Activin A inhibits vascular endothelial cell growth and suppresses tumour angiogenesis in gastric cancer

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BACKGROUND: Activin A is a multi-functional cytokine belonging to the transforming growth factor-β (TGF-β) superfamily; however, the effect of activin A on angiogenesis remains largely unclear. We found that inhibin βA subunit (INHBA) mRNA is overexpressed in gastric cancer (GC) specimens and investigated the effect of activin A, a homodimer of INHBA, on angiogenesis in GC.

METHODS: Anti-angiogenic effects of activin A via p21 induction were evaluated using human umbilical vein endothelial cells (HUVECs) in vitro and a stable INHBA-introduced GC cell line in vivo.

RESULTS: Compared with TGF-β, activin A potently inhibited the cellular proliferation and tube formation of HUVECs with induction of p21. A promoter assay and a chromatin immunoprecipitation assay revealed that activin A directly regulates p21 transcriptional activity through Smads. Stable p21-knockdown significantly enhanced the cellular proliferation of HUVECs. Notably, stable p21-knockdown exhibited a resistance to activin-mediated growth inhibition in HUVECs, indicating that p21 induction has a key role on activin A-mediated growth inhibition in vascular endothelial cells. Finally, a stable INHBA-introduced GC cell line exhibited a decrease in tumour growth and angiogenesis in vivo.

CONCLUSION: Our findings highlight the suppressive role of activin A, unlike TGF-β, on tumour growth and angiogenesis in GC.

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identified the overexpression of INHBA in the GC samples (Yamada et al, 2008 and unpublished data). Based on this finding of INHBA overexpression and accumulating evidence of the role of TGF-β in angiogenesis, we focused on the role of activin A in angiogenesis in GC in the present study.

MATERIALS AND METHODS

Antibodies and ligands
The following antibodies were used: anti-p21, anti-cdk2, anticyclin D, anti-phospho-Rb, anti-Smad2, anti-phospho-Smad2, anti-Smad3, anti-Smad4, and secondary antibodies (Cell Signaling, Beverly, MA, USA); anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and a mouse anti-CD31 monoclonal antibody (BD Biosciences, San Jose, CA, USA). Recombinant human activin A (Cruz, CA, USA); and a mouse anti-CD31 monoclonal antibody (BD Biosciences, San Jose, CA, USA). Recombinant human activin A and TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA). The Alk4/Alk5/Alk7-specific inhibitor SB341542 was purchased from Sigma (St Louis, MO, USA).

Cell lines and cultures
58As1, 44As3, Okajima, KATOIII, MKN1, MKN7, MKN28, and MKN74 cell lines were cultured in RPMI-1640 medium (Sigma) with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL). The HEK293 (Human Embryonic Kidney cell line 293) cell lines were cultured in DMEM medium (Sigma) with 10% heat-inactivated FBS. Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Osaka, Japan) and maintained in Humedia (Kurabo) supplemented with 2% FBS, 5 ng ml⁻¹ FGF-2, 10 ng ml⁻¹ EGF, 10 μg ml⁻¹ heparin, 1 μg ml⁻¹ hydrocortisone, and antibiotics. The cell lines were maintained in a 5% CO₂-humidified atmosphere at 37°C.

Patients and samples
The methods were described previously (Yamada et al, 2008). This study was approved by the institutional review board, and written informed consent was obtained from all the patients.

Plasmid construction, viral production, and stable transfectants
The methods used in this section have been previously described (Kaneda et al, 2010). Briefly, the cDNA fragment encoding human full-length INHBA was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa, Otsu, Japan) with 5'-GGG AAT TCG 3'TGG GCA GCC GTT AG-3' and 5'-GCC CTC ACC ACC CCT CC-3' forward and reverse, respectively. The results were normalised to co-transfected vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. The reaction was normalised to β-galactosidase activity and are representative of at least three independent experiments.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer’s protocol. The Smad-binding region (SBR) of the p21 promoter was subcloned into a luciferase reporter vector, pGL4.14 (Promega, Madison, WI, USA). All the sequences were verified using DNA sequencing. The empty and p21 promoter-containing reporter vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. The results were normalised to co-transfected β-galactosidase activity and are representative of at least three independent experiments.

Real-time reverse transcription PCR and western blot analysis
The methods used in this section have been previously described (Matsumoto et al, 2009). The primers used for real-time RT-PCR were as follows: INHBA forward, 5'-CAT TGC CCT CTC TGG CTA TGG T-3' and reverse, 5'-CTA GAG CTG CTC GAC GTC AGT TTT T-3'; GAPDH forward, 5'-GGG CCG TCA AGG CTC AGA AC-3' and reverse, 5'-ATG GTG GTG AAG ACC CCA GT-3'. The densitometry data from the western blot analysis were quantified automatically using Multigauge Ver. 3.0 (Fujifilm, Tokyo Japan). The densitometry data were normalised by β-actin and is shown above the western blot as a ratio of that in the control sample.

ELISA
A total of 10⁶ cells from each of the GC cell lines TK3/EGFP and TK3/INHBA were cultured in normal medium for 12 h and the medium was replaced with a serum-free medium. After 12 h of culture, the medium was collected, centrifuged to remove floating cells, and used for analysis. The concentration of activin A described above was determined using a human activin A DuoSet ELISA Development kit (R&D Systems), according to the manufacturer’s instructions.

Cell proliferation assay
HUVECs were plated at a density of 3 × 10⁴ cells in 96 wells in growth medium overnight. The cells were then stimulated with the vehicle, activin A, or TGF-β1 at the indicated concentrations for 72 h (Figures 2A and 5C) or the indicated time (Figure 5B). The experiment was performed using an MTT assay in triplicate. The methods have been previously described (Kaneda et al, 2010).

Tube formation assay
A 96-well plate was coated with Matrigel (BD Biosciences) avoiding bubble formation and was incubated at 37°C for 30 min to allow the Matrigel to solidify. HUVECs (2 × 10⁴ cells per well) were pretreated with 10 ng ml⁻¹ of activin A or 1 ng ml⁻¹ of TGF-β1 for 48 h and then were plated onto the Matrigel-coated plate. After 16 h of incubation, the HUVECs were photographed using fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

Luciferase reporter assay
The human p21 promoter-containing reporter vector was constructed according to a previously described method (Kaneda et al, 2010). Briefly, a 2.4-kb section of the p21 promoter region was subcloned into a luciferase reporter vector, pGL4.14 (Promega, Madison, WI, USA). All the sequences were verified using DNA sequencing. The empty and p21 promoter-containing reporter vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. The results were normalised to co-transfected β-galactosidase activity and are representative of at least three independent experiments.

Xenograft studies and immunohistochemical staining
Nude mice (BALB/c nu/nu; 6-week-old females; CLEA Japan Inc., Tokyo, Japan) were used for the in vivo studies and were cared for...
in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research, as compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the UKCCCR guidelines. To evaluate tumour growth and tumour angiogenesis, the cell suspensions of 1 x 10^7 TK3/EGFP or TK3/INHBA cells in 0.1 ml PBS were subcutaneously injected into the left or right flanks of nude mice (n = 5), respectively. The tumour volume was calculated as the length x width^2 x 0.5. The tumour volume was assessed every week. At the end of the experiment, the mice were killed and the xenografts were resected, fixed in 10% buffered formalin for 10 h, and processed for histological analysis. The immunohistochemical staining methods have been previously described (Kaneda et al, 2010).

**Statistical analysis**

The statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) to calculate the average, s.d., and results of a Student’s t-test. A P-value < 0.05 was considered statistically significant.

**RESULTS**

**INHBA mRNA is overexpressed in GC**

A real-time RT–PCR analysis revealed that *INHBA* mRNA was overexpressed an average of 37-fold higher in 24 GC specimens than in paired non-cancerous mucosa samples (p = 0.014; Figure 1A). The average levels of *INHBA* mRNA expression in the GC and paired non-cancerous mucosa samples were 236 ± 42 and 6.0 ± 16.0 (10^3/GAPD), respectively. Since *INHBA*/activin proteins are multi-functional ligands and its superfamily member, TGF-β, is closely involved in angiogenesis, we speculated that the overexpression of *INHBA* may have some role in tumour biology. Thus, we focused on the effect of activin A on tumour angiogenesis.

*INHBA* overexpression likely leads to the overexpression of its homodimer form, activin A; therefore, we evaluated the correlation between mRNA expression and secreted activin A protein expression in nine GC cell lines. The expressions of both *INHBA* mRNA and activin A protein were increased in 44As3, MKN1, and MKN7 cells but were very low in the other cell lines (Figure 1B).

These mRNA and protein expressions were strongly correlated (R = 0.82), indicating that *INHBA* overexpression in GC leads to the overexpression of activin A.

**Activin A potently inhibits cellular proliferation in vascular endothelial cells**

We examined the effect of activin A, compared with TGF-β, on cellular proliferation using HUVECs. TGF-β slightly decreased cellular proliferation at a dose of 1 ng ml^-1^, while a higher dose of TGF-β (10 ng ml^-1^) tended to increase proliferation (Figure 2A). In contrast, activin A potently and dose-dependently decreased cellular proliferation at a dose of 10–100 ng ml^-1^ (Figure 2A). In addition, a tube formation assay showed that activin A, but not TGF-β, inhibited tube formation in HUVECs (Figure 2B). These results indicate that activin A and TGF-β have quite different effects on the cellular proliferation of vascular endothelial cells.

**Activin A mediates the persistent phosphorylation of Smad2 and p21 induction in HUVECs**

Activin A and TGF-β inhibited the cellular growth of HUVECs in different manners; therefore, we examined the downstream signalling under activin A or TGF-β stimulation. Based on the many previous studies and our data for cellular growth inhibition (Figure 2A), we used 10 ng ml^-1^ of activin A and 1 ng ml^-1^ of TGF-β as moderate doses to compare the effects of activin A and TGF-β.

Activin A (10 ng ml^-1^) strongly increased the phosphorylation levels of Smad2, compared with TGF-β (1 ng ml^-1^), and the effects were cancelled by the Alk4/Alk5/Alk7 inhibitor SB341542 (Figure 3A). Interestingly, activin A persistently increased the phosphorylation of Smad2 from 5 min to over 3 h, while TGF-β increased the phosphorylation during a shorter period of from 15 to 60 min (Figure 3B). In addition, activin A strongly induced the nuclear translocation of phosphorylated-Smad2 and Smad2, while TGF-β induced a milder effect (Figure 3C). These results suggest that activin A activates Smad signalling more potently than TGF-β in HUVECs.

Next, we evaluated the expression levels of cell cycle-related proteins to investigate the difference in the growth inhibitory effects. A western blot analysis revealed that the expression of cyclin D1 and the phosphorylation levels of Rb were decreased by activin A stimulation after 48 h of stimulation, while TGF-β showed a weak effect consistent with the results for growth inhibition (Figure 3D).
p21CIP1/WAF1 is a major cdk inhibitor and the hallmark of the cytostatic role of TGF-β (Weiss, 2003). TGF-β is known to increase p21 expression (Datto et al., 1995; Hu et al., 1998; Pardali et al., 2000), but the regulation of p21 by activin A remains largely unclear, especially in vascular endothelial cells. We found that p21 expression was increased by both activin A and TGF-β stimulation at 12–48 h in HUVECs (Figure 3D); therefore, we speculated that p21 may have a role in activin A-mediated growth inhibition and cell-cycle progression in vascular endothelial cells.

Figure 2  Activin A potently inhibits the proliferation and tube formation of HUVECs. (A) HUVECs were cultured in 96-well plates and stimulated with the indicated doses of activin A or TGF-β for 72 h. The cell proliferation was assayed using an MTT assay. Columns: mean of independent triplicate experiments. Bars: s.d. *P < 0.05. (B) Effect of activin A on tube formation in HUVECs. HUVECs were cultured with normal medium (untreated) or activin A (10 ng ml⁻¹) or TGF-β (1 ng ml⁻¹) containing medium for 48 h and the cells were seeded in 96-well culture plates (1.5 x 10⁴ cells per well) precoated with 80 μl Matrigel and cultured with normal medium (untreated) or activin A (10 ng ml⁻¹) or TGF-β (1 ng ml⁻¹). After 16 h of incubation, the wells were photographed using a microscope.

Figure 3  Activin A mediates the persistent phosphorylation of Smad2 and p21 induction in HUVECs. (A) HUVECs were treated with or without 2 μM of SB341542 for 30 min, then stimulated with activin A (10 ng ml⁻¹) or TGF-β (1 ng ml⁻¹) for 1 h. The phosphorylation and expression levels of Smad2 were evaluated using western blot. (B) Time-course analysis with activin A or TGF-β-induced Smad2 phosphorylation. HUVECs were stimulated with 10 ng ml⁻¹ activin A or 1 ng ml⁻¹ TGF-β for the indicated time periods. (C) Activin A or TGF-β-induced nuclear translocation of Smad2. HUVECs were stimulated with or without activin A (10 ng ml⁻¹) or TGF-β (1 ng ml⁻¹) for 1 h. Nuclear and cytosolic protein fractions were then analysed using a western blot analysis. C = cytosolic fraction; N = nuclear fraction. (D) Expression changes of cell cycle-related proteins by stimulation with activin A (10 ng ml⁻¹) or TGF-β (1 ng ml⁻¹) for the indicated time period in HUVECs. A western blot analysis was performed using anti-p21, cyclin D, and phosphorylated Rb antibodies. β-Actin was used as an internal control.
Activin A directly regulates p21 transcriptional activity through Smads

A recent report demonstrated that TGF-β increases the binding of Smad2/3 and Smad4 to a distal portion of the p21 promoter, in which a SBR contains four Smad-binding elements (Seoane et al., 2004). To determine whether activin A regulates p21 expression at the transcriptional level, we performed a luciferase reporter assay. Activin A stimulation markedly increased the p21 promoter activity to 16.9-fold, compared with a control (Figure 4A). TGF-β stimulation markedly increased the p21 promoter activity to 16.9-fold, compared with a control. Activin A stimulation strongly increased the binding of Smad2/3 and Smad4 to the SBR on the p21 promoter (Figure 4B). These results indicate that activin A directly regulates the p21 transcriptional activity through Smads.

p21 induction has a key role on activin A-mediated growth inhibition in vascular endothelial cells

To evaluate the role of p21 induction on activin A-mediated growth inhibition, we examined the cellular growth of stable p21-knockdown (HUVEC/sh-p21) or control (HUVEC/sh-Scr) HUVECs using viral shRNA-targeting p21 or shRNA-scramble vectors (Kaneda et al., 2010). Interestingly, the knockdown of p21 significantly increased the cellular proliferation, compared with a control, in HUVECs, indicating that p21 has a growth inhibitory role in HUVECs (Figure 5B). Furthermore, stable p21-knockdown HUVECs were significantly resistant to activin A-mediated growth inhibition, compared with a control, although not completely resistant (Figure 5C). Our new findings demonstrate that p21 induction at least partially has a key role in activin A-mediated growth inhibition in vascular endothelial cells.

Effect of activin A activity on cellular proliferation in GC cell lines

Since whether activin A stimulation inhibits the cellular proliferation of GC cell lines as well as vascular endothelial cells remains unclear, we evaluated this effect. Activin A stimulation strongly upregulated the expression levels of p-smad2 in KATOIII cells, while it slightly upregulated them in MKN7 cells (Figure 6A). TGF-β potently upregulated the expression levels of p-smad2 in both cell lines. Regarding cellular proliferation, activin A inhibited cellular proliferation in KATOIII cells but did not inhibit...
proliferation in MKN7 cells, while TGF-β potently inhibited cellular proliferation when administered at a dose of 1–10 ng ml⁻¹ (Figure 6B). These results indicate that activin A has a weak or no effect on the cellular proliferation of GC cells, compared with that of vascular endothelial cells.

**Overexpression of activin A inhibits tumour growth and angiogenesis in vivo**

*In vitro* experiments showed that activin A inhibits the cellular growth of vascular endothelial cells. Next, we evaluated the effects of activin A overexpression in GC using an *in vivo* experiment. The INHBA or EGFP genes were stably introduced to TU-KATOIII, a low activin A-expressing GC cell line, to produce cell lines that were designated as TK3/INHBA and TK3/EGFP, respectively. The TK3/INHBA cells markedly secreted activin A (12.1 ng ml⁻¹) protein into the culture medium, compared with a control cell line (Figure 7A). The conditioned medium from the TK3/INHBA cells, but not from the TK3/EGFP cells, induced the phosphorylation of Smad2 on HUVECs (Figure 7B). These results indicate that exogenous INHBA actually functions as activin A.

TK3/INHBA and TK3/EGFP cells were inoculated into mice, and tumour growth and angiogenesis were evaluated. The tumour volume of the TK3/INHBA cells on day 39 was significantly smaller (104.9 ± 86.2 mm³) than that of the TK3/EGFP cells (245.1 ± 34.7 mm³; Figure 7C). These results clearly indicated that the overexpression of activin A in GC significantly inhibited tumour growth. Next, we evaluated angiogenesis in these tumour xenografts using CD31 staining. The microvessel density was significantly reduced in the tumours of TK3/INHBA cells, but not from the TK3/EGFP cells, compared with the indicated doses of activin A or TGF-β for 72 h. Cellular proliferation was assayed using an MTT assay. Columns: mean of independent triplicate experiments. Bars: s.d.

**DISCUSSION**

TGF-β is almost definitively considered to be an angiogenic factor (Wakefield and Roberts, 2002), but the role of activin A remains largely unclear. Our results showed that activin A and TGF-β have different effects on the proliferation of vascular endothelial cells in *vivo*. In line with this difference, previous knockout mice studies have demonstrated that embryos lacking any one of the TGF-β signalling components die during mid-gestation as a result of impaired vascular development, exhibiting hyper-dilated, leaky vessels, and highlighting the importance of TGF-β signalling in the vascular system (Goumans et al, 2009). Meanwhile, activin-βA-deficient mice did not develop any defects in angiogenesis (Matzuk et al, 1995). Although we did not directly compare the effect on angiogenesis between activin A and TGF-β *in vivo*, our findings support the inhibitory role of activin A on tumour angiogenesis, unlike TGF-β.

The relation between activin A expression and clinical outcome remains controversial. A high expression of INHBA was associated with a favourable prognostic outcome, exerting a tumour suppressor and anti-angiogenic role in neuroblastoma patients (Schramm et al, 2005). Meanwhile, its expression was correlated with tumour aggressiveness and a poor clinical outcome in patients with oesophageal carcinoma (Yoshinaga et al, 2004). This discrepancy may be explained by the dual role of TGF-β signalling as a tumour suppressor and pro-oncogenic factor. The TGF-β signalling pathway has a complicated role in cancer cells, mediating the ability of the cells to participate negatively or positively in growth inhibition, proliferation, replication, invasion, metastasis, apoptosis, immune surveillance, and angiogenesis (Jakowlew, 2006). For example, a defect of function in the TGF-β signalling component leads to carcinogenesis by acting as a definite tumour suppressor during the early phase, but it may exhibit an oncogenic function during later clinical stages.
The anti-angiogenic activity of activin A

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Figure 7 Overexpression of INHBA potently inhibited tumour growth and angiogenesis in GC in vivo. (A) TU-KATOIII GC cells were stably transfected with an INHBA (TK3/INHBA cells) or control vector (TK3/EGFP cells). Activin A secretion in the conditioned medium was analysed using an ELISA. B Activity of exogenous activin A on phosphorylation of smad2. Conditioned media from TK3/INHBA or /EGFP cells were exposed to HUVECs for 1 h and the phosphorylation of Smad2 in the HUVECs was assessed using a western blot analysis. β-Actin was used as an internal control. (C) Effect of overexpression of INHBA on tumour growth. TK3/EGFP and TK3/INHBA cells (1 x 10^7 cells) were subcutaneously inoculated into mice and evaluated for tumour growth in vivo. The data indicate the mean ± s.d. *P<0.05. (D and E) CD31 staining for tumour specimens. Microvessel density (MVD) was evaluated using CD31-positive endothelial cells in tumour specimens using a computer-assisted image analysis. *P<0.05. (F) Diagram of the proposed mechanism of activin A on vascular endothelial cells and in angiogenesis.

(Jakowlew, 2006). This context dependency of TGF-β signalling and differences in the origins of the cancer tissue may lead to such discrepancies. We plan to clarify the clinical meaning of activin A expression in GC in a future study.

Our findings and other reported data indicate that activin A inhibits the cellular proliferation of vascular endothelial cells (Panopoulou et al, 2005; Schramm et al, 2005): however, the underlying mechanism in endothelial cells has not been fully elucidated. p21 is a member of the cip/kip family of cyclin kinase inhibitors, and initial reports have demonstrated that p21 functions as a G1 cyclin kinase inhibitor and a downstream molecule of p53 (el-Deiry et al, 1993). p21 possesses a variety of cellular functions, including the negative modulation of cell-cycle progression, cellular differentiation, and the regulation of p53-dependent anti-apoptosis (reviewed in Garner and Raj, 2008). We demonstrated that activin A directly regulates p21 expression at the transcriptional level, and the knockdown of p21 increased the cellular proliferation and mediated the resistance to activin A-mediated growth inhibition. Our findings have thus shed light on p21 as an activin A-mediated growth inhibitor in vascular endothelial cells.

In conclusion, our findings indicate that activin A inhibits vascular endothelial cell growth via the direct induction of p21 and highlight the suppressive role of activin A in tumour growth and angiogenesis in GC.

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