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Pectenotoxin-2 represses telomerase activity in human leukemia cells through suppression of hTERT gene expression and Akt-dependent hTERT phosphorylation

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Abstract In this study, we found that pectenotoxin-2 (PTX-2) decreased cell viability and inhibited telomerase activity with downregulation of hTERT expression in human leukemia cells. PTX-2 treatment also reduced c-Myc and Sp1 gene expression and DNA binding activity. Further chromatin immunoprecipitation assay demonstrated that PTX-2 attenuated the binding of c-Myc and Sp1 to the regulatory regions of hTERT. We also observed that PTX-2 treatment attenuated the phosphorylation of Akt, thereby reducing the phosphorylation and nuclear translocation of hTERT. We concluded that PTX-2 suppressed telomerase activity through the transcriptional and post-translational suppression of hTERT and this process precedes cellular differentiation of human leukemia cells.

Structured summary:
MINT-6742762:
hTERT (uniprotkb:O14746) *physically interacts* (MI:0218) with *AKT* (uniprotkb:P31749) by *anti bait coimmunoprecipitation* (MI:0006)

Keywords: Pectenotoxin-2; hTERT; c-Myc; Sp1; Akt

1. Introduction

Telomeres are responsible for maintaining chromosomal stability by preventing attrition as well as end-to-end fusions and chromosomal rearrangements [1–3]. Actually, most normal human somatic cells progressively lose their telomeres with each cell division [4,5]; however, telomerase activity is reactivated through an unknown mechanism in over 85% of human cancer cells and spontaneously immortalized cells, allowing these cells to survive with sustained telomeres [6,7]. Therefore, this enzyme offers an attractive target for chemoprevention and other anticancer strategies.

The telomerase complex consists of two essential components: hTERT [8,9] and hTR [10]. Although hTR is ubiquitously expressed in most cells [10], expression of hTERT is limited in germinal and cancer cells [6,7]. As a result, many studies have focused on relationships between apoptosis and telomerase activity using anticancer agents, in order to find the precise mechanism of hTERT expression, which is thought to be a more attractive target than hTR [11,12]. The hTERT promoter region includes two typical E-boxes and several GC-boxes for transcription factors c-Myc and Sp1, respectively [13]. c-Myc directly binds with E-box and induces hTERT transcription and subsequent cell proliferation [14]. The core promoter for hTERT expression also contains Sp1 binding sites. Particularly, Sp1 works in conjunction with c-Myc to activate transcription of hTERT [15]. In addition, the regulation of hTERT activity is controlled in post-translational alterations [16–18], as well as at the transcriptional level.

Pectenotoxins are a group of natural toxins that can be extracted from marine sponges, also found in shellfish and intoxicated humans [19]. The most toxic compound in this group is pectenotoxin-2 (PTX-2), which is isolated from *Dinophysis* spp. [20,21]. Previous studies have been also shown that PTX-2 may modify actin cytoskeletons due to its depolymerization through their binding sites with actin in vitro and in vivo [22,23]. In addition, this compound was reported to display selective and potent cytotoxicity against human lung, colon, and breast cancer cells [24,25].

This study was designed to investigate the underlying mechanisms involved in the induction of apoptosis by PTX-2 in human leukemia cells, with special emphasis on its role on the regulation of telomerase, an important molecular marker for carcinogenesis.

2. Materials and methods

2.1. Reagents and antibodies

PTX-2 was prepared as described previously [24]. Antibodies against c-Myc, Sp1, DNA repair enzyme poly-(ADP-ribose) polymerase (PARP), caspase-3, hTERT, and nucleolin were purchased from Santa

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Abbreviations: PTX-2, pectenotoxin-2; TRAP, telomeric repeat amplification protocol; ELISA, enzyme-linked immunosorbent assay; ChIP, Chromatin immunoprecipitation; PARP, DNA repair enzyme poly-(ADP-ribose) polymerase

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Cruz Biotechnology (Santa Cruz, CA), and antibodies against α-tubulin, phospho (p)-Akt, Akt, and Akt substrate were purchased from Cell Signaling (Beverly, MA).

2.2. Cell culture

Human leukemia U937, THP-1, HL-60 cells, human prostate cancer cell PC-3, and human colorectal cancer cell HCT116 were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in an RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin (Sigma) in 5% CO_2 at 37°C.

2.3. Flow cytometric analysis

In order to analyze the percentage of apoptotic cells with annexin V staining, cells were washed in phosphate-buffered saline and then incubated with annexin V-fluorescein isothiocyanate (R&D Systems; Minneapolis, MN). Normal murine splenocytes were pooled, washed with PBS, fixed in 70% ethanol for 20 min at −20 °C, and incubated with 40 μg/ml propidium iodide (PI; Sigma) and 100 μg/ml RNase A for 30 min at 37 °C in the dark. In a parallel experiment, GFP-conjugated CD11b antibody (Pharmingen; Sin Diego, CA) was used for the detection of cell differentiation. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickenson; San Jose, CA).

2.4. In vitro caspase-3 activity assay

The activity of caspase-like protease was measured using a caspase activation kit (R&D System) according to the manufacturer's protocol.

2.5. RNA extraction and RT-PCR

Total RNA was isolated using the Trizol reagent (GIBCO-BRL; Gaithersburg, MD) according to the manufacturer's recommendations. Genes of interest were amplified from cDNA which was reverse transcribed from 1 μg of total RNA using the One-Step RT-PCR Premix (iNtRON Biotechnology; Sungnam, Republic of Korea). Primers and conditions for the amplification of hTERT, GAPDH, c-Myc, and Sp1 have been described previously [26].

2.6. Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology; Sungnam, Republic of Korea). The preparation of cytoplasmic and nuclear extracts was conducted using the NE-PER nuclear and cytosolic extraction reagents (Pierce; Rockford, IL). Total cell extracts were separated on 10% polyacrylamide gels, and then transferred to nitrocellulose membranes using standard procedures. The membranes were developed using an ECL reagent (Amersham; Arlington Heights, IL).

2.7. Immunoprecipitation

The total cell extracts were immunoprecipitated with anti-hTERT overnight at 4 °C. Immune complexes were collected using protein A/G-Sepharose beads (Santa Cruz Biotechnology), washed, and eluted in sample buffer. Samples were run on 8% SDS–PAGE gels, transferred to nitrocellulose membranes, and probed with the Akt-substrate antibody or hTERT antibody. Blots were developed using the ECL reagent.

2.8. Telomerase activity assay

Telomerase activity was measured using a TRAP-ELISA kit (Boehringer Mannheim; Mannheim, Germany) according to the manufacturer's instructions.

2.9. Electrophoretic mobility shift assay (EMSA)

DNA–protein binding assays were carried out with nuclear extract. Synthetic complementary c-Myc (5′-GGA AGC AGA CCA **CGT** GGT CTG CTT CC-3′) and Sp1 (5′-ATT CGA TC**G GGG CGG GGC** GAG C-3′) binding oligonucleotides (Santa Cruz) were 3′-biotinylated using the biotin 3′-end DNA labeling kit (Pierce).

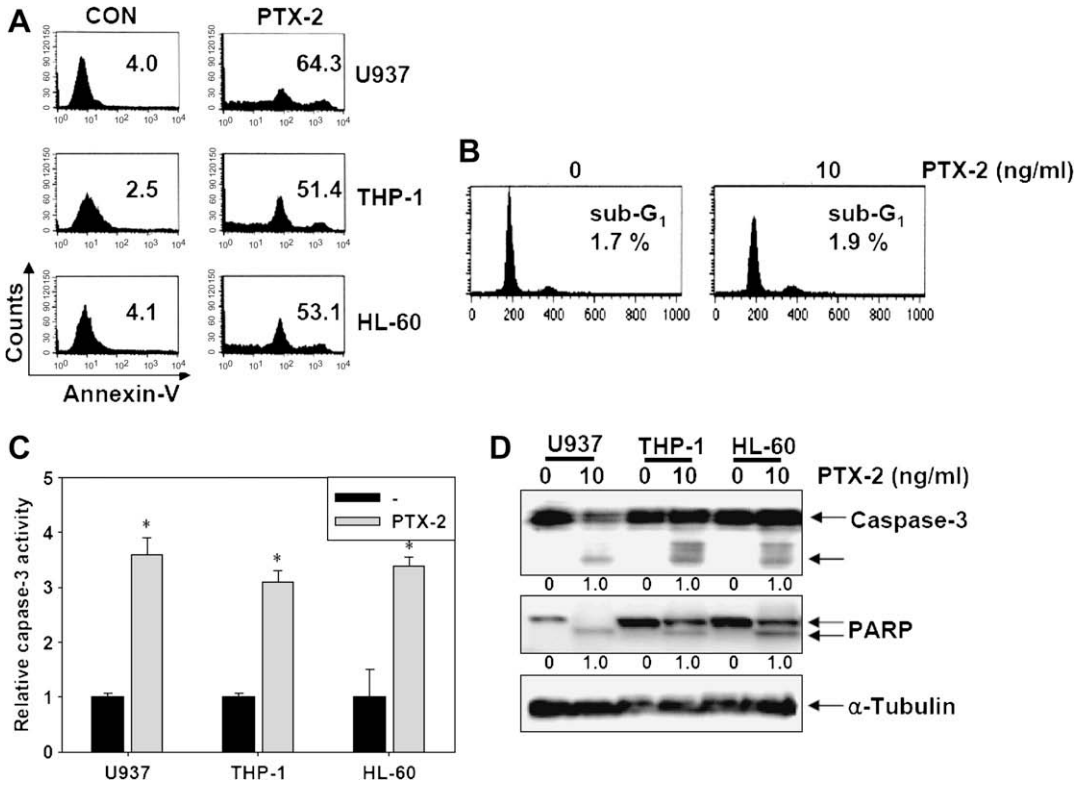

Fig. 1. PTX-2-induced apoptosis in human leukemia cells. Cells were seeded at 4×10^4 cells/ml and treated with the indicated concentrations of PTX-2 for 72 h. (A) Annexin V^+ cells are represented on the *x*-axis, while the number of cells counted is represented on the *y*-axis. (B) DNA content was analyzed in normal murine splenocytes by flow cytometry. Arrows indicate the population of cells in the sub-G_1 phase. (C) Caspase-3 activity was determined by the manufacturer's protocol. (D) Western blot was performed by standard procedure. Data are expressed as overall means ± S.D. from three independent experiments. Statistical significance was determined by Student's *t*-test ($^*P < 0.05$ vs. vehicle control).

2.10. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the EZ-Chip™ assay kit according to the manufacturer's protocol (Upstate Biotechnology; Lake Placid, NY). The primers used for the amplification of hTERT promoter region were Sp1 (forward): 5′-GGT ACC GAC CCC CGG GTC CGC CCG GA-3′ and Sp1 (reverse): 5′-AAG CTT GCT GCC TGA AAC TCG CGC CG-3′ (284 bp DNA product); and c-Myc (forward): 5′-AGT GGA TTC GCG GGC ACA GA-3′and c-Myc (forward): 5′-TTC CCA CGT GCG CAG CAG GA-3′ (reverse) (257 bp DNA product) [27,28].

2.11. Statistical analysis

Statistical analyses were conducted using SigmaPlot software (version 6.0), and Scion Imaging software (http://www.scioncorp.com) was used to quantify the results normalized to the control gene. Values were presented as means ± S.D. Significant differences between the groups were determined using the unpaired Student's *t*-test. Statistical significance was regarded at $P < 0.05$.

3. Results

3.1. PTX-2 inhibits cell proliferation of leukemia cells

To determine the effect of PTX-2 on cell growth, human myeloid leukemia cells were treated with increasing concentrations for 72 h, and apoptosis was analyzed using FACS flow cytometer. As shown in Fig. 1A, a 72 h exposure time of each cell line to 10 ng/ml PTX-2 resulted in an increase of more than 50%, indicating an apoptotic population. However, normal murine splenocytes cells had no effect on cytotoxicity and cell cycle distribution (Fig. 1B). In this process, PTX-2 exposure also increased caspase-3 activity, which was observed to increase over 3-fold as compared with control cells (Fig. 1C). Western blot analysis revealed that 10 ng/ml of PTX-2 treatment for 72 h resulted in the cleaved form of caspase-3 (Fig. 1D). The cleavage of PARP was consistently revealed by the appearance of the 89 kDa cleaved intermediate in PTX-2 treatment. Collectively, these data indicate that PTX-2 inhibits cell viability and leads to caspase-3 activation in leukemia cells.

3.2. PTX2 represses telomerase activity in leukemia cells

As shown in Fig. 2A, telomerase activity was significantly reduced in cells after 72 h of exposure with PTX-2 in a dose-dependent manner. To visualize the effect observed by TRAP-ELISA assay, we also investigated ladder formation by telomerase activity. As expected, the addition of PTX-2 (10 ng/ml) decreased ladder formation by telomerase (Fig. 2B). We next performed RT-PCR and Western blot analysis to examine changes in hTERT expression following treatment with PTX-2. As indicated in Fig. 2C, hTERT mRNA decreased in leukemia cells following PTX-2 treatment. Concordantly, the level of hTERT protein was also shown to undergo a decrease in the whole cell extraction fraction (Fig. 2D). The PTX-2-induced downregulation in telomerase

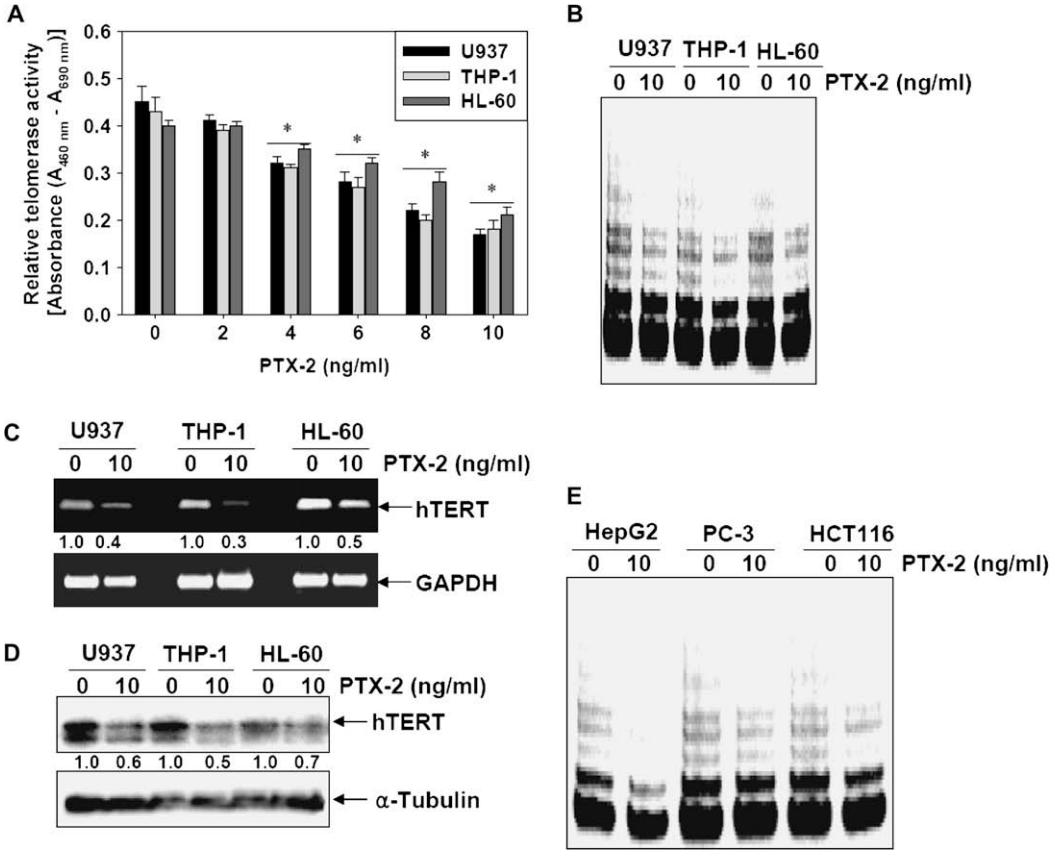

Fig. 2. Downregulation of telomerase activity by PTX-2. Leukemia cells were incubated with the indicated concentration of PTX-2 for 72 h. Telomerase activity was measured using a TRAP-ELISA kit (A) and TRAP assay (B). (C) Total RNA was isolated using a Trizol reagent and RT-PCR was performed. (D) Western blot was performed by standard procedure. (E) Telomerase activity using TARP assay was assessed in HepG2, PC-3, and HCT116. Data are expressed as overall means ± S.D. from three independent experiments. Statistical significance was determined by Student's *t*-test (*$P < 0.05$ vs. vehicle control).

activity was not cell line specific because a similar effect was observed in another human cancer cell lines (Fig. 2E). These results suggest that PTX-2 treatment induces the downregulation of telomerase activity through suppression of hTERT gene expression at the transcriptional levels.

3.3. PTX-2 downregulates c-Myc- and Sp1-dependent hTERT gene expression in leukemia cells

We next examined whether PTX-2 treatment alters the expression and DNA binding activity of c-Myc and Sp1 in leukemia cells. As shown in Fig. 3A and B, the levels of c-Myc mRNA and protein were markedly reduced in PTX-2-treated cells. Sp1 was also downregulated in mRNA levels, but its proteins were degraded at the same conditions. Additionally, PTX-2 treatment resulted in a significant decrease of c-Myc and Sp1 DNA binding activity in leukemia cells (Fig. 3C). To further investigate the exact mechanism of PTX-2 on the regulation of hTERT expression, ChIP assay was performed to examine the binding of c-Myc and Sp1 on the hTERT promoter regions. As shown in Fig. 3D, our results showed that PTX-2 attenuated c-Myc (left panel) and Sp1 (right panel) binding to the promoter regions of hTERT. PTX-2 was shown to significantly suppress the nuclear expression of c-Myc and Sp1 in other two cancer cell lines tested, indicating that the effect of PTX-2 was not cell type specific (Fig. 3E). These results indicate that PTX-2 attenuates hTERT gene expression through suppression of c-Myc- and Sp1-binding on the regulatory regions of hTERT.

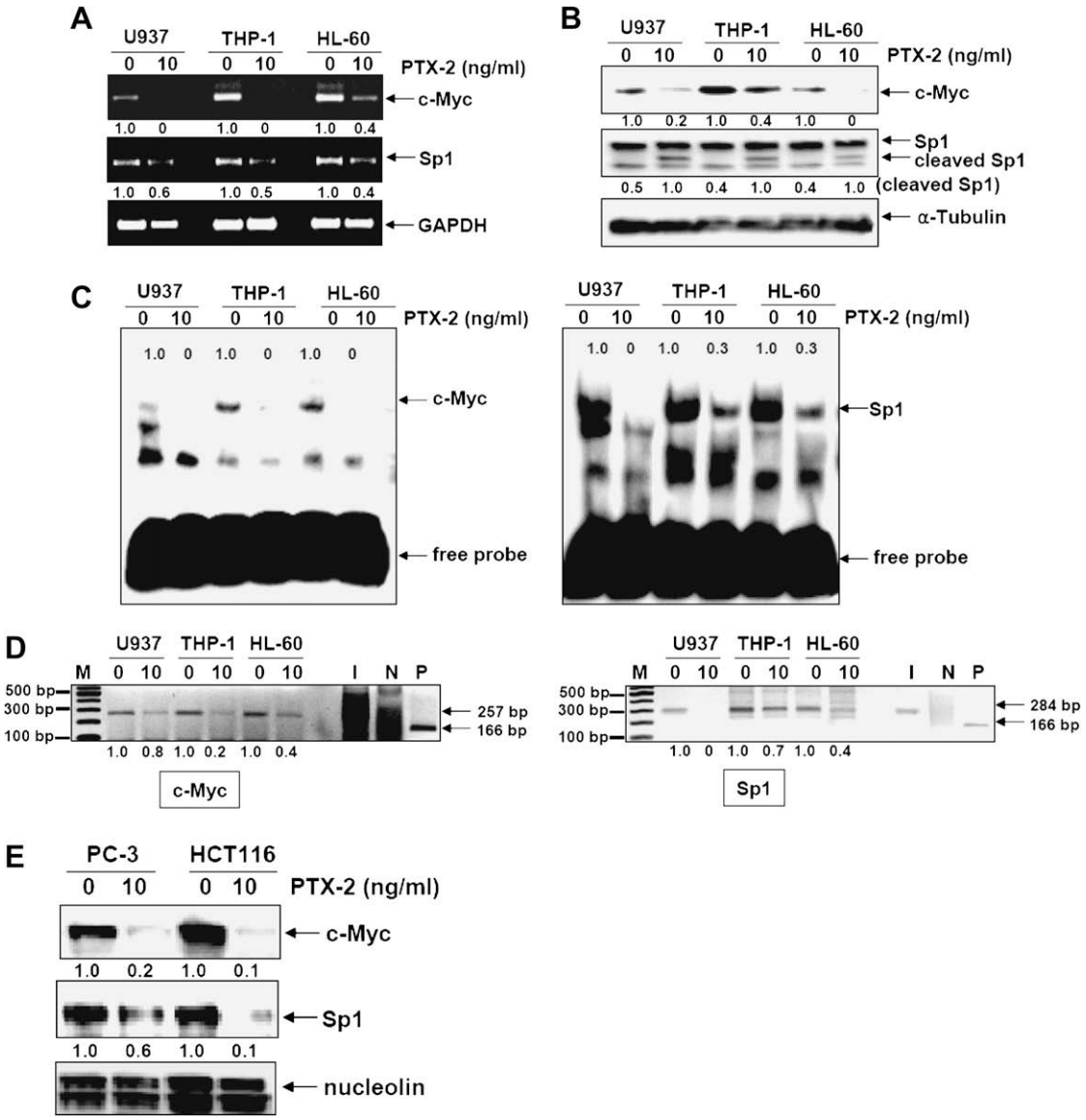

Fig. 3. Downregulation of c-Myc and Sp1 by PTX-2. Cells were treated with 10 ng/ml of PTX-2 for 72 h. (A) Total RNA was isolated using a Trizol reagent and RT-PCR was performed. (B) Western blot was performed by standard procedure. (C) c-Myc and Sp1 DNA binding activity was analyzed by a LightShiftTM chemiluminescent EMSA kit as described in Section 2. (D) ChIP assay was performed using antibodies against c-Myc (left panel) and Sp1 (right panel). Total sonicated chromatin was used to use as input. Positive and negative controls were performed using antibody against histone-H3 and rabbit IgG, respectively. I, input; N, negative control; and P: positive control. (E) Nuclear proteins were subjected to Western blotting using anti-c-Myc and anti-Sp1 antibodies in PC-3 and HCT116.

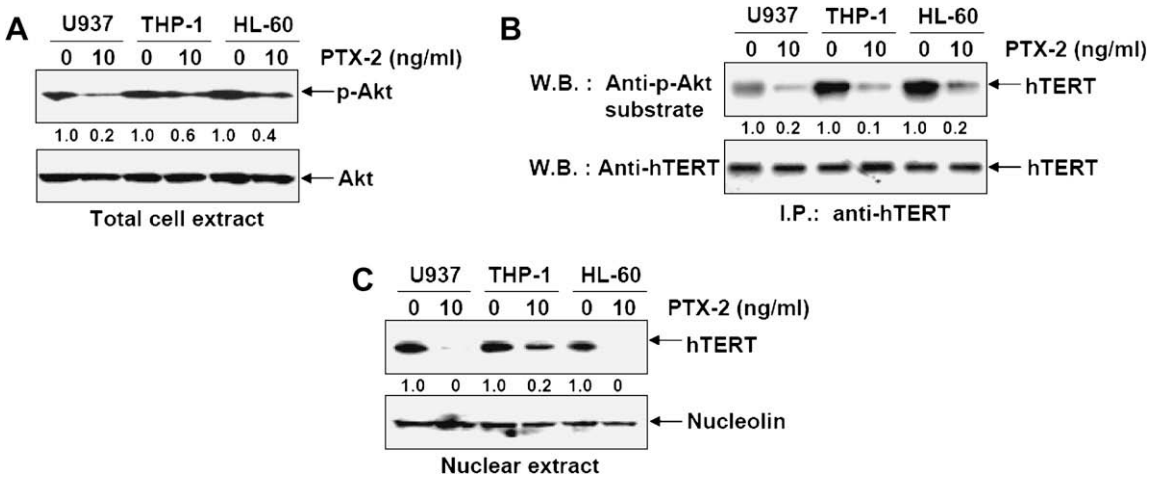

Fig. 4. Suppression of Akt-dependent hTERT phosphorylation by PTX-2. Cells were incubated with the indicated concentrations of PTX-2 for 72 h. (A) Western blot was performed by standard procedure. (B) The lysates were subjected to immunoprecipitation with the anti-hTERT antibody and then blotted with anti-p-Akt substrate or anti-hTERT antibody. (C) Nuclear proteins were subjected to Western blotting using anti-hTERT antibodies, and then normalized to nucleolin.

3.4. PTX-2 attenuates nuclear translocation of hTERT through dephosphorylation of Akt

Because Akt is reported to enhance human telomerase activity through the phosphorylation and nuclear translocation of hTERT [17], we investigated whether PTX-2 treatment inactivates the Akt pathway, resulting in hTERT dephosphorylation and nuclear translocation. As shown in Fig. 4A, total leukemia cell Akt levels did not change following treatment with 10 ng/ml PTX-2 for 72 h, while p-Akt levels significantly decreased. Since the activation of Akt is known to directly phosphorylate hTERT, which could subsequently translocate into the nucleus, we also investigated whether phosphorylation and nuclear translocation of hTERT was inhibited by PTX-2 treatment. The cells were treated with PTX-2 and then used to prepare lysates that were immunoprecipitated with the anti-hTERT antibody. The cell lysates were subjected to Western blotting with the anti-p-Akt substrate antibody or anti-hTERT antibody. As can be seen in Fig. 4B, PTX-2 decreased hTERT phosphorylation at a putative Akt phosphorylation site. As already highlighted, because phosphorylation of hTERT is necessary for its nuclear translocation, we determined that PTX-2 also blocked the nuclear translocation of hTERT (Fig. 4C). These results suggest that PTX-2 decreases phosphorylation of hTERT and thereby possibly inhibits its translocation to the nucleus through the dephosphorylation of Akt.

3.5. PTX-2 induces cytodifferentiation of leukemia cells

Expression of the CD11b antigen was analyzed at 72 h during the suppression of telomerase activity by PTX-2 in leukemia cells. Cells were stained with FITC-conjugated mouse monoclonal antibody against human CD11b after 72 h of PTX-2 treatment. As shown in Fig. 5, untreated cells were grown in parallel and included as controls (black-shade line). Following PTX-2 treatment, expression of CD11b significantly increased in three leukemia cells (white-shade line). These results indicate that telomerase transcription and activity may be reduced in all these cell lines upon induction of differentiation.

4. Discussion

Telomerase is composed of a catalytic subunit, hTERT, hTR, and TEP-1 [3–5]. In particular, hTERT has received considerable attention for its role in regulating telomerase activity [11,12]. The hTERT core promoter contains numerous transcription factor binding sites, including two for c-Myc, five for Sp1, one for Ets, and two for Inr [29,30]. Of all these transcription factors, c-Myc binds directly to E-boxes at the promoter of hTERT and activates hTERT transcription [14]. In addition to the c-Myc recognition sequence (E-box), Sp1 is involved in the regulation of hTERT promoter activity in various human cells [13,15]. Similar to these findings, our results indicate that PTX-2 suppresses telomerase activity in human leukemia cells via the transcriptional downregulation of hTERT through a reduction of c-Myc and Sp1 activity. Nevertheless, we could not rule out the possibility that other transcriptional factors might also be involved in the suppression of

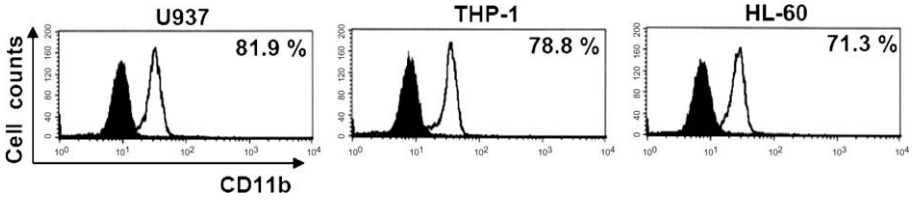

Fig. 5. Differentiation of leukemia cells by PTX-2. To investigate cellular differentiation, HL-60 cells were cultured in the presence (white-shaded line) or absence (black-shaded line) of 10 ng/ml PTX-2. After 72-h incubation, cells were harvested and analyzed by flow cytometry. The percentage within each histogram represents the incidence of $CD11b^{+}$ cells. The results are representative of three independent experiments.

hTERT gene expression by PTX-2, because Ets and NF-κB are implicated in the repression of telomerase activity [18]. It is also known that telomerase inhibition precedes differentiation of leukemia cells and may contribute to terminal differentiation [31]. We found that ablation of telomerase may be companied with the differentiation of leukemia cells. Therefore, further investigations are needed to identify inactivation mechanisms of telomerase and differentiation of leukemia cells by PTX-2.

Akt is a potent inhibitor of apoptosis through a blocking of caspase activation and the inhibition of chromatin condensation [32,33]. It has also been reported that constitutive and inducible Akt activity promotes resistance to chemotherapy in cancer cells [34]. Akt phosphorylation could also be a potent inducer for telomerase activation via hTERT phosphorylation linked to nuclear localization [17]. Nuclear translocation of hTERT from a presumably nonfunctional cytosolic location to a physiologically relevant nuclear compartment may be one important mechanism involved in the regulation of telomerase function in cells. The loss of PTEN, which can act as a counterpart of Akt, may allow malignant cells to inhibit telomerase activity through decreasing hTERT mRNA levels [35]. In this study, PTX-2 was observed to downregulate p-Akt, and phosphorylation and translocation of hTERT. These results clearly suggest that PTX-2 treatment regulates hTERT at the post-translational level by downregulating its phosphorylation by the Akt pathway. Recently, mitogen-activated protein kinases and serine/threonine kinases have also been shown to phosphorylate hTERT and regulate telomerase activity [36,37]. Therefore, further studies will be necessary to determine whether these kinases are related to the PTX-2–induced downregulation of hTERT.

In conclusion, our study demonstrated that PTX-2 suppresses cell viability and telomerase activity in human leukemia cells. Overall results suggest that PTX-2 can be used to effectively inhibit telomerase activity via the transcriptional and post-translational suppression of hTERT.

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