Selective inhibition of cell growth by activin in SNU-16 cells

Young Il Kim, Hee Joo Lee, Inkoo Khang, Byung-Nam Cho, Ha Kyu Lee

INTRODUCTION

Inhibins and activins, members of the transforming growth factor-β (TGF-β) superfamily, are polypeptides that were originally isolated from ovarian fluid, based on their effect on pituitary follicle-stimulating hormone (FSH) production. Inhibins are heterodimers that are composed of a common α subunit and one of the two homologous β subunits (βA and βB). Activins are either heterodimers or homodimers of inhibin β subunits (βAβA, βBβB), and βAβB[1,2].

Activin plays an important role in the proliferation, differentiation, and apoptosis of target cells. The biological activity of activin is mediated by receptor complexes consisting of two different types of receptors, the type I (ActR I) and type II (ActR II) activin serine/threonine kinase receptors[3]. ActR II binds to activin independently of ActR I, but is unable to signal without ActR I. The formation of heteromeric complexes of ActR I and ActR II is required for the mediation of the cellular signal[4]. ActR I and ActR II mRNAs have been widely detected in several human tissues, including prostate cancer, breast cancer, and epithelial ovarian cancer[5,6].

Smad proteins have been known as intracellular signaling mediators of the activin[7,8]. Smad1, Smad2, Smad3, and Smad5 proteins are phosphorylated by activated specific type I serine/threonine kinase receptors, and thus act in a pathway-restricted fashion. Smad4 protein forms hetero-oligomeric complexes with pathway-restricted Smad proteins, which translocate into the nuclei and activate transcriptional responses. Smad6 and Smad7 function as inhibitors of TGF-β family signaling, including activin[9,10]. Smad7 selectively interferes with different
activin signaling pathways and inhibits erythroid leukemia cells and liver Hep3B cells by blocking intracellular activin signaling (14,15). In addition, the mitotic inhibitor p21cip/raf (activin) thas suppresses the growth of malignant cells in vitro and in vivo can be up-regulated by activin.

This study was to investigate whether activin can affect cell proliferation in human gastric cancer cell line SNU-16 through the changes in activin receptors, Smads and p21 cip/raf mRNA levels.

MATERIALS AND METHODS

Cell culture and reagents

Human gastric cancer cell lines AGS and KATO III were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). SNU-1, SNU-5, SNU-16, SNU-484, SNU-601, SNU-638, SNU-668, and SNU-719 cells were supplied from the Korean Cell Line Bank. Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified 5% CO₂ environment.

MTT assay

Cell proliferation was measured with CellTiter 96 Aqueous One Solution (Promega, Madison, WI). Cells were seeded at 5 × 10⁴ cells/well in 6-well plates and incubated with different concentrations of activin A (0, 10, 50, and 100 ng/mL) at 37 °C for 24, 48, and 72 h respectively. Cell viability was determined by a colorimetric assay with PMS/MTS (20 µL/well). The absorbance was determined at 492 nm with background subtraction at 650 nm.

Cell number assay

For the cell viability assay, cells were seeded at 5 × 10⁴ cells/mL in 6-well plates. The cells were cultured with 100 ng/mL of activin A for 24, 48, and 72 h respectively. Then cells were washed with phosphate buffered saline (PBS) and resuspended in PBS. Viable cells were counted by the trypan blue exclusion method at each time using a hemocytometer.

RNA extraction and RT-PCR procedures

Total RNA was extracted from cultured cells using the RNAzol B solution kit following the manufacturer’s protocol (Tel-test, Friendswood, Texas). First-strand cDNA synthesis was performed using a cDNA synthesis kit (Roche, Mannheim, Germany). cDNA synthesis was performed by reverse transcription in a total volume of 20 µL reaction mixture containing 1 µg RNA, 1× reaction buffer, 1 mM dNTP, 5 µM oligo(dT), 20 units of RNase inhibitor, and 20 units of AMV reverse transcriptase. The reaction mixture was incubated at 42 °C for 1 h, terminated by heating at 95 °C for 5 min. Polymerase chain reaction (PCR) was performed with 5 µL (α, βA, βB subunits, and p21 cip/raf) or 1 µL (activin receptors, Smads and β-actin) cDNA in a 50 µL reaction mixture of 1× PCR buffer, 0.2 mM dNTP of each dNTP, 20 pmol primer, and 1 unit of Taq DNA polymerase (Roche). Primer sequences are shown in Table 1. The conditions for amplification were as follows: first denaturation at 95 °C for 4 min, then denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min.

Table 1 Oligonucleotide sequences of PCR primers

| Oligo     | Sequence               | Annealing temperature (°C) | Size (bp) |
|-----------|------------------------|-----------------------------|-----------|
| Inhibin α | 5'-AGGAAGAGGAGGTGCTCC-3' | 50                          | 823       |
| ActR IIA | 5'-GCCCAAGGTCAACCCCAAACTCT-3' | 60                          | 265       |
| ActR IIB | 5'-ACCAGCTGTCCTCCAGCCCAAATCT-3' | 62                          | 456       |
| Smad2    | 5'-TAGGTCAGTGATGTGGATCT-3' | 62                          | 730       |
| Smad4    | 5'-AAGGGAAGGATGTTGATCT-3' | 60                          | 509       |
| Smad7    | 5'-GCTGGGAGGCTCTACTGCTG-3' | 58                          | 477       |
| β-actin  | 5'-GCTTCTTCGGCCCAGTGGAC-3' | 50                          | 267       |

ActR: activin receptor.

Statistical analysis

Values were expressed as mean ± SD. Student’s t test was used to evaluate differences between control and activin A-treated samples. *P < 0.05 and **P < 0.01 were considered statistically significant.

RESULTS

Effects of activin A on cell proliferation

Ten human gastric cancer cell lines were treated with various concentrations of activin A (10-100 ng/mL) for 24,
48, and 72 h respectively. The effect of activin on cell proliferation was assessed by MTT assay (Table 2). Activin induced a significant decrease in SNU-16 cell proliferation in a dose- and time-dependent manner, whereas other cells showed no detectable response. To evaluate the growth inhibition of activin in SNU-5 and SNU-16 cells, the cells were cultured with 100 ng/mL of activin A for 24, 48, and 72 h respectively. The cell number was counted by a hemocytometer. Activin A treatment resulted in a significant decrease in the number of SNU-16 cells in a time-dependent manner, whereas no effect was observed in the number of SNU-5 cells (Figure 1).

Expression of inhibin/activin subunit, activin receptor, Smads, and p21\(^{CIP1/WAF1}\) mRNAs
Basal expressions of inhibin/activin subunits, activin receptors, Smads, and p21\(^{CIP1/WAF1}\) mRNA in human gastric cancer cell lines were investigated by RT-PCR (Figure 2). The expression of inhibin \(\alpha\) mRNA was detectable only in SNU-638 cells whereas the activin \(\beta A\) mRNA was expressed in the majority of cells tested, but not in SNU-5 or SNU-719 cells. The activin \(\beta B\) was not detected in all cells. The activin receptor IA and IB mRNAs were expressed in all the cell lines tested. The primers for ActR IIA and IIB amplification were designed to locate within intracellular kinase domains of ActR IIA and IIB. The ActR IIA and IIB mRNAs were expressed in all the cell lines tested. Interestingly, significantly higher expressions of ActR IIA and IIB mRNAs were observed in SNU-16 cells when compared to other cells. The Smad2, 4, and 7 mRNAs were expressed in all cells and p21\(^{CIP1/WAF1}\) mRNAs were not expressed in SNU-1, SNU-484, and SNU-668 cells.

Effects of activin A on expression of activin receptors, Smad and p21\(^{CIP1/WAF1}\) genes
To detect the effects of activin on ActR I and ActR II mRNA expression in SNU-16 cells, RT-PCR analysis was performed using the cells treated with 100 ng/mL of activin A for various durations (Figure 3A). Expression of

---

Table 2 Effects of activin A on cell proliferation in human gastric cancer cell lines (mean ± SD, ng/mL)

| Times | 24 h | 48 h | 72 h |
|-------|------|------|------|
|       | 0    | 10   | 50   | 100  | 0    | 10   | 50   | 100  | 0    | 10   | 50   | 100  |
| Activin A |      |      |      |      |      |      |      |      |      |      |      |      |
| AGS   | 100  | 92.7 ± 5.1 | 93.6 ± 4.3 | 93.6 ± 4.2 | 100  | 93.6 ± 2.9 | 94.1 ± 3.1 | 92.9 ± 3.9 | 100  | 94.0 ± 3.9 | 94.2 ± 3.7 | 91.7 ± 3.8 |
| KATO III | 100  | 94.2 ± 3.3 | 92.9 ± 4.6 | 92.1 ± 4.8 | 100  | 93.1 ± 3.6 | 89.8 ± 4.2 | 90.9 ± 3.7 | 100  | 91.1 ± 4.0 | 85.6 ± 4.2 | 84.1 ± 6.8 |
| SNU-1 | 100  | 94.2 ± 2.6 | 94.0 ± 2.8 | 94.5 ± 2.9 | 100  | 94.6 ± 2.0 | 93.4 ± 5.7 | 94.7 ± 3.3 | 100  | 94.0 ± 3.5 | 94.0 ± 2.8 | 93.9 ± 4.1 |
| SNU-5 | 100  | 93.9 ± 0.6 | 94.7 ± 2.4 | 94.1 ± 1.8 | 100  | 94.1 ± 3.3 | 94.2 ± 5.6 | 94.8 ± 4.0 | 100  | 94.7 ± 2.6 | 94.0 ± 4.3 | 94.8 ± 4.0 |
| SNU-16 | 100  | 87.3 ± 2.3 | 81.7 ± 3.3 | 77.2 ± 4.5 | 100  | 87.8 ± 2.6 | 74.3 ± 3.4 | 61.5 ± 2.9 | 100  | 81.2 ± 4.6 | 62.2 ± 4.5 | 45.2 ± 4.0 |
| SNU-484 | 100  | 94.3 ± 3.9 | 94.0 ± 3.0 | 92.1 ± 2.9 | 100  | 94.9 ± 1.5 | 92.6 ± 3.6 | 94.8 ± 1.8 | 100  | 93.4 ± 4.8 | 92.5 ± 5.5 | 91.8 ± 6.8 |
| SNU-601 | 100  | 94.2 ± 2.0 | 94.2 ± 1.8 | 91.6 ± 2.3 | 100  | 94.6 ± 3.4 | 94.5 ± 2.1 | 94.0 ± 2.9 | 100  | 94.9 ± 4.7 | 91.9 ± 6.1 | 91.3 ± 6.7 |
| SNU-638 | 100  | 91.1 ± 2.6 | 92.5 ± 1.1 | 91.5 ± 2.5 | 100  | 93.3 ± 3.7 | 92.6 ± 4.2 | 91.8 ± 4.2 | 100  | 93.9 ± 3.3 | 92.0 ± 3.8 | 91.3 ± 4.1 |
| SNU-668 | 100  | 94.2 ± 2.8 | 94.3 ± 2.3 | 94.4 ± 3.1 | 100  | 91.7 ± 1.4 | 91.8 ± 1.6 | 92.3 ± 4.8 | 100  | 93.2 ± 1.5 | 94.0 ± 2.2 | 94.9 ± 2.3 |
| SNU-719 | 100  | 92.8 ± 2.8 | 91.6 ± 4.0 | 91.4 ± 1.2 | 100  | 91.7 ± 2.2 | 92.4 ± 5.2 | 91.7 ± 3.4 | 100  | 91.8 ± 2.0 | 88.7 ± 2.8 | 89.2 ± 3.1 |

Values are the percentage for three individual experiments, each with triplicate samples. *\(p < 0.05\) and **\(p < 0.01\) vs the control values.
Activin has several biological functions, including regulation of cell proliferation and inhibition of tumor cells. Activin inhibits cell growth in human prostate cancer LNCaP cells, human HepG2 hepatoma cells, and mouse B-cell hybridoma cells. In this study, treatment with activin A (10-100 ng/mL) induced a significant decrease in the proliferation of SNU-16 cells in a dose- and time-dependent manner. In contrast, some human gastric cancer cell lines were relatively resistant to the growth inhibition induced by exogenous activin treatment. Interestingly, activin A (100 ng/mL) decreased the number of SNU-16 cells in a time-dependent manner. The mechanism of growth inhibition by activin in SNU-16 cells remains uncertain, but activin may act as a growth inhibitor in SNU-16 cells, and the subsequent loss of this autocrine growth inhibitory pathway may lead to the development of cancer.

Basal expressions of inhibin/activin subunits, activin receptors, Smads, and p21\(^{CIP1/WAF1}\) mRNAs in the human gastric cancer cell lines demonstrated to have differential expression patterns for inhibin \(\alpha\), activin \(\beta\)A subunits, activin receptors, and Smads, as well as the presence of p21\(^{CIP1/WAF1}\) and the absence of activin \(\beta\)B subunits. Interestingly, significantly higher expressions of ActR IIA and IIB mRNAs were observed in SNU-16 cells when compared to other cells.

To determine the time-course of activin’s effects on mRNA levels of ActRs, Smads, and p21\(^{CIP1/WAF1}\), RT-PCR was performed using SNU-16 cells treated with activin A (100 ng/mL) for different time durations. Activin A decreased ActR I A, IB, and IIA mRNA levels whereas increased ActR IIB mRNA level in SNU-16 cells. Treatment with activin A (10-100 ng/mL) could not affect ActR IIA and IIB mRNA expression for 24 h in OVCAR-3 cells. ActRIs act primarily through Smad2, possibly in partnership with Smad4, which forms heteromeric complexes with ligand-specific Smads after activation. In OVCAR-3 cells, no detectable change has been induced in Smad4 mRNA expression within 72 h of activin treatment, but the Smad2 mRNA level gradually increases and is significantly higher than that in the control at 72 h. Activin–induced HepG2 liver cell apoptosis involves ActRs and Smad proteins. Overexpression of ActR I B and IIB or Smad2 and Smad4 stimulates apoptosis, whereas the dominant negative mutant forms of ActR IIB or Smad2 block activin-stimulated apoptosis. Signal transduction from the cell surface to the nucleus through Smad proteins is required for activin-induced cell death in liver cells. In SNU-16 cells, no difference was observed in Smad2 mRNA levels but the Smad4 mRNA level increased for up to 48 h after activin treatment. Taken together, these results suggest that enhanced levels of ActR IIB and Smad4 mRNAs likely contribute to activin signal transduction. A novel activity of Smad7 is the inhibition of erythroid differentiation by blocking the intracellular activin signaling. Smad7 expression can be induced by activin, and the overexpression of Smad7 suppresses activin-induced apoptosis in mouse B hybridomas. In the present study, expression of Smad7 mRNA increased vigorously after 24 h of activin stimulation in SNU-16 cells, and then decreased at 48 h to a level significantly below the initial basal level. These results indicate that Smad7 acts as a selective blocker of activin and provides feedback regulation in the activin-signaling pathway. In human pituitary tumors with activin-inhibited proliferation, p21\(^{CIP1/WAF1}\) gene expression is up-regulated in a dose-
Figure 3  Effects of activin A on activin receptor mRNA expression (A), smad mRNA expression (B), and p21\textsuperscript{CIP1/WAF1} mRNA expression (C) in SNU-16 cells. Cells (5 × 10\textsuperscript{4} cells/mL) were cultured with activin A (100 ng/mL) in a time-dependent manner and mRNA levels were measured by RT-PCR. Values are mean ± SD of three individual experiments and reported as the ratio of activin receptors to β-actin signals, the ratio of Smad to β-actin signals, and the ratio of p21\textsuperscript{CIP1/WAF1} to β-actin signals, respectively. aP < 0.05 and bP < 0.01 vs the control values.

In conclusion, inhibin/activin subunits, activin receptors, Smads, and p21\textsuperscript{CIP1/WAF1} are expressed in human...
gastric cancer cell lines. Moreover, the inhibition of cell growth by activin is regulated by the negative feedback effect of Smad7 on the activin signaling pathway, and is mediated through p21\(^{CIP1/WAF1}\) activation in SNU-16 cells. Further investigation is needed to determine the molecular mechanisms of activin’s inhibitory effects on cell growth, apoptosis, and cancer progression.

REFERENCES

1. Ying SY. Inhibins and activins: chemical properties and biological activity. Proc Soc Exp Biol Med 1987; 186: 253-264
2. Ying SY. Inhibins, activins, and follistatins: gonadal proteins mediating the secretion of follicle-stimulating hormone. Endocr Rev 1988; 9: 267-293
3. Vale W, Hseau A, Rivier C, Yu J. The inhibin/activin family of hormones and growth factors. In: Sporn MB, Roberts AB, eds. Peptide Growth Factors and Their Receptors. New York: Springer-Verlag, 1990; 211-246
4. Mathews LS. Activin receptors and cellular signaling by the activin family. Endocr Rev 1994; 15: 310-325
5. Attisano L, Wrana JL, Montalvo E, Massagué J. Activation of signalling by the activin receptor complex. Mol Cell Biol 1996; 16: 1066-1073
6. Dalkin AC, Gilrain JT, Bradshaw D, Myers CE. Activin inhibition of prostate cancer cell growth: selective actions on androgen-responsive LNCaP cells. Endocrinology 1996; 137: 5230-5235
7. Ying S, Zhang Z, Huang G, Hsieh F. Inhibin/activin subunits and activin receptors in human breast cell lines and lactating rat mammary-glands. Int J Onco 1995; 7: 481-485
8. Liu QY, Niranjan B, Gomes P, Gomm JJ, Davies D, Coombes RC, Buluwela L. Inhibitory effects of activin on the growth and morphogenesis of primary and transformed mammary epithelial cells. Cancer Res 1996; 56: 1155-1163
9. Yingling JM, Wang XF, Bassing CH. Signaling by the transforming growth factor-beta receptors. Biochim Biophys Acta 1995; 1242: 115-136
10. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 1997; 390: 465-471
11. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA Jr, Wrana JL, Falb D. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell 1997; 89: 1165-1173
12. Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K. Smad6 inhibits signalling by the TGF-beta superfamily. Nature 1997; 389: 622-626
13. Nakao A, Afrahtke M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawaabata M, Heldin NE, Heldin CH, ten Dijke P. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature 1997; 389: 631-635
14. Kitamura K, Aota SI, Sakamoto R, Yoshikawa SI, Okazaki K. Smad7 selectively interferes with different pathways of activin signalling and inhibits erythroid leukemia cell differentiation. Blood 2000; 95: 3371-3379
15. Kanamaru C, Yasuda H, Fujita T. Involvement of Smad proteins in TGF-beta and activin A-induced apoptosis and growth inhibition of liver cells. Hepatol Res 2002; 23: 211-219
16. el-Deiry WS, Tokino T, Velecescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KD, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993; 75: 817-825
17. Skapek SX, Rhee J, Spicer DB, Lassar AB. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science 1995; 267: 1022-1024
18. Yang ZY, Perkins ND, Ohno T, Nabel EG, Nabel GJ. The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo. Nat Med 1995; 1: 1052-1056
19. Zauberman A, Oren M, Zipori D. Involvement of p21\(^{CIP1/WAF1}\) CDK4 and Rb in activin A mediated signaling leading to hematoma cell growth inhibition. Oncogene 1997; 15: 1705-1711
20. Hashimoto O, Yamato K, Koseki T, Ohguchi M, Ishisaki A, Shoji H, Nakamura T, Hayashi Y, Sugino H, Nishihara T. The role of activin type I receptors in activin A-induced growth arrest and apoptosis in mouse B-cell hybridoma cells. Cell Signal 1998; 10: 743-749
21. Choi KC, Kang SK, Nathwani PS, Cheng KW, Auersperg N, Leung PC. Differential expression of activin/ inhibin subunit and activin receptor mRNAs in normal and neoplastic ovarian surface epithelium (OSE). Mol Cell Endocrinol 2001; 174: 99-110
22. Ito I, Minegishi T, Fukuda J, Shinozaki H, Auersperg N, Leung PC. Differential expression of activin/ inhibin subunit and activin receptor mRNAs in normal and neoplastic ovarian surface epithelium (OSE). Mol Cell Endocrinol 2001; 174: 99-110
23. Chen W, Woodruff TK, Mayo KE. Activin A-induced HepG2 liver cell apoptosis: involvement of activin receptors and smad proteins. Endocrinology 2000; 141: 1263-1272
24. Ishisaki A, Yamato K, Nakao K, Nonaka K, Ohguchi M, ten Dijke P, Nishihara T. Smad5 is an activin-inducible inhibitor of activin-induced growth arrest and apoptosis in mouse B cells. J Biol Chem 1998; 273: 24293-24296
25. Danila DC, Inder WJ, Zhang X, Alexander JM, Swearingen B, Hedley-Whyte ET, Klibanski A. Activin effects on neoplastic proliferation of human pituitary tumors. J Clin Endocrinol Metab 2000; 85: 1099-1015

S-Editor Wang J L-Editor Wang XL E-Editor Zhang Y