EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 39, No. 1, 121-127, February 2007

TNF-α upregulates PTEN *via* NF-κB signaling pathways in human leukemic cells

Young-Rae Lee[1], Hong-Nu Yu[1], Eun-Mi Noh[1], Hyun Jo Youn[1], Eun-Kyung Song[2], Myung-Kwan Han[2], Chang-Sik Park[3], Byung-Soo Kim[4], Young-Seok Park[4], Byung-Kwon Park[4], Sung-Ho Lee[4] and Jong-Suk Kim[1,5]

[1]Department of Biochemistry
[2]Department of Microbiology and Immunology
Chonbuk National University Medical School
Jeonju 561-180, Korea
[3]Division of Animal Science and Resources
Research Center for Transgenic Cloned Pigs
Chungnam National University
Daejeon 305-764, Korea
[4]Department of Companion and Laboratory Animal Science
Kongju National University
Yesan 340-702, Korea
[5]Corresponding author: Tel, 82-63-270-3085;
Fax, 82-63-274-9833; E-mail, jsukim@moak.chonbuk.ac.kr

Accepted 25 July 2006

Abbreviations: PDTC, pyrrolidine dithiocarbamate; PI phosphatidylinositol; PIP_3, phosphoinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homologue

Abstract

TNF-α plays a variety of biological functions such as apoptosis, inflammation and immunity. PTEN also has various cellular function including cell growth, proliferation, migration and differentiation. Thus, possible relationships between the two molecules are suggested. TNF-α has been known to down-regulate PTEN *via* NF-κB pathway in the human colon cell line, HT-29. However, here we show the opposite finding that TNF-α upregulates PTEN *via* activation of NF-κB in human leukemic cells. TNF-α increased PTEN expression at HL-60 cells in a time- and dose-dependent manner, but the response was abolished by disruption of NF-κB with p65 anisense phosphorothioate oligonucleotide or pyrrolidine dithiocarbamate. We found that TNF-α activated the NF-κB pathways, evidenced by the translocation of p65 to the nucleus in TNF-α-treated cells. We conclude that TNF-α induces upregulation of PTEN expression through NF-κB activation in human leukemic cells.

Keywords: leukemia; NF-κB; PTEN phosphohydrolase; tumor necrosis factor-α

Introduction

PTEN dephosphorylates the lipid second messenger, phosphoinositol 3,4,5-trisphosphate [PI(3,4,5)P_3], a product of phosphatidylinositol 3' kinase (PI3K) activity, and negatively regulates survival signaling mediated by PI3K/protein kinase B/Akt (PI3K/PKB/Akt) (Haas-Kogan *et al.*, 1998; Maehama and Dixon, 1998; Hwang *et al.*, 2005). A variety of human cancers, including brain, breast, endometrial, prostate, and kidney tumors are involved in mutations of *Pten* (Cantley and Neel, 1999; Simpson and Parsons, 2001). Mutations in this gene also are responsible for Cowden syndrome, a multiple hamartoma condition associated with high incidence of breast, brain, and thyroid neoplasia (Cantley and Neel, 1999; Simpson and Parsons, 2001). Disruption of *pten* induces a partial resistance to numerous apoptotic stimuli, including UV irradiation, osmotic shock, heat treatment, and tumor necrosis factor α stimulation (Stambolic *et al.*, 1998; Zhang *et al.*, 2005).

Although PTEN mutations are characterized in human cancers and molecular roles of PTEN are well studied in the control of cellular processes, a little is known about modes of PTEN regulation. The tumor suppressor p53 increases PTEN expression via a direct activation of PTEN promoter (Stambolic *et al.*, 2001; Sheng *et al.*, 2002). There is a negative report showing no direct relationship between p53 and PTEN (Tang and Eng, 2006). Nuclear transcription factor κ-B (NF-κB) also has been known to regulate PTEN expression even though the regulation is controversial depending on cell type and stimulants. NF-κB activated by tumor necrosis factor-α (TNF-α) induces downregulation of PTEN in colon cancer cell line H27 (Kim *et al.*, 2004). However, we previously demonstrated that NF-κB activated by dimethysulfoxide (DMSO) induces upregulation of PTEN in HL-60 cells (Lee *et al.*, 2005). In this study, we wished to examine effect of TNF-α induced activation of NF-κB on the PTEN expres-

sion. We found that TNF-α also induced expression of PTEN through NF-κB activation in HL-60 cells.

Materials and Methods

Materials

Anti-PTEN monoclonal antibody and anti-p65 monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and phospho-AKT (p-AKT) polyclonal antibody from Cell Signaling Technology (Minneapolis, MN). TNF-α was obtained from R&D Company (Minneapolis, MN). RPMI 1640 and FBS were obtained from Gibco-BRL (Gaithersburg, MD). Hanks' balanced salt solution (HBSS), pyrrolidine dithiocarbamate (PDTC), and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). The antisense and nonsense phosphorothioate oligonucleotides consisted of analogues to the 5' end of the p65 subunit of NF-κB, including the ATG initiation codon. The sequences of phosphorothioate oligonucleotides were designed and manufactured by GenoTech (Daejeon, Korea); p65 antisense, 5'-GGGGAACAGTTCCTCCATGGC-3'; p65 nonsense as a scrambled control, 5'-GTACTACTCTGAGCA-AGGA-3'.

Cell culture

HL-60 cells were obtained form American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 containing 10% FBS, 2 mM glutamine, 60 μg/l penicillin G, 100 μg/l streptomycin, and 50 μl/l amphotericin B at a humid atmosphere (5% CO_2, 95% air).

Preparation of nuclear extract for determination of p65 nuclear translocation

Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.9), and pelleted at 1,500 rpm for 3 min. The cell pellet was suspended in ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM KCl, 0.2 mM PMSF, 0.5 mM DTT), vortexed for 10 s and then centrifuged at 3,000 rpm for 5 min. The packed cells were resuspended with ice-cold hypotonic lysis buffer in presence of 50 μl of 10% Nonidet P-40 and then incubated on ice for 25 min. The nuclear fraction was precipitated by centrifugation at 4,000 rpm for 15 min at 4°C. The nuclei pellet was resuspended in 50-100 μl of low salt extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) and added to equal volume of high salt extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) in a dropwise fashion, and then incubated under continuous shaking at 4°C for 45 min. The sample was centrifuged for 20 min at 12,000 rpm at 4°C. The nuclear extract was aliquoted and stored at -80°C. Protein concentration was determined by the method of Bradford (1976).

Western blot analysis

HL-60 cells (1×10^6 cells) were seeded on 100-mm culture dishes and harvested in PBS. After washing with PBS, cell pellets were lysed with lysis buffer (20 mM Hepes pH 7.2, 1% Triton X-100, 150 mM NaCl, 0.1 mM PMSF, 1 mM EDTA, and 1 μg/ml aprotinin). After incubation for 30 min at 4°C, cellular debris was removed by centrifugation at 13,000 rpm for 10 min, and supernatants were analyzed by SDS-PAGE. Protein concentrations were determined by protein assay reagents (Bio-Rad Laboratories). Samples (50 μg) were prepared with the four volume of 0.5 M Tris buffer (pH 6.8) containing 4% SDS, 20% glycerol and 0.05% bromophenol blue for 5 min at 95°C. SDS-PAGE was performed in 10% slab gel according to Laemmli (1970). Proteins were transferred to nitrocellulose paper. The membrane was washed in blocking buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 5% fat-free milk) for 60 min at room temperature with shaking and then washed with Tris-buffered saline containing 0.01% Tween 20. Primary antibody was incubated for 4 h at 4°C. The reactive proteins were detected using enhanced chemiluminescence (ECL, Amersham Life Sciences, Arlington Heights, IL).

Reverse transcription-PCR (RT-PCR)

RT-PCR was performed by using RNA PCR Kit (GeneAmp, Applied Biosystem). Total RNA was isolated from cells by using TRIzol reagent following the manufacture's instructions. Five micrograms of total RNA were transcribed into cDNA in a 20 μl final volume of reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM each dNTP) and 2.4 M oligo-d(T)16-primer, 1 U RNase inhibitor, and 2.5 U M-MLV RNase H-reverse transcriptase by incubation for 15 min at 70°C and 50 min at 42°C. The reaction was stopped by incubation at 95°C for 10 min. PCR aliquots of the synthesized cDNA were added to a 45 μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, and 2 U *Taq* DNA polymerase, and 0.4 μM of each PCR primer: sense primer, human PTEN (5'-CCGGA-ATTCATGACAGCCATCATCAAAGA-3'), antisense primer, human PTEN (5'-CGCGGATCCTCAGACTTT-

TGTAATTTGTG-3'). Amplification for PTEN was initiated with 3 min of denaturation at 94°C followed by 30 cycles at 94°C for 1 min, 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After the last cycle of amplification, the samples were incubated for 5 min at 72°C. β-actin PCR was performed with 2.5 μl of aliquots of synthesized cDNA using primers at a concentration of 0.15 μM: human β-actin sense primer (5'-CCACGAAACTACCTTCAACTCC-3'), human β-actin antisense primer (5'-TCATACTCCTGC-TGCTTGCTGATCC-3'). The obtained PCR products were analyzed on ethidium bromide-stained agarose (2%) gels.

Statistical analysis

All experimental data are mean ± SD. Statistical analysis was performed using Student's *t*-test, and $P < 0.005$ was considered to be significant.

Results

TNF-α induces upregulation of PTEN expression in HL-60 cells

HL-60 cells, a human promyelocytic leukemic cell line, control the progression of its cell cycle through

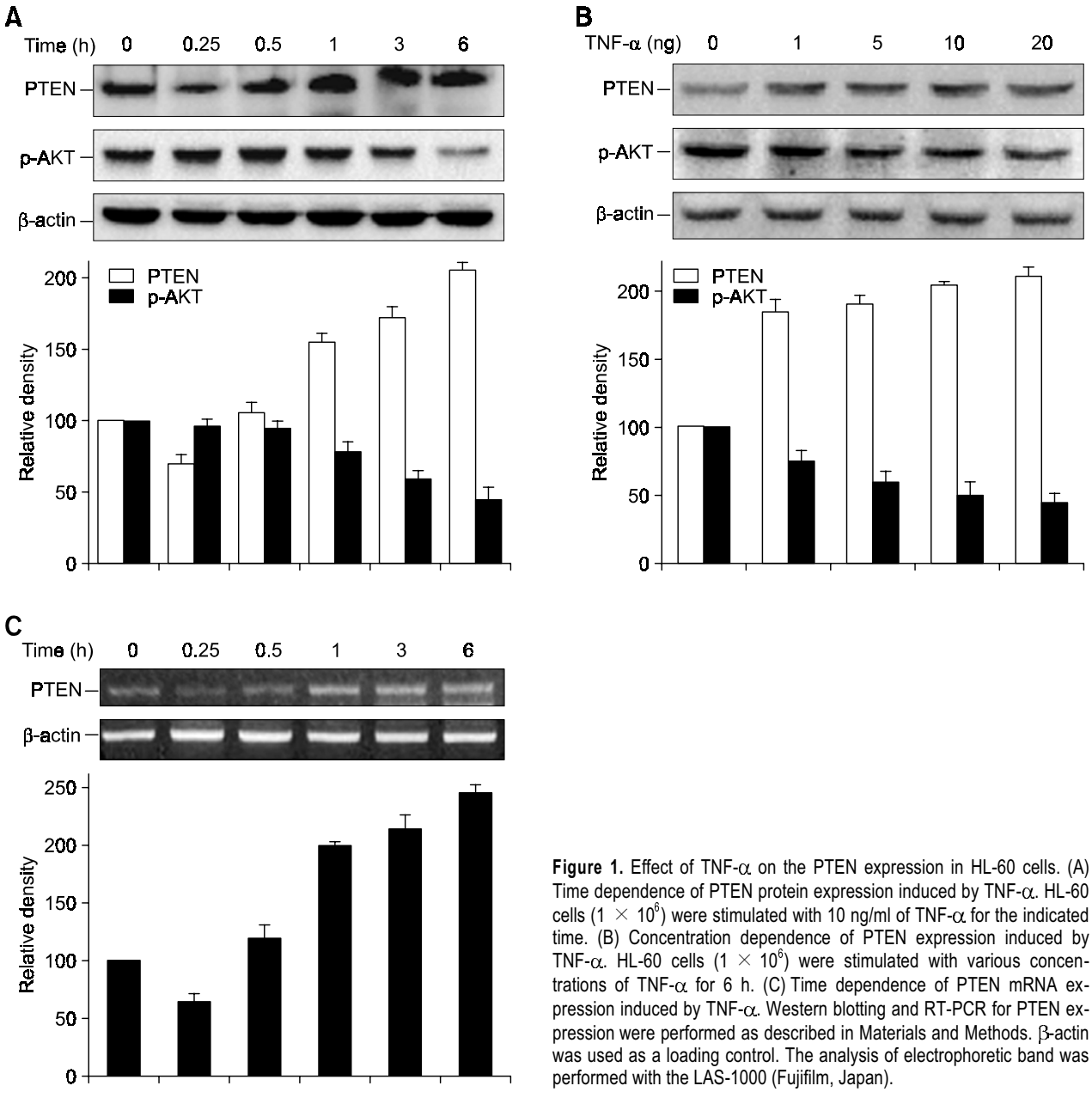

Figure 1. Effect of TNF-α on the PTEN expression in HL-60 cells. (A) Time dependence of PTEN protein expression induced by TNF-α. HL-60 cells (1×10^6) were stimulated with 10 ng/ml of TNF-α for the indicated time. (B) Concentration dependence of PTEN expression induced by TNF-α. HL-60 cells (1×10^6) were stimulated with various concentrations of TNF-α for 6 h. (C) Time dependence of PTEN mRNA expression induced by TNF-α. Western blotting and RT-PCR for PTEN expression were performed as described in Materials and Methods. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

the activation of PI3K/Akt signaling pathway (Cappellini *et al.*, 2003). Our previous result shows that PTEN expression is enhanced by DMSO through NF-κB activation in HL-60 cells. This finding suggests that TNF-α, a strong NF-κB activator, could affect PTEN expression in HL-60 cells. To address this possibility, HL-60 cells were treated with TNF-α, and then RT-PCR and western blot analysis were performed. HL-60 cells (1×10^6 cells) were treated with 10 ng/ml of TNF-α for various periods of time and western blotting for PTEN was performed. TNF-α caused an increase of PTEN protein level in a time-dependent manner (Figure 1A). PTEN level was significantly increased within 1 h after treatment with TNF-α and this response was persistent till 6 h. Viabilities of HL-60 cell treated with TNF-α were declined after 12 h treatment (data not shown).

To determine whether TNF-α stimulates expression of PTEN, we incubated HL-60 cells (1×10^6 cells) with various concentrations of TNF-α (0-20 ng/ml) for 6 h. TNF-α also induced PTEN expression dose-dependently (Figure 1B). At the same time, we also analyzed mRNA levels of PTEN. As shown at Figure 1C, PTEN mRNA level in HL-60 cells was also increased from 60 min after TNF-α treatment. The decrease of Akt phosphorylation was accompanied with the increase of PTEN expression (Figure 1A and B). This reduction in phosphorylated Akt correlated with the increase in PTEN expression, and occurred at the same time point and the same dose. These data indicate that TNF-α-induced increase of PTEN mediates inhibition of the PI3K/Akt signaling pathway. Taken together, we conclude that TNF-α upregulates PTEN expression in HL-60 cells.

TNF-α induces NF-κB activation in HL-60 cells

NF-κB is a member of the Rel family of proteins and is typically a heterodimer composed of p50 and p65 (RelA) subunits. TNF-α activates I-κB kinase (IKK) that phosphorylates IκBα, causing IκBα degradation by proteasome and allowing NF-κB to translocate to the nucleus to activate transcription (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997). To test whether TNF-α activates NF-κB in HL-60 cells, we measured the translocation of NF-κB p65 subunit from the cytosol to the nucleus, and levels of IκBα in the cytosol. The translocation of p65 was clearly enhanced from 3 h after TNF-α treatment and the enhancement persisted till 6 h (Figure 2). Cytosolic IκBα level was transiently increased within 1 h, but decreased at 6 h, indicating that degradation of cytosolic IκBα coincidentally occurred with translocation of NF-κB p65 (Figure 2). Thus, these results indicate that TNF-α activates NF-κB in HL-60 cells.

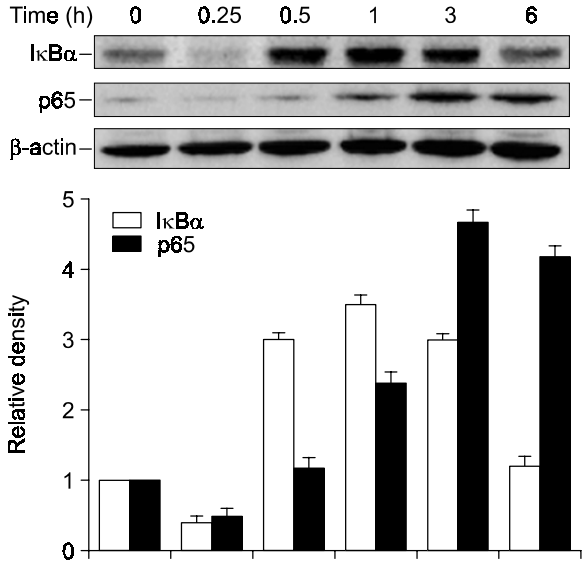

Figure 2. The activation of NF-κB by TNF-α in HL-60 cells. HL-60 cells were treated with 10 ng/ml TNF-α for the indicated time. After incubation, the nuclear extract preparation for assay of p65 nuclear translocation and the cytosolic extracts for assay of IκBα expression were prepared and analyzed by Western blot using antibody for p65, and IκBα, respectively. Western blotting was performed as described in Materials and Methods. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

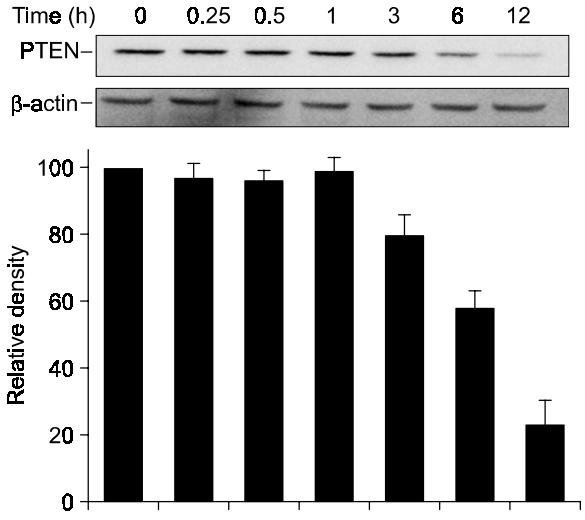

Figure 3. Effect of PDTC on TNF-α-induced PTEN expression in HL-60 cells. HL-60 cells (1×10^6) were stimulated with 10 ng/ml of TNF-α in the presence of 10 μM PDTC for the indicated time. Cell extraction and western blotting for PTEN level were performed as described in Materials and Methods. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

NF-κB activation is essential for TNF-α-induced upregulation of PTEN in HL-60 cells

To determine whether TNF-α-induced upregulation of PTEN is induced by NF-κB activation, we analysed PTEN expression induced by TNF-α after inhibition of NF-κB activation using pyrrolidine dithiocarbamate (PDTC), a chemical inhibitor of NF-κB and antisense phosphorothioate oligonucleotide for p65 subunit of NF-κB. HL-60 cells (1×10^6 cells) were treated with 10 ng/ml of TNF-α in the presence of 10 μM PDTC for various time. PDTC completely blocked the stimulation of PTEN expression induced by TNF-α (Figure 3). In addition, basal PTEN levels of HL-60 cells were decreased by PDTC treatment. We further confirmed whether TNF-α-induced PTEN expression is decreased by inhibition of NF-κB activation using antisense phosphorothioate oligonucleotide for p65. HL-60 cells (1×10^6 cells) were treated with 10 μM antisense phosphorothioate oligonucleotide of p65 (manufactured by GenoTech, Daejon, Korea) for 6 h. The antisense treatment did not affect cell viability of HL60 cells. For the determination of p65 translocation into the nucleus, p65 in nuclei was analysed by western blotting after TNF-α stimulation. TNF-α-treated cells showed increase of p65 level in nuclei compared to that of control cells, but p65 antisense treated cells showed

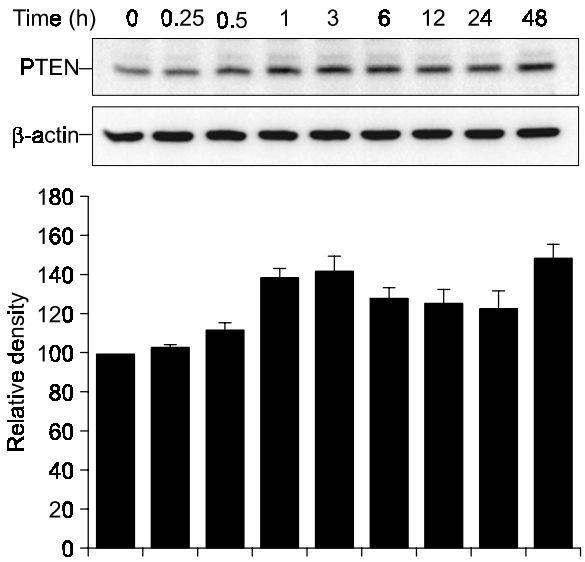

Figure 5. Effect of TNF-α on the PTEN expression in U937 cells. U937 cells (1×10^6) were stimulated with 10 ng/ml of TNF-α for the indicated time. Western blotting for PTEN expression was performed as described in Materials and Methods. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

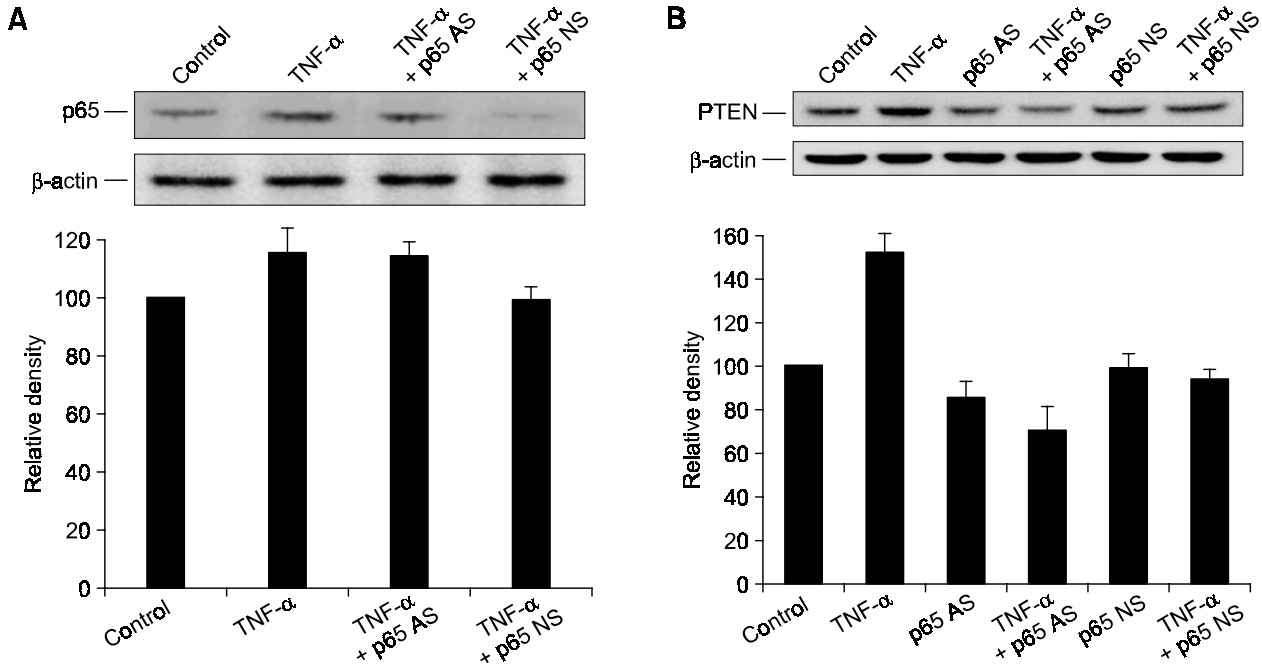

Figure 4. Effect of antisense phosphorothioate oligonucleotide for p65 on TNF-α-induced PTEN expression in HL-60 cells. HL-60 cells (1×10^6) were stimulated with 10 ng/ml TNF-α in the presence of 10 μM p65 antisense for 6 h. (A) Effect of antisense phosphorothioate oligonucleotide for p65 on p65 expression (B) Block of TNF-α-induced PTEN expression by antisense phosphorothioate oligonucleotide of p65. β-actin was used as a loading control. The analysis of band was performed with the LAS-1000 (Fujifilm, Japan).

much less p65 band than control cells or nonsense treated cells (Figure 4A). As shown at Figure 4B, antisense phosphorothioate oligonucleotide of p65 completely blocked TNF-α-induced PTEN expression. We also examined whether TNF-α upregulates PTEN in another leukemic human cell line, U937. TNF-α induced upregulation of PTEN in U937 cells (Figure 5), suggesting that TNF-α might induce PTEN in human leukemic cells.

Discussion

In the present study we show that TNF-α-induced PTEN expression is dependent on NF-κB activation. However, the role of NF-κB in PTEN expression is controversial; it has been reported that NF-κB inhibits PTEN expression in the human colon or cervix cancer cell lines (Kim *et al.*, 2004; Vasudevan *et al.*, 2004), whereas our previous finding suggested that NF-κB is a positive regulator of PTEN expression in HL-60 cells (Lee *et al.*, 2005). These inconsistencies may originate from the involvement of other factor in the pathway leading to PTEN expression after NF-κB activation. Vasudevan *et al.* reported the fine result that PTEN is downregulated by TNF-α *via* NF-κB (2004). They claimed that the mechanism underlying suppression of PTEN expression by NF-κB is independent of p65 DNA binding or transcription function and rather involves sequestration of limiting pools of transcriptional coactivators CBP/p300 by p65 (Vasudevan *et al.*, 2004). The report suggests that the coactivator sequestration could be mediated by p65 phosphorylation *via* protein kinase A. The controversial results between the results and ours might be due to the different regulation of PKA activity or PKA levels depending on the type of cell and its stimulator.

The PTEN promoter contains two potential NF-κB binding sites (GGGAATCTCT at nucleotide position -1565 and GGGTATTCCC at nucleotide position -1441) in the far upstream region, suggesting that NF-κB binds to PTEN promoter and regulates its expression. Although our previous report and this study support this possibility, binding assay of NF-κB to PTEN promoter and analysis of promoter activity is needed for the exact elucidation of PTEN regulation by NF-κB.

In conclusion, we found that TNF-α induces NF-κB-dependent PTEN expression in HL-60 cells. Our result is the first evidence that PTEN is a target of TNF-α/NF-κB pathways in leukemia cells. This paper might help us to understand the mechanism by which TNF-α induces differentiation of leukemia cells.

Acknowledgement

This work was supported by grants of the Korea Science and Engineering Foundation (No. R01-2002-000-00493-0, R11-2002-100-02007-0, M10528010003-05N2801-00310) and the post-BK21 project for Medical Science program for 21st century, Chonbuk National University Medical School.

References

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54

Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 1999;96:4240-5

Cappellini A, Tabellini G, Zweyer M, Bortul R, Tazzari PL, Billi AM, Fala F, Cocco L, Martelli AM. The phosphoinositide 3-kinase/Akt pathway regulates cell cycle progression of HL60 human leukemia cells through cytoplasmic relocalization of the cyclin-dependent kinase inhibitor p27(Kip1) and control of cyclin D1 expression. Leukemia 2003;17:2157-67

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 1997;388:548-54

Haas-Kogan D, Shalev N, Wong M, Mills G, Yount G, Stokoe D. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. Curr Biol 1998;8:1195-8

Hwang PH, Kim SY, Lee JC, Kim SJ, Yi HK, Lee DY. PTEN/MMAC1 enhances the growth inhibition by anticancer drugs with downregulation of IGF-II expression in gastric cancer cells. Exp Mol Med 2005;37:391-8

Kim S, Domon-Dell C, Kang J, Chung DH, Freund JN, Evers BM. Down-regulation of the tumor suppressor PTEN by the tumor necrosis factor-alpha/nuclear factor-kappaB (NF-kappaB)-inducing kinase/NF-kappaB pathway is linked to a default IkappaB-alpha autoregulatory loop. J Biol Chem 2004;279:4285-91

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5

Lee YR, Shim HJ, Yu HN, Song EK, Park J, Kwon KB, Park JW, Rho HW, Park BH, Han MK, Kim JS. Dimethylsulfoxide induces upregulation of tumor suppressor protein PTEN through nuclear factor-kappaB activation in HL-60 cells. Leuk Res 2005;29:401-5

Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1998;273: 13375-8

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science 1997;278:860-6

Sheng X, Koul D, Liu JL, Liu TJ, Yung WK. Promoter analysis of tumor suppressor gene PTEN: identification of minimum promoter region. Biochem Biophys Res Commun 2002; 292:422-6

Simpson L, Parsons R. PTEN: life as a tumor suppressor. Exp Cell Res 2001;264:29-41

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 1998;95:29-39

Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y, Benchimol S, Mak TW. Regulation of PTEN transcription by p53. Mol Cell 2001;8:317-25

Tang Y, Eng C. PTEN autoregulates its expression by stabilization of p53 in a phosphatase-independent manner. Cancer Res 2006;66:736-42

Vasudevan KM, Gurumurthy S, Rangnekar VM. Suppression of PTEN expression by NF-kappa B prevents apoptosis. Mol Cell Biol 2004;24:1007-21

Zhang SY, Park KW, Oh S, Cho HJ, Cho HJ, Park JS, Cho YS, Koo BK, Chae IH, Choi DJ, Kim HS, Lee MM. NF-kappaB decoy potentiates the effects of radiation on vascular smooth muscle cells by enhancing apoptosis. Exp Mol Med 2005; 37:18-26