

## Bis induces growth inhibition and differentiation of HL-60 cells via up-regulation of p27

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Abbreviations: PI, propidium iodide; RA, retinoic acid

### Abstract

**Bis (Bag-3, CAIR), a Bcl-2-interacting protein, promotes the anti-apoptotic activity of Bcl-2 and increased levels of Bis have been observed in several disease models. The involvement of Bcl-2 and some Bcl-2-binding proteins in differentiation has recently been reported. However, the relevance of Bis to cellular differentiation remains unknown. The findings herein show that Bis expression is up-regulated during the differentiation of HL-60 cells. To investigate the effect of Bis expression on differentiation, we established Bis-overexpressing HL-60 cells (HL-60-*bis*). HL-60-*bis* cells have a low nuclear:cytoplasmic ratio and indented nucleus in Wright-Giemsa staining, and an increased expression of CD11b in immunofluorescence study, indicating the promotion of differentiation. The overexpression of Bis also resulted in a retarded cell growth rate, accompanied by the accumulation of HL-60 cells at the G0/G1 phase of the cell cycle, which was sustained during the differentiation process. Western blot analysis revealed that the expression of p27, a representative inducer of cell cycle arrest at the G1**

**phase, was increased 2.5-fold in HL-60-*bis* cells compared to HL-60-*neo* cells. These results suggest that the Bis induced growth inhibition of HL-60 cells promotes G0/G1 phase arrest via up-regulation of p27, which seems to be a prerequisite for differentiation. Further studies will be required to define the exact roles of Bis on cellular differentiation more precisely.**

**Keywords:** BAG3 protein, human; cell differentiation; cyclin-dependent kinase inhibitor p27; HL-60 cells

### Introduction

Bis has been identified as a novel Bcl-2 binding protein that enhances the anti-apoptotic activity of Bcl-2 (Lee *et al.*, 1999). Bis is also known as Bag-3 or CAIR-1, which has been reported to bind to Hsp70 or phospholipase C $\gamma$ , respectively (Takayama *et al.*, 1999; Doong *et al.*, 2000). The Bis protein shows no significant homology with the Bcl-2 family proteins and the transfection of *bis* DNA indicates that Bis itself serves as only a weak anti-apoptotic protein, but synergistically acts with Bcl-2 to protect from Bax and Fas-mediated cell death (Lee *et al.*, 1999). The pro-survival activity of Bis is further supported by findings showing that the expression of Bis is increased in pancreatic cancer where Bcl-2 is frequently overexpressed and the down regulation of Bis protein levels using specific antisense nucleotides results in B-CLL cell apoptosis (Liao *et al.*, 2001; Romano *et al.*, 2003). In the brain, we previously observed that the expression levels of Bis protein were increased in reactive astrocytes, accompanied by increased Bcl-2 levels in the hippocampus after forebrain ischemia and kainic acid-induced seizure (Lee *et al.*, 2002a; b). Collectively, these results suggest that Bis modulates Bcl-2 activity under the certain patho-physiological conditions.

It has recently been suggested that Bcl-2, in addition to its ability to protect against apoptosis, might also be involved in the regulation of cell differentiation such as neural differentiation and myeloid cell differentiation (Seite *et al.*, 2000; Liang *et al.*, 2003; Zhang *et al.*, 2003). Immunohistochemistry experiments also revealed that Bcl-2 is highly expressed in several organs during morphogenesis and differentiation (Delia *et al.*, 1992; Sun *et al.*, 2002). In addition, the overexpression of Bag-1, a

Bcl-2 binding protein that shares the Bag domain with Bis (Takayama *et al.*, 1995; Takayama *et al.*, 1999), slowed the proliferation rates of CSM14.1 neuronal cells (rat nigro-striatal cell line) and accelerated neuronal differentiation (Kermer *et al.*, 2002). Based on the findings that the Bis modulates anti-apoptotic activity of Bcl-2 *in vitro* (Lee *et al.*, 1999) and Bis is co-expressed with Bcl-2 *in vivo* after an ischemic injury or excitotoxic brain injury (Lee *et al.*, 2002a; b; Schwarz *et al.*, 2002), it is possible that Bis also influences the differentiation process in which Bcl-2 is involved.

In the present study, therefore, we examined the Bis protein expression during the differentiation of HL-60 cells and the effect of Bis expression on the growth and differentiation of HL-60 cells to determine if a causal relationship exists between expression of Bis and differentiation.

## Materials and Methods

### Cell cultures

Human Promyelocytic Leukemia HL-60 cells obtained from the American Type Culture Collection were maintained in RPMI 1640 medium (Gibco-BRL, Grand island, LY) supplemented with 10 mM HEPES (Gibco-BRL), 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and antibiotic solutions (100 U/ml of penicillin and 100 mg/ml streptomycin, Gibco-BRL) and incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Differentiation was induced by 1 µM of retinoic acid (RA; Sigma-aldrich, St. Louis, MO), 1.3% dimethyl sulfoxide (DMSO; Sigma-aldrich), or 20 ng of phorbol 12-myristate 13-acetate (PMA; Sigma-aldrich) for the indicated days.

### Preparation of Bis-overexpressing HL-60 cells

HL-60 cells, adjusted to a density of  $2.5 \times 10^5$  cells in 800 µl of phosphate buffered saline (PBS; Sigma-aldrich), were transfected with 25 µg of neomycin-resistance expression vector (pCXN, Niwa *et al.*, 1991) or pCXN-*bis* for HL-60-*neo* or HL-60-*bis*, respectively, using a Gene Pulser (BioRad, Munchen, Germany) under the conditions of 975 µF and 300 V. The selection for stable transfectants was started after 3 days with 1 mg/ml of neomycin (G418; Gibco-BRL) and neomycin resistant cells were pooled.

### Assessment of differentiation

Differentiation was determined by morphological observation after staining using Wright-Giemsa stain solution (Sigma-aldrich) of cytopins of HL-60-*neo* and HL-60-*bis*. Cell surface immunofluorescence staining was also performed for both cells. Briefly,

the cells were washed with cold PBS containing 1% bovine serum albumin, and labeled with PE-conjugated anti-human CD11b (BD Pharmingen, San Diego, CA) for 30 min at 4°C in the dark. The cells were then washed twice with PBS and fixed in 1% paraformaldehyde/PBS solution. The cells were analyzed on FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) using Cell Quest software (Becton Dickinson).

### Cell growth analysis

Twenty thousand cells were plated on a 48-well plate in 200 µl of medium and incubated with various agents for the indicated times. Cell growth was monitored by the classical 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 50 µl of MTT reagent (500 µg/ml) (Sigma-aldrich) was added to each well. After 3 h at 37°C, the cell supernatants were discarded, MTT crystals were dissolved in acid isopropanol and the absorbance was measured at 570 nm using UV MR<sup>700</sup> (Dynatech Laboratories Inc., Chantilly, VA). All assays were performed in triplicate. The relative growth rate was defined as the percentage of the absorbance of treated cells versus untreated cells at 0 day. For the comparisons of growth rate, one-way ANOVAs were used. All results are presented as mean values  $\pm$  SEM. Statistical significance was accepted at  $P < 0.05$ .

### Analysis of cell cycle distribution

After treatment with 1.3% aqueous DMSO,  $1 \times 10^6$  HL-60-*neo* or HL-60-*bis* cells were harvested at the indicated times and washed twice with cold PBS and fixed in 70% ethanol at -20°C overnight. The cells were washed with PBS and incubated with 100 µg/ml RNase A at 37°C for 30 min. Propidium iodide (PI) solution (Sigma-aldrich) was added at a final concentration of 50 µg/ml. Analysis was performed immediately after PI staining by a FACS Calibur flow cytometer.

### Immunoblot analysis

Cells were harvested and lysed in ice-cold RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate). An equivalent protein extract from each sample was separated by 12.5% sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Immobilon-P, Milipore, Bedford, MA). The membranes were blocked with PBS containing 5% non-fat milk for 30 min and then incubated for 1 h at room temperature with primary antibodies such as anti-Bis

antiserum (1/1,000) (Lee *et al.*, 1999), anti-p27 antibody (1/400) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti- $\beta$ -actin antibody (1/5,000) (Sigma-aldrich). Blots were washed four times for 15 min with 0.3% Tween 20-containing PBS and then incubated for 1 h with peroxidase-labeled anti-mouse or anti-rabbit immunoglobulin (1/2000) (Promega Corporation, Madison, WI). The membranes were washed again four times. Blots were developed using an enhanced chemiluminescence detection system (ECL, Amersham Corp., Cardiff, UK). The resulting bands were quantified by densitometry using Scion image software program (Scion Corp., Frederick, MD).

## Results

### Expression of Bis during differentiation of HL-60 cells

To determine whether Bis expression is regulated during the HL-60 cell differentiation process, Bis protein levels were measured by Western blotting following the induction of differentiation using appropriate stimuli in HL-60 cells. Treatment of HL-60 cells with RA or DMSO is known to induce granulocytic differentiation while PMA leads to mono-

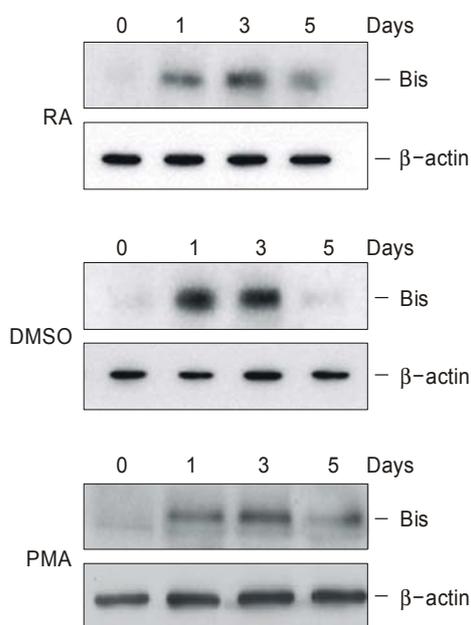
cytic differentiation (Collons *et al.*, 1978; Rovera *et al.*, 1979; Breitman *et al.*, 1980). As shown in Figure 1, all these three stimuli significantly increased the level of Bis expression in HL-60 cells and Bis induction was clearly detected as early as day 1 and decreased at day 5 after treatment. These results indicate that Bis is induced in the early phase of the differentiation process, raising the possibility that Bis is one of determinants of differentiation.

### Effects of Bis expression on the morphology and growth of HL-60 cells

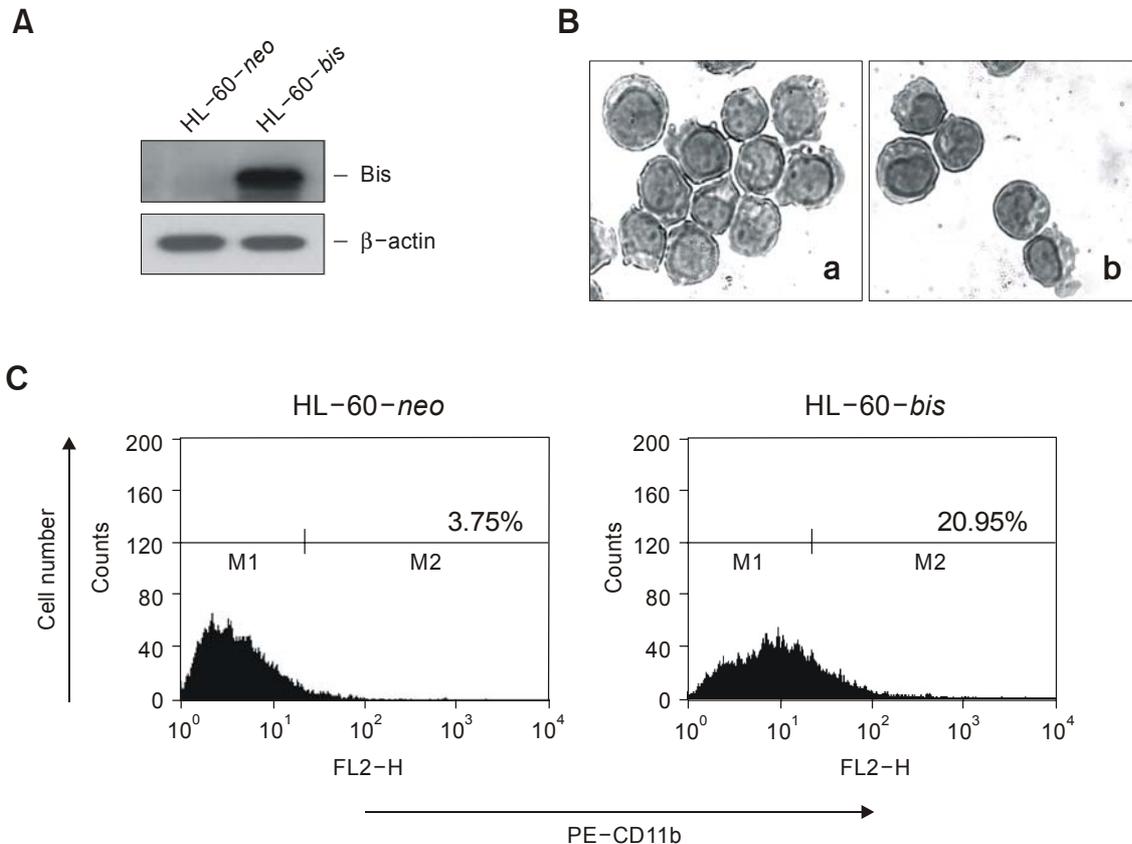
To investigate the significance of the Bis expression observed in the differentiation process, we established Bis-overexpressing HL-60 cells and examined the effect of Bis expression on differentiation. After transfection of the Bis-expressing vector or control vector, followed by three weeks of selection, the pooled populations from each preparation were analyzed for Bis expression by Western blotting. HL-60-*bis* cells showed a considerable increase in Bis expression, about a 13-fold higher level than in HL-60-*neo* cells (Figure 2A).

Wright Giemsa staining was performed to observe whether Bis expression induces morphological changes in HL-60 cells. HL-60-*neo* cells were predominantly promyelocytes with large nucleoli with dispersed nuclear chromatin and the nuclear: cytoplasmic ratio, an indicator of differentiation, was relatively high (Figure 2B). HL-60-*bis* cells showed a heterogeneous pattern of population. The main populations showed a low nuclear: cytoplasmic ratio and nucleoli were markedly reduced or had completely disappeared, and the nuclear membrane was indented or convoluted (Figure 2B), suggesting that Bis expression promotes differentiation in HL-60 cells. Furthermore, the immunofluorescence study revealed that the CD11b antigen was expressed in a larger population of untreated HL-60-*bis* cells than in HL-60-*neo* cells, 14% and 1.8%, respectively as shown in Figure 2C, suggesting that phenotypic differentiation was also induced by Bis expression in HL-60 cells.

Since a differentiated phenotype is frequently associated with the transition out of the mitotic pool (Yaroslavskiy *et al.*, 1999), we measured the growth kinetics of HL-60-*bis* cells. As shown in Figure 3A, HL-60-*neo* cells cultured for 3 days showed a 13-fold increase in viable cell populations while the HL-60-*bis* cells showed only a 5-fold increase for the same period, indicating that Bis expression caused an appreciable retardation in the rate of proliferation in HL-60 cells. When these two cells were induced to differentiate by RA or DMSO, HL-60-*bis* cells maintained their decreased growth rate, showing



**Figure 1.** Expression of Bis during differentiation of HL-60 cell. HL-60 cells were treated with 1  $\mu$ M of RA, 1.3 % of DMSO or 20 ng of PMA and harvested and cell lysates were prepared at the indicated time-points. Western blot analysis was performed using anti-Bis serum and subsequently with an anti- $\beta$ -actin antibody as a loading control.



**Figure 2.** Overexpression of Bis induced differentiation in HL-60 cells. (A) Establishment of Bis-expressing HL-60 cells. Expression of Bis protein in HL-60-*neo* and HL-60-*bis* was determined by Western blot analysis using an anti-Bis antiserum. Detection of  $\beta$ -actin served as loading control. (B) The morphologic changes of HL-60-*bis* cells were observed by Wright-Giemsa method under the light microscope ( $\times 1000$  magnification). (a) HL-60-*neo* cells, (b) HL-60-*bis* cells. (C) The CD11b expression in HL-60-*neo* or HL-60-*bis* cells was determined by staining with CD11b antibody and FACS analysis as described in Materials and Methods.

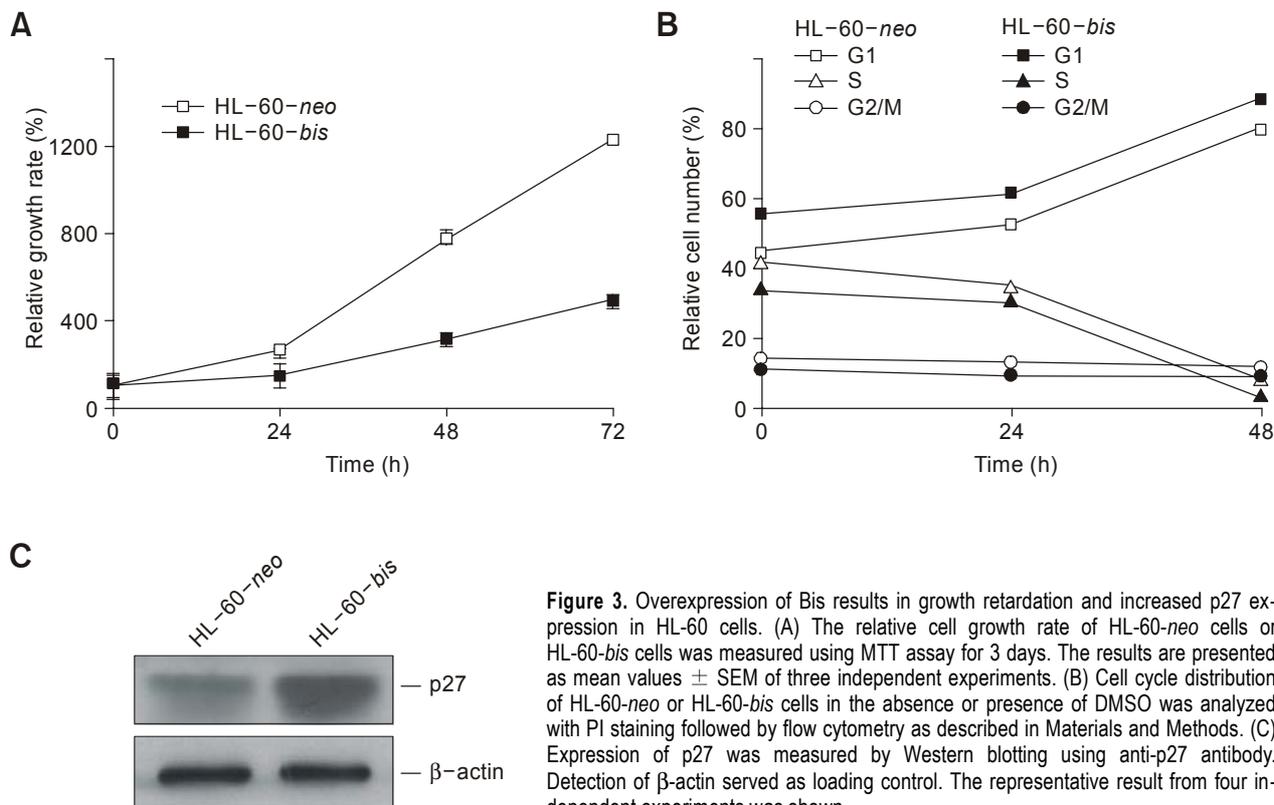
that the viable population of HL-60-*bis* cells is about 33.1% or 33.4% of HL-60-*neo* cells at 3 days of culture in the presence of RA or DMSO, respectively (data not shown).

HL-60-*neo* and HL-60-*bis* cells were also analyzed for cell cycle progression by flow cytometry. Figure 3B demonstrates that the proportion of HL-60-*bis* cells in the G<sub>0</sub>/G<sub>1</sub> phase was higher than that of HL-60-*neo* cells, 55.5% and 44.8%, respectively, in the absence of differentiation stimuli. When these two cells were treated with DMSO, to induce differentiation, the percentage of G<sub>0</sub>/G<sub>1</sub> phase of cell cycle was increased in HL-60-*neo* cells, as observed previously (Braverman *et al.*, 1986; Spector *et al.*, 1993), from 44.8% at day 0 to 52.1% at day 1 to 80.3% at day 2. In HL-60-*bis* cells, the G<sub>1</sub> accumulation was persistent during differentiation, from 55.5% at day 0 to 61.2% at day 1 to 88.3% at day 2 in the presence of DMSO. These results indicate that the growth retardation in HL-60-*bis* cells could be attributed to the increased accumulation of cells

at the G<sub>1</sub> phase, in the absence or presence of differentiation agents.

#### p27 is up-regulated in HL-60-*bis* cells

To analyze the molecular mechanism of cell growth inhibition of Bis, we investigated the expression level of p21 and p27 proteins in HL-60-*neo* and HL-60-*bis* cells. These two proteins are representative inducers of cell cycle arrest at the G<sub>1</sub> phase and also known to be increased by the induction of differentiation in hematopoietic cells (Taniguchi *et al.*, 1999). A Western blot analysis showed that p27 expression was significantly increased in HL-60-*bis* cells, about 2.5-fold, compared to HL-60-*neo* cells (Figure 3C). An increase in the expression of p21 was not detected in our experiments (data not shown). Therefore, the increased p27 may cause the cells to accumulate at G<sub>1</sub> phase, which contributes in part to the growth inhibition of HL-60-*bis* cells.



**Figure 3.** Overexpression of Bis results in growth retardation and increased p27 expression in HL-60 cells. (A) The relative cell growth rate of HL-60-*neo* cells or HL-60-*bis* cells was measured using MTT assay for 3 days. The results are presented as mean values  $\pm$  SEM of three independent experiments. (B) Cell cycle distribution of HL-60-*neo* or HL-60-*bis* cells in the absence or presence of DMSO was analyzed with PI staining followed by flow cytometry as described in Materials and Methods. (C) Expression of p27 was measured by Western blotting using anti-p27 antibody. Detection of  $\beta$ -actin served as loading control. The representative result from four independent experiments was shown.

## Discussion

The control of cellular differentiation is a complex process that depends on a variety of endogenous and exogenous factors. In the present report, we observed that Bis, a novel Bcl-2 binding protein, is induced during the differentiation of HL-60 cells with appropriate differentiation-inducing stimuli. The maximal increase in Bis protein was observed at 3 day after the induction of differentiation when the differentiation is not fully completed, indicating that Bis induction is related to the early stage of differentiation rather than a result or an accompanying event of differentiation. Considering that an increase in Bis protein level is not confined to the differentiation of specific cell lineages, Bis might be involved in the basic mechanism of differentiation of different cell lineage. Although the functional role of Bis in the differentiation of HL-60 cells is not known, our results using Bis-expressing HL-60 cells suggest that Bis expression triggers the progress of differentiation, based on the morphological findings as well as CD11b expression. In addition, HL-60-*bis* cells also showed a retarded growth rate in the absence or presence of differentiation inducing stimuli, which is probably due to the accumulation of cells at the G1 phase, as evidenced by cell cycle

analysis. Therefore, the increase in Bis expression during the differentiation seems to be involved in the inhibition of cell growth progress, which is known to be tightly coupled to steps forward differentiation (Defacque *et al.*, 1999; Cabodi *et al.*, 2000; Curtis *et al.*, 2000; Nakajima *et al.*, 2003).

While Bcl-2 has been recently reported to be involved in the differentiation of several cell types including neuronal cells (Mariano *et al.*, 1992; Hanada *et al.*, 1993; Zhang *et al.*, 1996; Suzuki *et al.*, 1998; Liang *et al.*, 2003; Schuller *et al.*, 2004), Bcl-2 levels decrease during the differentiation of myeloid cells (Naumovski *et al.*, 1994). In addition, bcl-2-transduced HL-60 cells showed same differentiative response to RA or PMA with a prolonged survival (McCarthy *et al.*, 1994; Park *et al.*, 1994; Robertson *et al.*, 1998). Thus, apoptosis and differentiation are regulated independently in myeloid cells. The induction of Bis observed in the present study is not correlated with the decreasing expression pattern of Bcl-2 in HL-60 cells (Delia *et al.*, 1992; Naumovski *et al.*, 1994). Therefore, the growth inhibitory activity of Bis may be a novel function of Bis which is not dependent on the presence of Bcl-2, even though Bis has been cloned as Bcl-2 binding protein (Lee *et al.*, 1999). It is also possible that Bcl-2 negatively regulates Bis protein stability which

is increased by the down-regulation of Bcl-2 during differentiation, as simply postulated by the inverse expression pattern of these two proteins.

Even though the molecular mechanisms by which Bis induces growth retardation in HL-60 cells is not clear, an elevated expression of p27 in HL-60-*bis* cells provide a possibility that p27 functions as a mediator that delays the cell cycle progression. Our presumption is supported by a recent paper showing that HL-60 cells expressing antisense RNA for p27 revealed a significant decrease in their ability to differentiate into granulocytes (Horie *et al.*, 2004), indicating that an increased expression of p27 is essential for cellular differentiation of HL-60 cells. However, differentiation was shown to be uncoupled from growth arrest in several cells such as primary keratinocytes or luteal cells. Therefore, it can not be excluded that Bis induces differentiation of HL-60 cells through uncharacterized mechanism which is not related with p27 (Tong *et al.*, 1998; Dransfield *et al.*, 2001). Consequently, an increased expression of p27 as well as the growth retardation observed in HL-60-*bis* cells might be an accompanying phenotype of differentiation, rather than a prerequisite for differentiation. Thus, the issue of whether p27 levels and growth rate are directly affected by Bis expression should be investigated in other types of cells to clarify the role of p27 in the differentiation of these cells in relation to Bis expression. And further studies using the systems in which Bis or p27 expression is genetically down-regulated will be necessary to define their roles in differentiation more precisely.

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