Commun., *246:* 873–880, 1998.
45. Moustakas, A., and Kardassis, D. Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. Proc. Natl. Acad. Sci. USA, *95:* 6733–6738, 1998.
46. Piek, E., and Roberts, A. B. Suppressor and oncogenic roles of transforming growth factor-β and its signaling pathways in tumorigenesis. Adv. Cancer Res., *83:* 1–54, 2001.
47. Pietras, K., Rubin, K., Sjöblom, T., Buchdunger, E., Sjöquist, M., Heldin, C-H., and Östman, A. Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. Cancer Res., *62:* 5476–5484, 2002.

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SB-431542 and Gleevec Inhibit Transforming Growth Factor-β-Induced Proliferation of Human Osteosarcoma Cells

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