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Tumor Necrosis Factor–Alpha (TNF-α) Induces PTEN Expression in HL-60 Cells

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백혈병세포에서 종양괴사인자에 의한 PTEN 발현증가

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ABSTRACT – Tumor necrosis factor-alpha (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 two molecules are suggested. TNF-α has been known to downregulate PTEN via nuclear factor–kappa B (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 HL-60 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 oligonucleotide or pyrrolidine dithiocarbamate (PDTC). 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-kB activation in HL-60 cells.

Key words: TNF-α, PTEN, NF-kB, p65, HL-60, apoptosis, inflammation, immunity

Various physiological stimulants including growth factors and cytokines activate receptor tyrosine kinases, activated Ras, or G proteins and then lead to the stimulation of the key modulator of cellular function, Akt, via the activation of phosphatidylinositol 3-kinase (PI3K).[1-5] The function of PI3K/Akt axis can be antagonized by PTEN (phsophatase and tension homologue) that dephosphorylates the main PI3K product phosphatidylinositol 3,4,5-triphosphate (PIP_3).[1-6] The modulation of Akt activation by the enzymatic balance between PI3K and PTEN controls a variety of cellular functions such as cell growth, differentiation, migration and apoptosis.[6-9] It has been well known how PI3K is activated and modulated by many physiological stimulators and which effecter molecules are involved in the process in multiple cellular systems.[6-9] However, the way to regulate PTEN has been little known compared to that of PI3K. There are a few reports about the regulator of PTEN expression. Some reports demonstrated that p53 increases PTEN expression via the direct activation PTEN promoter.[10-11] Other report suggests no direct relationship between p53 and PTEN.[12] Nuclear transcription factor kappa-B (NF-κB) also has been known to regulate PTEN expression even though the regulation is controversial depending on cell type and stimulants. Tumor necrosis factor-alpha (TNF-α) downregulates PTEN via NF-κB in colon cancer cell line H27,[13] whereas dimethysulfoxide (DMSO) increases PTEN via NF-kB activation.[14]

TNF-α is a pleotropic cytokine that regulates immunity, cell growth, and differentiation.[15-18] TNF-α also induces programmed cell death (apoptosis) of many can-

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cer cells. TNF-α binding to the subtype of TNF receptors containing a death domain (TNFR1) recruits TRADD (TNFR1-associated death domain protein), FADD (Fas-associated–death domain protein, and caspase 8 to induce apoptosis).[19-20] TNFR1 recruits RIP (receptor-interacting protein) to activate nuclear factor kappa-B signaling (NF-κB),[19] whereas TRADD can interact with TRAF2 (TNFR-associated factor 2) and activate the NF-κB pathway through NIK (NF-κB-inducing kinase,[22] indicating that NF-κB is directly involved in the apoptosis induced by TNF-α. Indeed, inhibition of NF-κB activity blocks TNF-α-induced apoptosis.[23] These results strongly suggest that NF-κB activation is necessary factor for TNF-α-induced apoptosis. However, in contrast to the evidence in favor that activation of NF-κB could lead to apoptosis, its activation is also related, under certain circumstances, to induction of antiapoptosis. In many cell types, TNF-α-induced apoptosis is prevented by NF-κB-mediated production of anitapoptotic proteins, such as cellular inhibitor of apoptosis protein (c-IAP) and cellular FADD-like interleukin-1-β-converting enzyme-like inhibitory protein (c-FLIP).[22-29] Indeed, the activation of NF-κB inhibits the apoptosis of TNF-α through FADD and by the other stimuli.[28-31]

It has been reported that NF-κB inhibited PTEN expression, resulted in prevention of apoptosis of normal and cancer cells.[13,32] However, we previously found that DMSO upregulated PTEN via NF-κB activation.[14] Therefore, we raised the question why DMSO and TNF-α showed the different effect on the PTEN expression via NF-κB. Consequently, we investigated whether TNF-α could upregulate PTEN in HL-60 cells where we found the upregulation of PTEN by DMSO via NF-κB. We found that TNF-α also induced expression of PTEN through NF-κB activation in HL-60 cells.

Materials and Methods

Materials

The anti-PTEN monoclonal mouse antibody and The anti p65 monoclonal mouse antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the phospho-AKT (p-AKT) polyclonal rabbit antibody from Cell Signaling Technology (Minneapolis, MN, USA). Recombinant human cytokine, tumor necrosis factor-á (TNF-α) was obtained from R & D Company (Minneapolis, MN, USA). RPMI 1640 and fetal calf serum were obtained from Gibco-BRL (Gaithersburg, MD, USA). HBSS (Hanks' balanced salt solution), pyrrolidine dithiocarbamate (PDTC), and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 5' end p65 antisense oligonucleotide of NF-κB gene (5'-GAAACAGATCGTCCATGGT-3') and p65 nonsense (scrembled control) oligonucleotide (5'-GTAC-TACTCTGAGCAAGGA-3') was designed and manufactured by GenoTech (Daejon, Korea).

Cell culture

Human leukemia cell line, HL-60 cells were obtained form the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, antibiotics (Penicillin G 60mg/L, Streptomycin 100 mg/L, Amphotericin B 50 ml/L) 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 3min. 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 phenylmethylsulphonylfluoride (PMSF), 0.5 mM dithiothreitol (DTT)), voltexed for 10 sec and then centrifuged at 3,000 rpm for 5 min. The packed cells were resuspended with ice-cold hypotonic lysis buffer in presence of 50ìl 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 ìl 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.[33]

Western blot analysis

Human leukemia cells (1×10^6 cells) were seeded in 100-mm culture dishes and harvested in phosphate buffered saline (PBS). After washing with PBS, cell pellets were lysed with the lysis buffer (20 mM Hepes pH 7.2, 1% TritonX-100, 150 mM NaCl, 0.1 mM PMSF, 1mM 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 sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the Bio-Rad protein assay regent (Bio-Rad Laboratories, USA). 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 at 95°C for 5 min. SDS-PAGE was performed in 10% slab gel according to Laemmli, (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 TBST (TBS, 0.01 % Tween 20). Primary antibody incubated 4°C for 4h. The secondary HRP-conjugated antidodies were goat-anti-mouse IgG (PTEN, p65, β-actin), and goat anti-rabbit IgG (p-AKT) (Santa Cruz, CA, USA). The reactive proteins were detected using enhanced chemiluminescence (ECL, Amersham Life Sciences, Arlington Heights, IL).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To examine the contribution of transcriptional control in PTEN regulation, RT-PCR was performed by using RNA PCR Kit (GeneAmp, Applied Biosystem, USA). Total RNA was isolated from cells by using TRIzol reagent following the manufacture's instructions. Five microgram of total RNA was transcribed into cDNA in a 20 ml 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 units RNase inhibitor, and 2.5 units M-MLV RNase H-reverse transcriptase by incubation for 15 minutes at 70°C and 50 minutes at 42°C. The reaction was stopped by incubation at 95°C for 10 minutes. 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 units Taq DNA polymerase, and 0.4 μM of each PCR primer: sense primer, human PTEN (5'-CCGGAATTCATGACAGCCATCATCAAAGA-3'), antisense primer, human PTEN (5'-CGCGGATCCTCAGACTTTTGTAATTTGTG-3'). Amplification for PTEN was initiated with 3 minutes of denaturation at 94°C followed by 30 cycles at 94°C for 1 minute, 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. After the last cycle of amplification, the samples were incubated for 5 minutes 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: sense primer, human β-actin (5'-CCACGAAACTACCTTCAACTCC-3'), antisense primer (5'-TCATACTCCTGCTGCTTGCTGATCC-3'). The obtained PCR products were analyzed on ethidium bromide-stained agarose (2%) gels.

Statistical Analysis

All experimental data are mean ± standard deviation (SD). Statistical analysis was performed using Student's test, and $p < 0.005$ was considered to be significant.

Results and discussion

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

HL60 cells, a human promyelocytic leukemic cell line, control the progression of its cell cycle through the activation of PI3K/Akt signaling pathway.[34] Our previous results show that PTEN expression is enhanced during DMSO-induced NF-κB activation in HL-60 cells. These results suggest that TNF, strong NF-κB activator, could affect the PI3K/Akt signaling pathway in HL-60 cells. Therefore, it was hypothesized that "the expression of PTEN may be upregulated by TNF".

To address such a possibility, HL-60 cells were treated with TNF-α, and then RT-PCR and western blot analysis was performed. HL-60 cells (1×10^6 cells) were treated with 10 ng/ml of TNF-α for various periods of times and used to prepare whole cell extracts for western blotting of PTEN. TNF-α caused an increase of PTEN protein level in a time-dependent manner (Fig. 1A). PTEN level was significantly increased within 1hr after treatment with TNF-α and this response was persistent till 6hr. Cell viabilities of leukemia cells receiving TNF-α were declined after 12hr treatment (data not shown).

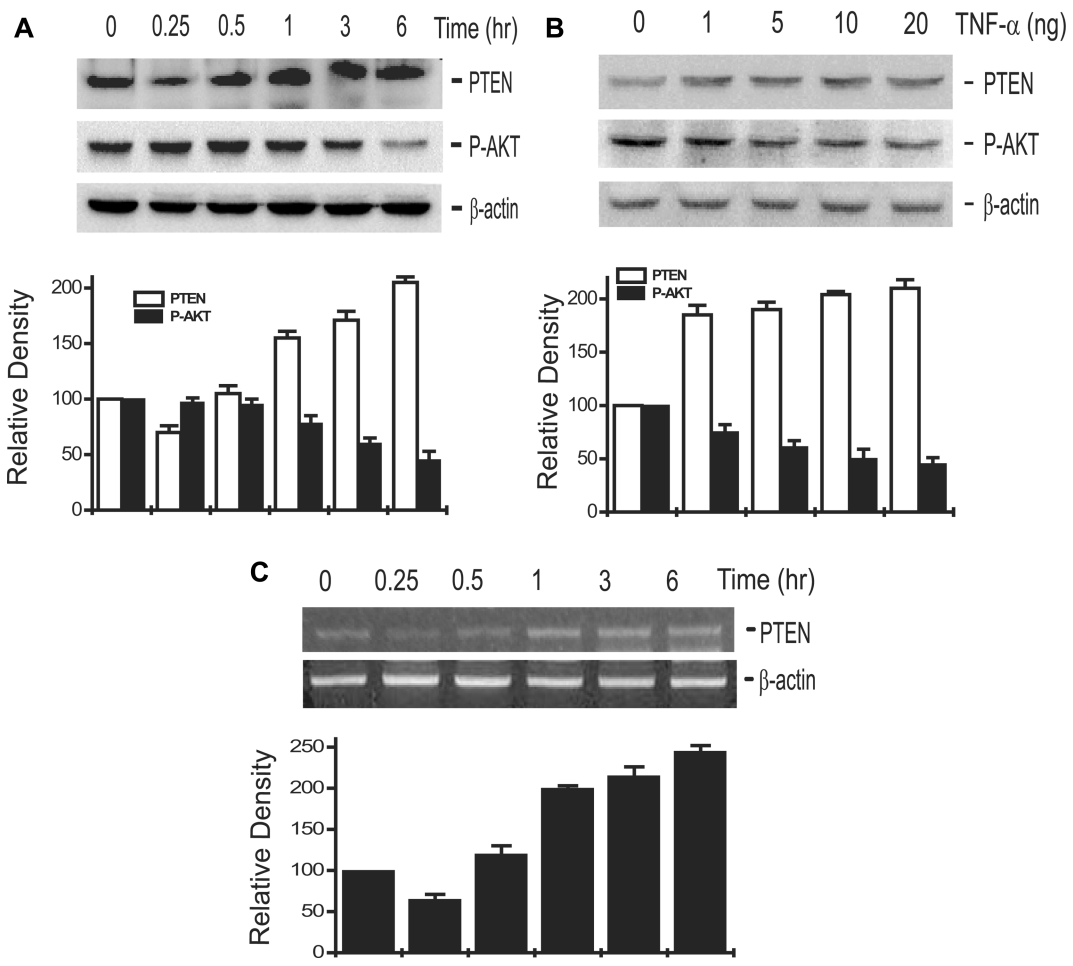

Fig. 1. Effect of TNF-α on the PTEN expression in human leukemia cells. (A) Kinetics of PTEN protein expression stimulated by TNF-α. HL-60 cells (1×10^6) were stimulated with 10 ng/ml of TNF-α for the indicated times. (B) The concentration effect on TNF-α-induced PTEN expression. HL-60 cells (1×10^6) were stimulated with TNF-α at the indicated concentrations for 6hr. (C) PTEN mRNA expression was analyzed by RT-PCR. HL-60 cells (1×10^6) were stimulated with 10 ng/ml of TNF-α for the indicated times. 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).

To determine whether TNF-α stimulates expression of PTEN in a dose dependent manner, we incubated Leukemia cells (1×10^6 cells) with various concentrations of TNF-α (0-20 ng/ml) for 6hr. TNF-α also dose-dependently stimulated PTEN expression (Fig. 1B). At the same time, we also determined the mRNA levels of PTEN in a function of incubation times. As shown at Fig. 1C, PTEN mRNA level in leukemia cells was also increased from 60 min after treatment of TNF-α. An increased expression of PTEN could induce the inhibition of Akt phosphorylation. Therefore, Western blotting was performed to detect the phosphorylated Akt in the TNF-α-treated HL-60 cells (Fig. 1A and B). This reduction in phosphorylated Akt correlated with the increase in PTEN expression, and occurred at the same time point and with the same dose. Thereby, we confirmed that PTEN activity was enhanced within TNF-α-treated leukemia cells. Furthermore, it has been already shown that retinoic acid, a strong upstream of TNF-α-induced signal pathway, can increase PTEN expression in leukemia cells.[35-36] These findings strongly suggest that TNF-α upregulate 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 I-κBα, causing I-κBα degradation by proteasome and allowing NF-κB to translocate to the nucleus to activate transcription [37-38]. To test whether TNF-α activates NF-κB in HL-60 cells, we measured the translocation of NF-κB p65 subunit from cytosol to nucleus, and the level of I-κBα in cytosol. The translocation of p65 was clearly enhanced from 3 hr after the treatment of TNF-α and the enhancement persisted till 6hr (Fig. 2). Cytosolic I-κBα level was transitionally increased within 1hr, but decreased at 6hr, indicating that degradation of cytosolic I-κBα was coincidentally occurred with translocation of NF-κB p65 (Fig. 2). Thus, these results indicate that TNF-α activates NF-κB in HL-60 cells. From above results, it was suggested that TNF-α-induced upregulation of PTEN was correlated with NF-κB activation. Therefore, we next examined whether TNF-α stimulates upregulation of PTEN through NF-κB activation.

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

In order to determine definite role of NF-κB in TNF-α-induced upregulation of PTEN, two methods were used to inhibit the activation of NF-κB in leukemia cells treated with TNF-α; first, pyrrolidine dithiocarbamate (PDTC), a chemical inhibitor of NF-κB; second, antisense oligonucleotide of 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 mM PDTC for various periods of times. PDTC completely blocked the stimulation of PTEN expression by TNF-α (Fig. 3). In addition, PTEN levels of leukemia cells in the presence of PDTC were even less than those of control cells. As the inhibition of NF-κB by PDTC is sometimes controversial, this study was confirmed by additional antisense oligonucleotide of p65. Leukemia cells (1×10^6 cells) were treated with 10 mM antisense oligonucleotide of p65 (manufactured by GenoTech, Daejon, Korea) for 6 hr. For the determination of p65 translocation into nucleus, nuclei were isolated for western blotting of p65 after TNF-α stimulation. TNF-α-treated cells showed thick band compared to those of control cells, but p65 antisense treated cells showed much less p65 band than control cells or nonsense treated cells (data not shown). As shown at Fig. 4, antisense of p65 completely blocked the stimulation of PTEN expression by TNF-α. These results show that TNF-α-induced PTEN expression is depen-

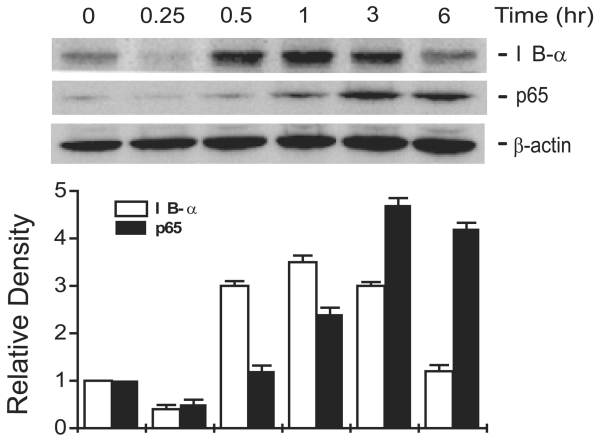

Fig. 2. The activation of NF-βB by TNF-α in human leukemia cells. HL-60 cells were treated with 10 ng/ml TNF-α for the indicated times. After incubation, the nuclear extract preparation for p65 nuclear translocation and cytosolic extracts for IκBα expression were prepared and analyzed by Western blot using antibody for p65, and IêBa, 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).

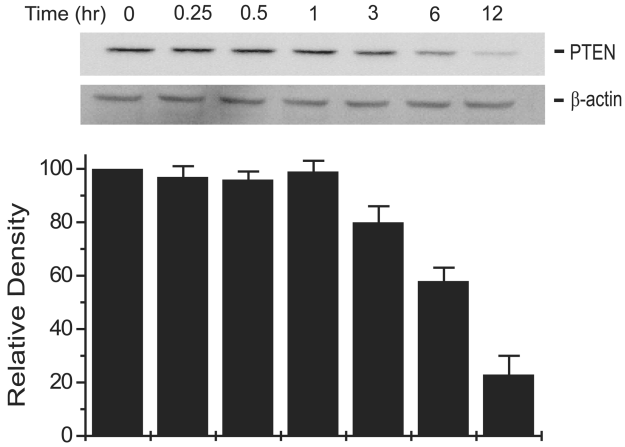

Fig. 3. Effect of PDTC on TNF-α-induced PTEN expression in human leukemia cells. HL-60 cells (1×10^6) were stimulated with 10 ng/ml of TNF-a in the presence of 10 mM PDTC for the indicated times. Cell extraction and western blotting for PTEN level were performed as described in "Materials and methods".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

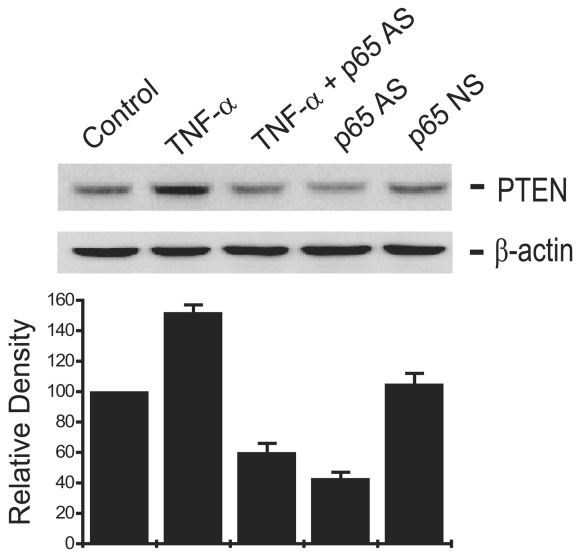

Fig. 4. Effect of antisense oligonucleotide of p65 on TNF-α-induced PTEN expression in human leukemia cells. HL-60 cells (1×10^6) were stimulated with 10 ng/ml TNF-α in the presence of 10 mM antisense for 6 hr. TNF-α-induced PTEN expression was blocked by antisense oligonucleotide of p65. The analysis of band was performed with the LAS-1000 (Fujifilm, Japan).

dent on NF-κB activation, and NF-κB is consequently a key regulator of the upregulation of PTEN by TNF-α. However, the role of NF-κB in PTEN expression is controversial; some results suggested that NF-κB inhibits PTEN expression in the human colon or cervix cancer cell lines,[13] our previous finding suggested that NF-κB activity is positive regulator of PTEN expression in leukemia cells.[14] Thus, these findings indicate that the profile of PTEN expression controlled by NF-κB is different by cell natures. Vasudevan *et al*. reported the fine result that PTEN is downregulated by TNF-α via NF-κB. The result suggest that the mechanism underlying suppression of PTEN expression by NF-kappa B was independent of p65 DNA binding or transcription function and rather involved sequestration of limiting pools of transcriptional coactivators CBP/p300 by p65.[32] Many reports suggest 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.

In conclusion, we found that TNF-α induces NF-κB-dependent PTEN expression in leukemia cells. Our result is the first evidence that PTEN is 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.

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국문요약

Tumor necrosis factor-alpha(TNF-α)는 세포의 고사, 염증 및 면역 등의 다양한 생물학적 기능에 대한 역할을 한다. PTEN 역시 세포의 성장과 증식 그리고 세포의 유주와 분화 등의 세포학적인 다양한 기능을 갖는다. 그러므로 이들 두 분자들 사이의 상호관계가 있을 것으로 제안되고 있으며, TNF-α는 사람의 대장세포 주인 HT-29에서 nuclear factor–kappa B(NF-κB) 경로를 통해 PTEN downregulate 기능이 있는 것으로 알려져 왔다. 그러나 저자 등은 본 연구에서 HL-60 cells에서 TNF-α가 NF-κB를 통해 PTEN를 upregulates하는 기존의 반대 현상을 확인하였다. TNF-α는 HL-60 cells에서 time과 dose의존성 방법으로 PTEN 발현을 증가시켰지만 반응은 p65 anisense oligonucleotide 또는 pyrrolidine dithiocarbamate (PDTC)으로 NF-κB를 분해함으로 파괴되었다. 따라서 저자 등은 TNF-α가 NF-κB경로를 활성화시킴을 확인하였고, TNF-α를 처리 할 경우 핵에 대하여 p65 전위에 의해 TNF-α가 활성화 됨을 증명하였다. 결국 HL-60세포에서 NF-κB의 활성화에 따라 PTEN 발현의 upregulation이 유도되는 것으로 결론지었다.

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