

## Effect of TIEG1 on apoptosis and expression of Bcl-2/Bax and Pten in leukemic cell lines

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**ABSTRACT.** We examined the effect of transforming growth factor- $\beta$  inducible early gene-1 (TIEG1) on the apoptosis of leukemic cell lines and expression of B-cell lymphoma 2 (Bcl-2) and phosphatase and tensin homolog (Pten). Four leukemic cell lines (HL-60, U937, Raji, and K562) were treated with 0, 1, 5, 10, and 20 ng/mL TIEG1, respectively. The cell growth inhibitory ratio was assessed using the MTT assay. An inhibitory curve was drawn, and half-maximal inhibitory concentration was calculated. Additionally, 1640 culture medium containing 10 ng/mL TIEG1 was used to culture leukemic cell lines for 0, 6, 12, 24, and 48 h. The apoptosis of each cell line at different time points was detected by flow cytometry. Total RNA was extracted before reverse transcription-polymerase chain reaction. The products of this reaction were analyzed by electrophoresis, and the expression of Bcl-2/Bcl-2-associated X protein (Bax) and Pten were detected. After treatment with TIEG1, proliferation of the 4 leukemic cell lines was inhibited both time- and dose-dependently. During apoptosis induction, the expression of Bcl-2 was decreased and the expressions of Bax and Pten were increased in the 4 leukemic cell

lines induced by TIEG1 ( $P < 0.05$ ). *TIEG1* can inhibit the proliferation of leukemic cells and induce their apoptosis in a time- and dose-dependent manner. A close relationship exists between Bcl-2/Bax and Pten expression and cell apoptosis induced by TIEG1.

**Key words:** Apoptosis; Bcl-2/Bax; Leukemic cells; Pten; TIEG1

## INTRODUCTION

Transforming growth factor- $\beta$  inducible early gene-1 (*TIEG1*), also known as Kruppel-like factor 10, is a TGF- $\beta$ 1-inducible early gene and was first detected in fetal osteoblasts using a polymerase chain reaction (PCR) assay. The *TIEG1* gene encodes a 72-kD protein of 480 amino acids and is located on chromosome band 8q22.2.TIEG1. The gene product is a transcription factor that regulates the expression of genes related to cell growth and differentiation (Reinholz et al., 2004). Recent studies have indicated that the over-expression of *TIEG1* is correlated with tumor development. The expression of *TIEG1* was increased in malignant tumor tissues, including breast, pancreatic, and liver cancers, among others (Jiang et al., 2011; Jiang et al., 2012; Jin et al., 2012; Song et al., 2012). Various anticancer drugs that upregulate the expression of *TIEG1* to induce cell apoptosis have been used to treat leukemia (Jin et al., 2004; Dosen-Dahl et al., 2008). These results of previous studies indicate that *TIEG1* may inhibit cell proliferation and induce cell apoptosis when it is over-expressed; however, this hypothesis requires further study.

The B-cell lymphoma 2 (Bcl-2) family is widely distributed in many organisms and can be either pro-apoptotic [Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter, Bcl-2 homologous antagonist killer (Bak), and Bcl-2-related ovarian killer, among others] or anti-apoptotic (Bcl-2, Bcl-xL, and Bcl-w, among others), and play an important role in mediating cell apoptosis. During tumor cell apoptosis, a correlation has been demonstrated between TIEG1 and Bcl-2/Bax, but the exact mechanism remains unknown (Duenker et al., 2005). Phosphatase and tensin homolog (Pten) acts as a tumor suppressor gene; its phosphatase protein product has been shown to have dual antitumor function (Dahia et al., 1999). Moreover, low expression of Pten may inhibit cell growth. Mutations or deletions of the Pten gene occur in most tumors, including breast carcinoma, prostatic carcinoma, endometrioid carcinoma, and glioblastoma, but low expression or deletion of Pten has been observed in leukemia (Dahia et al., 1999; Yilmaz et al., 2006).

In this study, we investigated the effect of *TIEG1* on the HL-60, U937, K562, and Raji4 cell lines and determined the expression of Bcl-2 and Pten.

## MATERIAL AND METHODS

### Cell culture

The HL-60, U973, and Raji cell lines (Boster Co., Wuhan, China) were cultured in a medium containing 10% fetal calf medium. K562 cell lines were cultured in 10% fetal serum medium. All cell lines were cultured in a humidified incubator at 37°C under 5% CO<sub>2</sub> and passaged every 3 days. All cell lines were grown to log phase. The cell concentration was 5.0 x 10<sup>4</sup> cells/mL.

### MTT assay

Four different leukemia cell lines were seeded into 96-well plates ( $1 \times 10^4$  cells/well) and 0, 1, 5, 10, or 20 ng/mL TIEG1 (Abcam Company, Hong Kong) was added to 1640 medium. The cells were grown at 37°C for 0, 6, 12, 24, and 48h. The 5 mg/mL 20  $\mu$ L MTT was added to the supernatant followed by incubation of the sample at 37°C for 4 h in an incubator. After centrifugation (100 g) for 10 min, the supernatant was discarded, and 200  $\mu$ L dimethyl sulfoxide was added. The OD value at a wavelength of 490 nm was measured using a microplate reader. The inhibitory ratio was calculated as follows: inhibitory ratio = (A control group - A test group)/A control group  $\times$  100%, the half maximal inhibitory concentration ( $IC_{50}$ ) was calculated using this formula.

### Flow cytometry

All cell lines were cultured in 1640 medium with 10 ng/mL TIEG1. Cells were collected at 0, 6, 12, 24, and 48 h. All tubes were centrifuged for 5 min (12000 g), washed 2 times with phosphate-buffered saline, and then centrifuged for 5 min in tubes containing 500  $\mu$ L binding buffer. The cells were then treated with 5  $\mu$ L Annexin-V-fluorescein isothiocyanate and stained with 5  $\mu$ L propidium iodide, followed by incubation at 37°C for 10 min in the dark. Samples were analyzed using a flow cytometer according to manufacturer instructions. All time point experiments were repeated 3 times.

### RT-PCR

All cell lines were cultured in 1640 medium containing 10 ng/mL TIEG1, and cells were collected at 0, 6, 12, 24, and 48 h. After total RNA extraction, RNA samples were subjected to reverse transcription-PCR (RT-PCR) and amplified products were analyzed by gel electrophoresis.

Primers sequences were as follows: Bcl-2: 5'-CAAGTGTTCCGCGTGATTG AA-3' (sense) and 5'-CCCGGTTATCGTACCCTGTTC-3' (antisense); Bax: 5'-CTCAG GATGCGTC ACCAAGAA-3' (sense) and 5'-AACCCGGCCCCAGTTGAAGT T-3' (antisense); Pten: 5'-TACAATTTCTGGGCACCGCATAT-3' (sense) and 5'-AACCCCT CATTGACACCTTCACA T-3' (antisense). Primers were synthesized by Shanghai Bio-Engineering Company (Shanghai, China). PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles at 95°C for 20 s, and 60°C for 20 s, with extension at 72°C for 5 min. Amplified products were visualized using a UVP BioSpectrum device (Upland, CA, USA) following 1.5% agarose gel electrophoresis. All experiments were repeated 3 times.

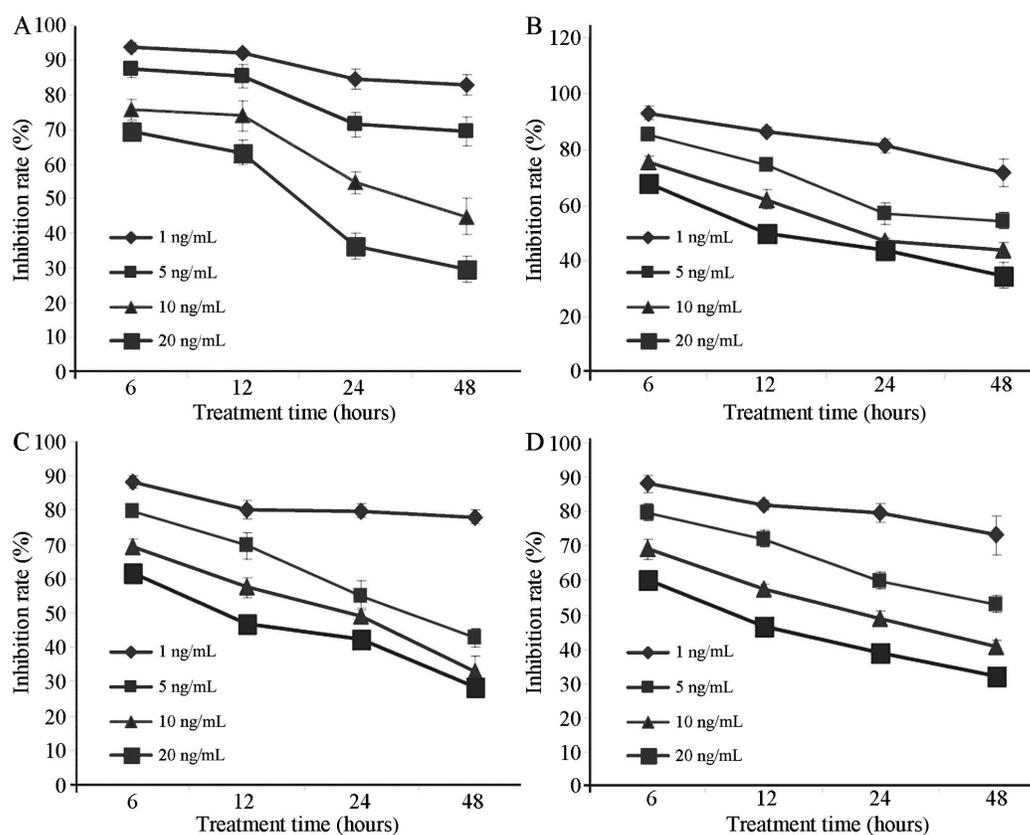
### Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and all results are reported as means  $\pm$  standard deviation (SD). One-way analysis of variance was used to compare groups. A P-value  $< 0.05$  was considered to be statistically significant.

## RESULTS

### Cell proliferation

TIEG1 inhibited the proliferation of the 4 leukemia cell lines proliferation in a dose- and time-dependent manner ( $P < 0.05$ ). All leukemia cell lines curves are shown in Figure 1 as determined using the MTT method. The  $IC_{50}$  (ng/mL) values of leukemia cells at different time points are presented in Table 1.



**Figure 1.** Inhibitory effect of 4 leukemia cell lines treated with TIEG1. **A.** Inhibitory effect of HL-60 cells treated with TIEG1. **B.** Inhibitory effect of U937 cells treated with TIEG1. **C.** Inhibitory effect of Raji cells treated with TIEG1. **D.** Inhibitory effect of K562 cells treated with TIEG1.

**Table 1.**  $IC_{50}$  (ng/mL) of leukemia cells at different time points.

Time (h)	HL-60	U937	Raji	K562
6	71.32	69.33	54.32	48.19
12	54.77	22.27	18.23	18.72
24	12.03	10.02	9.76	9.50
48	8.45	6.35	4.53	3.85

## Cell apoptosis

Based on the results of the MTT assay, the 4 leukemia cell lines treated with TIEG1 (10 ng/mL) showed different rates of cell apoptosis at 6, 12, 24, and 48 h. The apoptosis rates of the different groups were compared (Table 2), and all groups showed a significant difference ( $P < 0.05$ ), except for one group.

**Table 2.** Apoptosis rate of leukemia cells at different time points (%), means  $\pm$  SD, N = 3).

Time (h)	HL-60	U937	Raji	K562
0	4.70 $\pm$ 0.87*	2.03 $\pm$ 0.28*	2.21 $\pm$ 0.35*	2.13 $\pm$ 0.42
6	5.34 $\pm$ 0.71*	2.54 $\pm$ 0.52*	2.38 $\pm$ 0.73*	7.79 $\pm$ 0.71
12	8.32 $\pm$ 0.68	10.88 $\pm$ 0.46	8.92 $\pm$ 1.42	11.17 $\pm$ 1.37
24	15.16 $\pm$ 1.64	27.11 $\pm$ 0.79	26.12 $\pm$ 1.57	24.66 $\pm$ 0.29
48	19.92 $\pm$ 0.95	62.32 $\pm$ 1.35	52.59 $\pm$ 0.89	48.60 $\pm$ 1.38

All groups showed a significant difference ( $P < 0.05$ ), except for one group (marked with an asterisk).

## mRNA expression of Bcl-2, Bax, and Pten genes

Four leukemia cell lines were treated with TIEG1 (10 ng/mL) and total cell RNA was extracted at 6, 12, 24, and 48 h. Total RNA concentration was determined using an ultraviolet spectrophotometer. The relative mRNA expression of the Bcl-2, Bax, and Pten gene was measured using gray-scale analysis. The results showed that the mRNA expression of Bcl-2 was decreased, but the mRNA expression of Bax and Pten was increased (Table 3).

**Table 3.** mRNA expression level of Bcl-2, Bax, and Pten detected by RT-PCR.

Gene	Time (h)	HL-60	U937	Raji	K562
Bcl-2	0	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
	6	0.83 $\pm$ 0.11	0.81 $\pm$ 0.32	0.81 $\pm$ 0.32	0.76 $\pm$ 0.34
	12	0.78 $\pm$ 0.23	0.65 $\pm$ 0.18	0.48 $\pm$ 0.18	0.56 $\pm$ 0.29
	24	0.72 $\pm$ 0.28	0.43 $\pm$ 0.17	0.31 $\pm$ 0.16	0.38 $\pm$ 0.21
	48	0.63 $\pm$ 0.12	0.19 $\pm$ 0.09	0.13 $\pm$ 0.03	0.21 $\pm$ 0.11
Bax	0	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
	6	1.17 $\pm$ 0.18	1.21 $\pm$ 0.39	1.16 $\pm$ 0.18	1.06 $\pm$ 0.37
	12	1.85 $\pm$ 0.21	1.48 $\pm$ 0.18	1.38 $\pm$ 0.41	1.21 $\pm$ 0.51
	24	2.52 $\pm$ 0.16	1.79 $\pm$ 0.41	1.59 $\pm$ 0.24	1.42 $\pm$ 0.19
	48	2.71 $\pm$ 0.33	2.11 $\pm$ 0.36	1.86 $\pm$ 0.19	1.76 $\pm$ 0.38
Pten	0	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
	6	1.25 $\pm$ 0.31	1.18 $\pm$ 0.19	1.30 $\pm$ 0.44	1.18 $\pm$ 0.42
	12	1.58 $\pm$ 0.36	1.34 $\pm$ 0.43	1.68 $\pm$ 0.17	1.41 $\pm$ 0.32
	24	1.88 $\pm$ 0.27	1.59 $\pm$ 0.23	2.07 $\pm$ 0.33	1.68 $\pm$ 0.19
	48	2.13 $\pm$ 0.42	1.95 $\pm$ 0.33	2.55 $\pm$ 0.21	1.96 $\pm$ 0.27

$P < 0.05$ , compared between groups of one type of leukemia cells.

## DISCUSSION

Many biological functions of *TIEG1* remain unclear. *TIEG1* inhibits cell proliferation and induces apoptosis. Over-expression of exogenous *TIEG1* rather than endogenous *TIEG1* induced by TGF- $\beta$  was shown to increase the activity of alkaline phosphatase and decrease mRNA and protein levels of osteocalcin, as well as suppress cell growth in the osteosarcoma cell model transfected with *TIEG1* (Hefferan et al., 2000). Previous studies detected higher *TIEG1* protein expression in normal mammary epithelial cells than in breast tumor cells;

moreover, TIEG1 protein was not expressed in infiltrative tumor cells, indicating that TIEG1 plays an inhibitory role in regulating tumor growth (Subramaniam et al., 1998).

There have been some studies to examine the effect of TIEG1 on hematological tumor cells. The expression of *TIEG1* was clearly increased in leukemia cell apoptosis, implying that TIEG1 inhibits leukemia cell proliferation and induces cell apoptosis. Our previous study demonstrated that the expression of *TIEG1* was gradually enhanced during HL-60 apoptosis induced by TGF- $\beta$ 1. In this study, we found that *TIEG1* induced apoptosis in 4 different leukemia cell lines, including HL-60, U973, Raji, and K562, in a dose- and time-dependent manner. This suggests that *TIEG1* inhibits leukemia cell to induce cell apoptosis.

Bcl-2 is central to the regulation of apoptosis, a process vital for proper tissue development and cellular homeostasis. Members of the Bcl-2 protein family are critical regulators of apoptosis and include 3 subgroups of proteins that either promote cell survival or initiate cell death. The possible mechanisms of Bcl-2 induction of apoptosis involve the release of intracellular  $\text{Ca}^{2+}$ , oxygen free radical metabolism, and signal transduction, among others (Mathai et al., 2005; Brooks and Dong, 2007). The BCL2 family of proteins is composed of both anti- and pro-apoptotic proteins. Anti-apoptotic BCL2 proteins such as BCL2 and Bcl-xL induced myeloid leukemia cell differentiation protein 1, and Bcl-2-associated athanogene 1 in mammals share 4 BCL2 homology or 'BH' domains (BH1-BH4). Pro-apoptotic BCL2 proteins (such as bax, Bak, Bcl-2-associated death promoter, and Bcl-xs) can be separated into 2 subclasses: the Bax-like proteins (such as Bax and Bak in mammals - no Bax-like proteins have been identified in *Caenorhabditis elegans*), which contain 3 BH domains (BH1-BH3) and the BH3-only proteins (such as Puma or Noxa in mammals and EGL-1 in *C. elegans*), which contain a single BH domain (BH3). After replacing important amino acids in the BH domains (BH1-BH3) of Bcl-2, Bcl-2 lost its anti-apoptosis function; additionally, mutational Bcl-2 molecules formed homodimers with themselves, but did not form heterodimers with Bax. This indicated that Bcl-2 inhibited apoptosis to protect against cell survival by forming heterodimers with Bax to prevent its release (Cory and Adams, 2002). It was confirmed that Bcl-2 and Bax are associated with apoptosis in many tumor cells and are apoptosis-related factors in tumor cells. Various studies have also provided evidence that a correlation exists between TIEG1 and Bcl-2/Bax, but the exact mechanism remains unclear (Duenker et al., 2005).

Pten, the first tumor suppressor gene, was discovered in the phosphatase family and was shown to block signaling pathways by activating protein phosphatase and lipid phosphatase to inhibit tumor cell growth, invasion, and metastasis (Myers et al., 1997; Tamura et al., 1998). Conditionally deleting the Pten tumor suppressor gene was found to cause myeloproliferative disease within days and transplantable leukemia within weeks, while *Pten* deletion promoted hematopoietic stem cell proliferation. Thus, *Pten* plays a critical role in distinguishing between leukemia stem cells and hematopoietic stem cells (Yilmaz et al., 2006). In a hematopoietic system, malignant tumors (e.g., leukemia, lymphoma, and myeloma), low expression, or deletion of the *Pten* gene or protein was related to poor patient prognosis (Wu et al., 2012). Furthermore, the *Pten* gene was inactivated by transcriptional silencing, post-transcriptional modification, and methylation (Xu et al., 2003; Yang et al., 2007).

Various studies have confirmed that Bcl-2/Bax and Pten are associated with apoptosis of many tumor cells and are apoptosis-related factors in tumor cells. In this study, we observed decreased Bcl-2 expression and increased Bax and Pten expression induced by TIEG1 in 4 different leukemia cell lines. These results demonstrate that Bcl-2/Bax is related to leukemia cell apoptosis induced by TIEG1, but the details regarding this process require further investigation.

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