p27^{KIP1} and GATA-1 are potential downstream molecules in activin A-induced differentiation and apoptosis pathways in CML cells

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Abstract. p27^{KIP1} is known as a regulator of cellular differentiation and apoptosis in human cancer cells. We have previously reported that human chronic myeloid leukemia (CML) KU812 and K562 cells show inhibited cellular proliferation in response to treatment with activin A, a member of TGF-ß superfamily. Apoptosis and erythroid differentiation can be induced in KU812 and K562 cells, respectively. We report herein that activin A induced the expression of p27^{KIP1} in CML cells along with the induction of cellular differentiation and apoptosis. There are putative binding sequences of erythroid-related transcription factor GATA-1 in the promoter region of the human p27^{KIP1} gene. Expression of GATA-1 protein in activin A-treated KU812 and K562 cells showed dissimilar regulation in these two cell lines. Induction of p27^{KIP1} was commonly observed, but it did not correspond to the expression levels of GATA-1 in either line of activin A-treated CML cells. In addition, ERK protein was rapidly and transiently activated with activin A in both types of CML cells, suggesting that phosphorylation of ERK is required for activin A signaling in CML cells. These results indicate that both p27^{KIP1} induction and regulation of GATA-1 play essential roles in activin A-induced erythroid differentiation and apoptosis.

Introduction

Cyclin-dependent kinase (cdk) inhibitors are known to be involved in the control of cellular differentiation, proliferation, and apoptosis (1). TGF-ß superfamily cytokines including TGF-ß, activin A, and bone morphogenetic protein (BMP) are also known to be related to and regulate the diverse cellular responses (2). There have been many studies connecting the TGF-ß superfamily with the regulation of cdk inhibitors, and with the induction of cellular differentiation and apoptosis (3,4). It has been reported that p21$^{CIP1/WAF1}$ is associated with myeloid differentiation via transcriptional activation by p53, c-Jun, and TGF-ß (5-11). p21$^{CIP1/WAF1}$ and p15^{INK4} are both activated by TGF-ß; however, there is no direct evidence of a relationship between the cellular differentiation and the p21$^{CIP1/WAF1}$ regulation by TGF-ß.

p27^{KIP1} is also known to induce apoptosis and to behave as a target during cellular differentiation (13,14). Up-regulation of p27^{KIP1} was observed in the differentiation of various cells including osteoclast, neuron, myeloid, and erythroid cells (15-19). For TGF-ß signaling, p27^{KIP1} and p15^{INK4} coordinate their inhibitory interactions with cdk4 and cdk2 (20). In contrast, neutralization of autocrine TGF-ß increased p27^{KIP1} and affected the differentiation states of human primitive progenitor cells in a cell cycle-independent manner (21). Although p27^{KIP1} plays important roles in cellular differentiation and apoptosis (22), the regulation of this molecule has not been investigated in detail.

We have previously reported that activin A, a commitment factor in erythroid differentiation, induces apoptosis and differentiation in human CML cells (23). In this study, we examined the role of p27^{KIP1} in activin A-induced differentiation and apoptosis in CML cells.

Materials and methods

Cells and cytokine. Human CML cell lines (KU812 and K562 cells) were obtained from the Japanese Cancer Research Resources Bank (JCRB; Tokyo, Japan) and were maintained in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma) in a humidified atmosphere with 5% CO_2. Recombinant human activin A was kindly provided by

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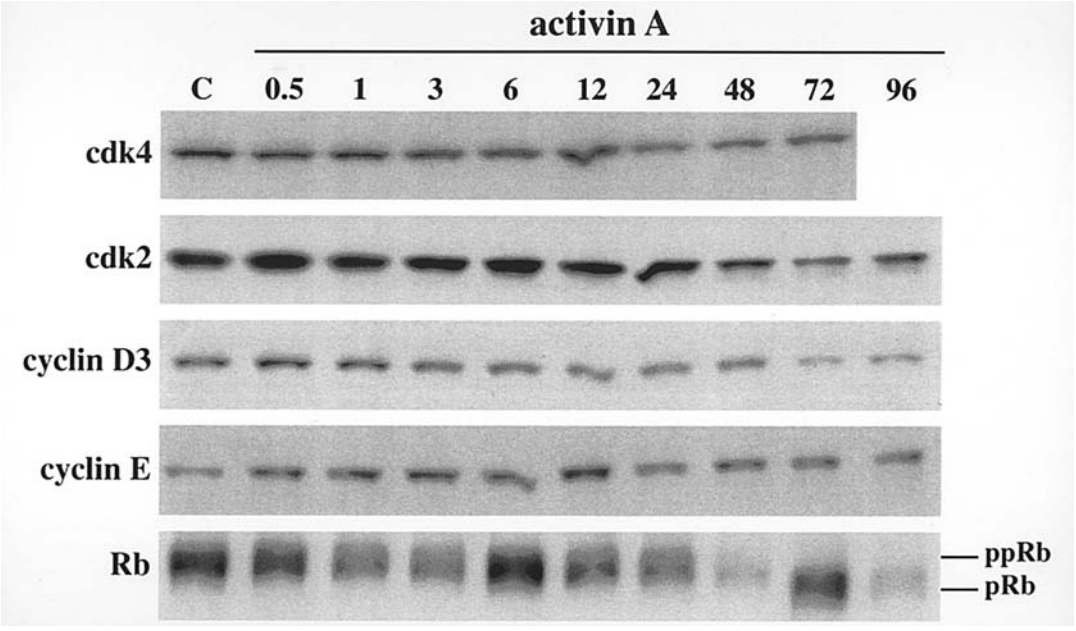

Figure 1. Expression of cell cycle-associated proteins. KU812 cells were treated with 50 ng/ml of activin A for the indicated times. Cell lysates (20 μg/lane) were fractionated on SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against cell cycle-associated proteins (cdk4, cdk2, cyclin D3, cyclin E, and phospho Rb).

Dr Y. Eto (Ajinomoto Co. Inc., Tokyo, Japan), and dissolved in RPMI-1640 medium at a stock concentration of 250 μg/ml.

Cell lysates and Western blotting. After stimulation with activin A (50 ng/ml) for various indicated times, cells were washed with 25 mM TBS (pH 7.4) and suspended in lysis buffer [1% NP-40, 1 mM PMSF, 40 mM Tris-HCl (pH 8.0), 150 mM NaCl] at 4˚C for 15 min. After centrifugation, supernatants were collected and protein concentrations were determined by using a Bio-Rad protein assay system (Bio-Rad, Richmond, CA). Cell lysates (20 μg of protein per lane) were fractionated in 12.5% SDS-polyacrylamide gel prior to transfer to the membrane (Immobilion-P membrane, Millipore, Bedford, MA) using the standard protocol. For the analysis of Rb protein, we used 6% SDS-polyacrylamide gel. Membranes were blocked overnight with 10% defatted milk in PBS containing 0.1% Tween-20 (PBS-T) or in 25 mM TBS containing 0.1% Tween-20 (TBS-T) at 4˚C. Antibodies were used at 1:300-2500 dilution in 0-10% defatted milk in PBS-T or in TBS-T for 1 h at 25˚C. Subsequently, membranes were incubated with a second antibody conjugated with horseradish peroxidase (HRP) using 1:1000-3000 dilution in 10% defatted milk. Antibody binding was detected by using an enhanced chemiluminescence kit for Western blot detection with Hyper ECL (Amersham, Buckinghamshire, UK). Blots were stained with Coomassie brilliant blue and confirmed to contain an equal amount of protein extract on each lane.

Antibodies. Antibodies used in this study were: anti-cdk4, -cyclin E, -cyclin D1, -cyclin D2, -GATA-1, -p21$^{CIP1/WAF1}$, -p15^{INK4}, -goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), -p27^{KIP1} (Calbiochem, La Jolla, CA), -cdk2, and -cyclin D3 (Transduction Lab., Lexington, KY), -Rb (Phar-Mingen, San Diego, CA), -rabbit Ig-HRP, -mouse Ig-HRP (Amersham). PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) antibody kit was obtained from Cell Signaling Technology (Beverly, MA). ß-actin was used to confirm that equal amounts of protein were loaded in each lane (data not shown).

Cell cycle analysis. Cells (1x10^6) were suspended in hypotonic solution [0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, 0.1 mM EDTA] and stained with 50 μg/μl of propidium iodide (PI). DNA contents were analyzed by a FACScan (Becton Dickinson, San Jose, CA). The proportion of cells in each cell cycle phase was determined by the use of Cell modiFIT software (Becton Dickinson).

Results

Expression of cell cycle-associated proteins. Levels of cdk (cdk2 and cdk4) and cyclin D3 in activin A-treated KU812 cells were all down-regulated after 12 to 48 h of treatment (Fig. 1). Expression of cyclin E protein was increased in 0.5 to 12 h with treatment of activin A, but the levels were returned to basal level after 24-h stimulation (Fig. 1). Phosphorylation of Rb protein, which was reported to be involved in the development of mature erythrocytes (19,24), was dephosphorylated after 48-h exposure to activin A (Fig. 1). Expression of cyclin D1, D2, p15^{INK4}, and p21$^{CIP1/WAF1}$ did not change during the treatment with activin A (data not shown).

Cell cycle analysis. KU812 cells were incubated with activin A (50 ng/ml) for various periods of times (0-96 h), and then cells were analyzed in terms of cell cycle distribution by means of flow cytometry. Cultivation of activin A (50 ng/ml) did not induce cell cycle arrest of KU812 cells (data not shown). At 24 h after treatment, an increase was observed in the fraction of hypodiploid cells, which indicated that these cells had undergone apoptosis (data not shown). These

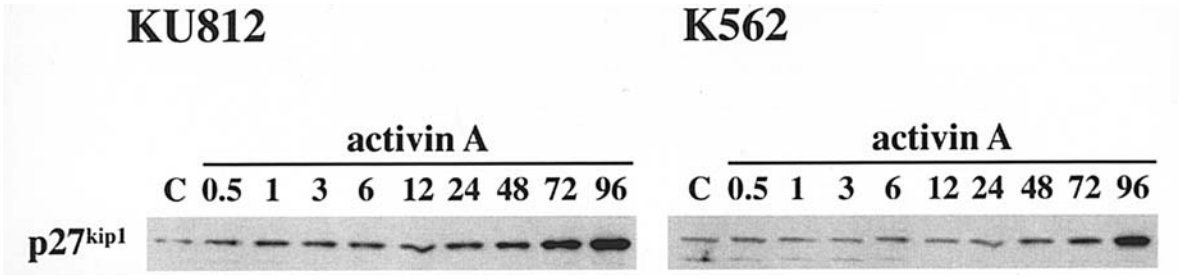

Figure 2. Effect of activin A on the expression of p27^{KIP1}. KU812 and K562 cells were treated with 50 ng/ml of activin A for the indicated times, and then the expression of p27^{KIP1} was analyzed by Western blotting.

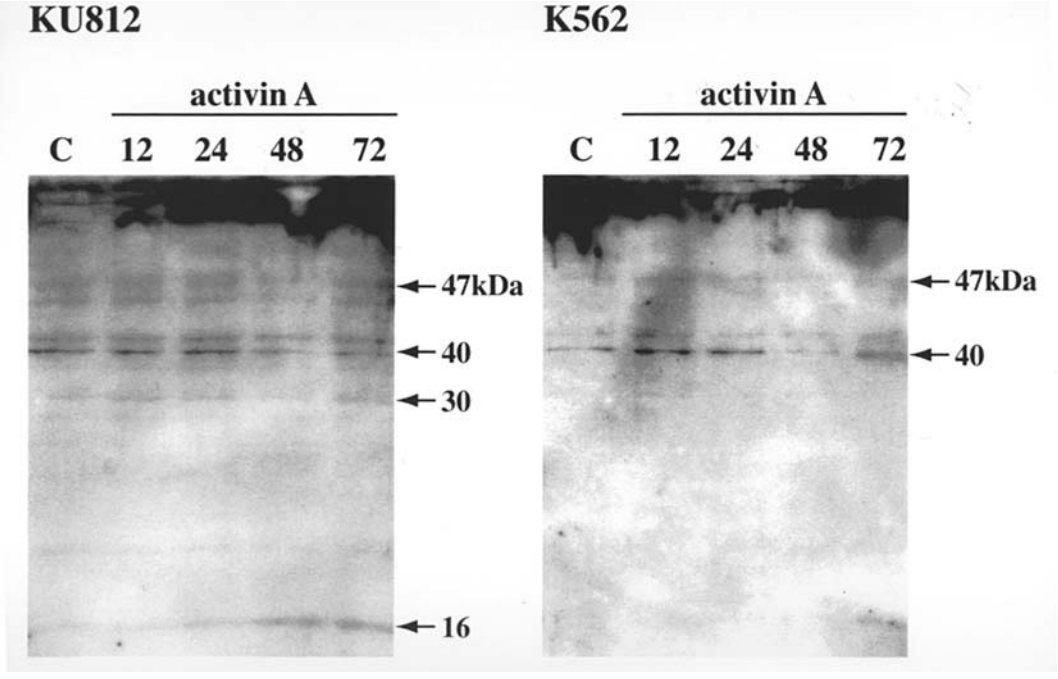

Figure 3. Expression of GATA-1 protein in activin A-treated CML cells. KU812 and K562 cells were cultured with 50 ng/ml of activin A for various times (0-72 h). Cell lysates (20 μg/lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with antibody against GATA-1.

results suggest that activin A induced apoptosis in a cell cycle-independent manner.

Expression of p27^{KIP1} protein in activin A-treated CML cells. To address the mechanism of activin A-induced apoptosis and erythroid differentiation in CML cells, we next examined the expression of p27^{KIP1} protein in activin A (50 ng/ml)-treated cells. Interestingly, p27^{KIP1} protein was induced and increased in both KU812 and K562 cells in a time-dependent manner (Fig. 2), suggesting that p27^{KIP1} is involved in the signaling pathway of activin A.

Expression of GATA-1 protein in activin A-treated CML cells. We searched for the human p27^{KIP1} promoter region using GenBank database analysis and found that there were several putative binding sequences of c-Myb, Sp1, p300, CRE-BP1/c-Jun, and GATA-1. Activin A induced erythroid differentiation of K562 cells (23); therefore, we investigated the expression of erythroid-related transcription factor GATA-1 in both lines of activin A-treated CML cells. In KU812 cells, two forms of GATA-1 (47 kDa and 40 kDa) (25) were both degraded, but cleaved forms (30 kDa and 16 kDa) (26) were newly produced (Fig. 3). In contrast, 47 kDa and 40 kDa forms of GATA-1 were both up-regulated during the treatment with activin A, but we did not detect cleaved forms in K562 cells (Fig. 3).

Activin A-induced ERK phosphorylation in CML cells. We commonly observed the induction of p27^{KIP1} by activin A treatment in both KU812 and K562 CML cells (Fig. 2), although the expression pattern of GATA-1 is accepted for distinct regulation (Fig. 3). It has been reported that phosphorylation levels of ERK are associated with the modulation of p27^{KIP1} expression and erythroid differentiation (27-29), so we examined the phophorylation state of ERK in activin A-treated CML cells. In both CML cells, ERK protein was rapidly and transiently up-regulated at 5 min for KU812 cells, and 5 to 10 min for K562 cells, respectively (Fig. 4). These results

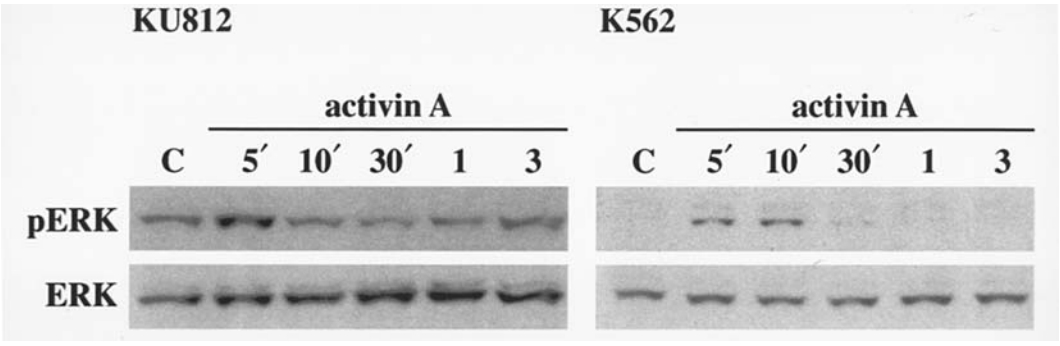

Figure 4. Activin A-induced phosphorylation of ERK. Expression of ERK and phospho-ERK in activin A (50 ng/ml)-treated KU812 and K562 cells was examined by Western blotting.

suggest that phosphorylation of ERK and the induction of p27^{KIP1} protein are necessary for inducing apoptosis and erythroid differentiation in activin A-treated CML cells.

Discussion

p27^{KIP1} induction is involved in cells undergoing differentiation in various types of cells (16,24), and overexpression of this molecule induces apoptosis of cancer cells (13). We have previously shown that activin A, a member of the TGF-ß superfamily, induced growth inhibition of KU812 and K562 human CML cells (23). Activin A treatment induced apoptosis in KU812, but it induced erythroid differentiation in K562 cells. In this study, we found that p27^{KIP1} induction was associated with both events; therefore, we further examined the relationship between the expression of p27^{KIP1} and activin A-induced apoptosis and cellular differentiation. In the TGF-ß signaling pathway, p21$^{CIP1/WAF1}$ and p15^{INK4} were both transactivated through coactivator p300CREB-binding protein (30), and p21$^{CIP1/WAF1}$ promoter was activated by Smads, which cooperate with Sp1 (8). Using the GenBank database analysis system, we found that the human p27^{KIP1} promoter region has several putative sites of c-Myb, Sp1, p300, CRE-BP1/c-Jun, but not Smads. In addition, the erythroid-related transcription factor GATA-1 was also involved in the promoter region of p27^{KIP1}. Analysis of GATA-1 protein showed the distinct regulation of activin A-treated KU812 and K562 CML cells. Accumulating evidence exists on the regulation of erythropoiesis by caspases (31,32), including caspase-mediated cleavage of GATA-1 (26). Our previous study showed that activin A-induced apoptosis in KU812 cells preceded via the activation of caspase-3 and caspase-9, and we predicted that the apoptotic signal is preceded by a differentiation signal in activin A-treated KU812 cells (23). Furthermore, co-stimulation with activin A and erythropoietin, a survival factor for erythroid cells and a downstream target of GATA-1 promoter (33,34), overrode activin A-induced apoptosis and stimulated erythroid differentiation in KU812 cells (23). Although the GenBank database analysis did not show the putative Smad binding site in the human GATA-1 promoter region, there have been many studies on the regulation of erythropoiesis by the TGF-ß superfamily (35-39). In addition, expression of GATA-1 also activates the induction of several erythroid-related genes (40). These results suggest that GATA-1 may be one of the key molecules for the activin A-induced erythroid differentiation signal.

Induction of p27^{KIP1} by activin A, but not GATA-1, was commonly observed in both KU812 and K562 CML cells. Recent studies have reported that the Ras/MAP kinase pathway is closely related to the regulation of cdk inhibitors (7,12) or erythroid differentiation (28,29). Additionally, the Ras/MAP kinase pathway is predominantly required for the stimulation of p21$^{CIP1/WAF1}$ by TGF-ß (7). In activin A-treated KU812 and K562 cells, ERK protein was rapidly and transiently upregulated. Although more direct evidence would be necessary to clarify the relationship between ERK phosphorylation and p27^{KIP1} induction in activin A-treated cells, our observation might have an important implication for the activin A signaling pathway related to erythroid differentiation in CML cells. In addition, the induction or degradation of GATA-1 may be a deciding factor in the fate of cells with respect to apoptosis or differentiation in the activin A signaling pathway.

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