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# Ciglitazone, in Combination with All *trans* Retinoic Acid, Synergistically Induces PTEN Expression in HL-60 Cells

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# 백혈병세포에서 PTEN 발현에 대한 Ciglitazone과 retinoic Acid의 항진 작용

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**ABSTACT** – Peroxisome proliferator-activated receptor-gamma (PPARγ) must form a heterodimer with the retinoid-X receptor (RXR) to bind DNA, and its transcriptional activity is thought to be maximized by ligands specific for either receptor. Activated PPARγ and PPARγ ligands may influence tumor growth through regulation of the tumor suppressor PTEN. Our aim in this study was to determine whether co-stimulation with the PPARγ ligand, ciglitazone, and RXR ligand can synergistically upregulate PTEN in human acute promyelocytic leukemia (APL) cells and consequently potentate the inhibition of cell growth and cell cycle progression of these cells. Human leukemia cell line, HL-60 cells were exposed to all-trans-retinol and ciglutazone. The PTEN expression was measured as the level of PTEN mRNA expression by RT-PCR and as the level of PTEN expression by western blot analysis. Cell cycle analysis was carried out by a propidium iodide (PI) staining method and analyzed with a FACScan. The PPARγ ligand, ciglitazone, and the RXR ligand, retinoic acid, upregulated PTEN expression by HL-60 cells in time- and dose-dependent manners, respectively. This was significantly enhanced by a combination of both ciglitazone and retinoic acid. Moreover, these compounds synergistically induced arrests of both cell growth and the $G_1$ phase of the cell cycle. Thus, the activation of the PPARγ: RXR heterodimer may represent a regulatory pathway for human leukemia cells and there may be important roles for PPARγ and RXR ligands in prophylactic and therapeutic approaches for controlling leukemia through the upregulation of PTEN.

**Key words:** PTEN, PPARγ, retinoid-X receptor, leukemia, ciglitazone, retinoic acid

The novel tumor suppressor gene *PTEN/MMAC1/TEP1*, a tyrosine phosphatase, on the chromosome 10q23 protein product (PTEN) is a lipid phosphatase that controls a variety of cellular functions including cell growth, cell survival, immune reaction, and cell differentiation.[1-3)]

*PTEN* gene mutations have been observed in a variety of human cancers including those of the breast, prostate, brain and in lymphoma and leukemia,[4-10)] and studies have focused on the invasiveness and metastatic properties of these tumors.[11)] *PTEN* loss might negatively influence apoptosis.[11)] It is well known that apoptosis serves as a cancer cell-death mechanism induced by anticancer chemotherapy. Thus, PTEN plays a pivotal role in the efficacy of anti-tumor chemotherapy.

Vitamin A (retinoid) suppresses carcinogenesis of a variety of tissues in experimental animals. Therefore, it is expected that retinoid would be clinically useful as a supplement of chemoprevention or chemotherapy against diverse human malignancies. Retinoic acid (RA) induces

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differentiation and growth arrest in myeloid precursor cells and in leukemia cells.[12,13] Especially, all-*trans*-retinoic acid (ATRA) therapy is also widely used in the treatment of acute promyelocytic leukemia (APL) patients, giving a remission rate of over 90%.[14]

Retinoid receptors belong to the greater steroid-receptor superfamily and are classified as RARs and RXRs. They function as dimeric, ligand-dependent transcription factors. RXRs can also form homodimers and activate the retinoid X response element or form heterodimers with other members of the steroid-receptor family, thus providing opportunities for cross-talk among different signaling pathways.[15]

One emerging therapeutic approach to cancer treatment is the induction of terminal differentiation through ligand activation of nuclear hormone receptors (NHRs). This family includes estrogen receptors, retinoic acid receptors (RARs), retinoid X receptors (RXRs), and the vitamin D receptor. The latest member of this superfamily to be identified is the peroxisome proliferator-activated receptor gamma, commonly known as PPARγ.[16] This is a transcriptional factor that plays an essential role in mediating the pharmacological actions of PPARγ ligands, and is highly expressed in normal monocytes, various leukemias, and epithelial malignancies.[17,18] PPARγ ligands have been developed to induce differentiation, growth arrest, and apoptosis.[19] Natural ligands for PPARγ are the hydroxyoctadecanoic acids and the cyclopentane prostaglandin 15-deoxy-D-12, 14 $PGJ_2$,[20] and synthetic ligands include triglutazone, rosiglitazone, and ciglutazone.[21]

Activation of NHRs has been identified as an approach to induce differentiation and inhibit proliferation of cancer cells. The best example of this paradigm is the induction of remission of patients with APL using ATRA.[22,23] ATRA has also been used to prevent the recurrence of head and neck cancers. Because normal pre-adipocytes can be induced to undergo terminal differentiation in the presence of ligands for PPARγ, investigators have been encouraged to use thiazolidinedione (TZD) in attempts to induce the differentiation of human liposarcoma cells.[24] Successes *in vitro* encouraged clinicians to give troglitazone to a series of patients with liposarcoma, which resulted in a retardation of growth and induction of differentiation of these tumor cells,[25] and these studies have spurred the examination of the effects of TZD on a number of cancers both *in vitro* and *in vivo*.

Abundant PPARγ, and PPARγ ligands can induce acute myelomonocytic leukemic cells (THP-1) to differentiate toward macrophages.[26] Furthermore, other studies have shown that PPARγ ligands can inhibit the clonal proliferation of U937 myeloid monocytic leukemic cells, and a PPARγ ligand plus 9-*cis*-retinoic acid was capable of synergistically inhibiting the clonal proliferation of HL-60, U937, and THP-1 human myeloid leukemic cell lines. In general, however, the effect of PPARγ ligands on myeloid leukemic growth and differentiation is modest.[27]

There have been many reports regarding the development of resistance to RA with the use of physiological differentiating agents alone or combination.[28-31] To date, there have been several reports of agents that combine with RA to overcome RA-resistant APL, and finding novel means to overcome this resistance remains an important goal in the treatment of this tumor type. Combination therapies that can overcome this resistance may act either by reactivating a blocked RA pathway or, alternatively, by way of stimulation of novel pathways that can mediate a differentiation response.[31]

Recently it has been shown that RA upregulates PTEN expression in leukemia cells,[32] suggesting that RA has tumor suppressive effects on tumor cells through upregulation of tumor suppressor PTEN. Interestingly, pharmacological ligands of PPARγ also induce upregulation of PTEN,[33] indicating that pharmacological ligands of PPARγ have tumor-suppressive effects through upregulation of the tumor suppressor PTEN. PPARγ forms a heterodimer with RXR to bind DNA and transcriptional activity is thought to be maximal in the presence of both PPARγ and RXR ligands.[17,34] Taken together, it is possible that a pharmacological PPARγ ligand in combination with RA might synergistically enhance the upregulation of novel tumor suppressor PTEN in cancer cells.

Our aim in this study was to determine whether co-stimulation with the PPARγ ligand, ciglitazone, and RXR ligand can synergistically upregulate PTEN in human APL cells and consequently potentiate the inhibition of cell growth and cell cycle progression of these cells.

## Materials and Methods

### Materials

Anti-*PTEN* monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ciglitazone [(+/–)-5-[4-(1-Methylcyclohexymethoxy)-thiazolidine-2, 4-dione] was purchased from ALEXIS Biochemicals (Lausen, Switzerland). M-MLV RNase H-reverse transcriptase, TRIzol reagent, RPMI 1640, and fetal calf serum were obtained from Gibco-BRL (Gaithersburg, MD, USA). ATRA, HBSS (Hanks balanced salt solution), propidium iodide, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### Cell culture

A human leukemia cell line (HL-60) was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, antibiotics (Penicillin G 60 mg/L, Streptomycin 100 mg/L, Amphotericin B 50 μl/L) under a humid atmosphere (5% $CO_2$, 95% air).

### Cell proliferation assay

The cell proliferation assay was performed using the Quick Cell Proliferation Assay Kit (BioVison, Mountain View, CA, USA), which is based on the cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenase. HL-60 cells ($1 \times 10^3$ cells/well) were incubated at 37°C in 96-well plates in 10% FCS medium and incubated with vehicle or drugs for 24 or 48 hours. Absorbance was measured at 440 nm by using a microplate reader (Molecular Probes, Eugene, OR, USA).

### Reverse Transcription Polymerase Chain Reaction (RT–PCR) for PTEN expression

RT–PCR was performed using RNA PCR Kits (GeneAmp, Applied Biosystems, USA). Total RNA was isolated from cells using TRIzol reagent following the manufacturer's instructions. Five micrograms of total RNA was 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 μM 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'-CCGGAATTCATGA-CAGCCATCATCAAAGA-3'), and antisense primer, human *PTEN* (5'-CGCGGATCCTCAGACTTTTGTAA-TTTGTG-3'). Amplification for *PTEN* or (PPAR) was initiated with 3 minutes of denaturation at 94°C followed by 26 cycles at 94°C for 1 minute, 94°C for 1 minute, 55°C for 1 minute, and 72°C for 3 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 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 gels (2%).

### Vector for PTEN over expression

An AdenoVector (Ad) system for PTEN overexpression was prepared by the methods developed by Kwak *et al.*.[36] The E1/E3-deleted replication-deficient recombinant Ad was made using the AdEasy system (Quantum Biotechnologies, Montreal, Quebec, Canada) as described by Hwang *et al* [37]. *Kpn*I-*Xho*I restriction fragments from pcDNA3/wild-type *PTEN* cDNA were ligated into *Kpn*I-*Xho*I-digested pShuttleCMV, as previously described.[36] To create AdLacZ, a *Sal*I-*Not*I restriction fragment from pcDNA3.1/LacZ (Invitrogen Corp., San Diego, CA, USA) was ligated to *Sal*I-*Not*I-digested pShuttleCMV. Recombination into the pAdEasy viral backbone was accomplished in bacteria (*E. coli* strain BJ5183) according to the manufacturer's instructions. Recombinant pAdEasy plasmids containing CMV-cDNA inserts were purified over QIAGEN columns (QIAGEN Inc., Valencia, California, USA), and 5 μg of *Pac*I-digested DNA was used to transfect QBI-293A cells using the calcium phosphate method (Promega Corp., Madison, Wisconsin, USA). Cells were seeded at $2 \times 10^6$ cells per 150 mm culture dish 24 hours before transfection. Following amplification, lysates containing clonal recombinant Ad were prepared from 150 mm cul-

ture dishes and purified by CsCl gradient centrifugation. The virus was aliquoted and stored at -20°C in 5 mM Tris (pH 8.0) buffer containing 50 mM NaCl, 0.05% BSA, and 25% glycerol. The virus was titrated by serial dilution infection of QBI-293A cells. HL-60 cells ($1 \times 10^7$ cells) were infected with recombinant adenovirus at a concentration of 10 plaque-forming units (pfu) per cell for 72 hours. LacZ-expressing cells were detected as blue-stained cells by β-gal staining.

### Western blot analysis for PTEN

HL-60 cells ($1 \times 10^6$ cells) were seeded in 100-mm culture dishes and harvested in phosphate buffered saline (PBS). After washing with PBS, cell pellets were lysed with lysis buffer (20 mM Hepes pH 7.2, 1% Triton X-100, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 1 mg/mL aprotinin). Total cellular proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). The protein concentration was determined using protein assay kits (Bio-Rad Laboratories, USA). Samples (50 mg) were prepared with four volumes of 0.5 M Tris buffer (pH 6.8) containing 4% SDS, 20% glycerol and 0.05% bromophenol blue at 95°C for 5 minutes. SDS-PAGE was performed in 10% slab gels. Proteins were then transferred to nitrocellulose paper. Blots were incubated with the primary antibodies against PTEN followed by alkaline phosphatase-conjugated secondary antibody.

### FACS analysis for cell cycle

Distribution of cellular DNA content was determined by hypotonic propidium iodide (PI) staining. Briefly, HL-60 cells ($1 \times 10^6$ cells) were suspended in 1 mL of PI solution (50 mg/mL of 0.1% sodium citrate and 0.2% Nonidet P-40) and analyzed with a FACScan flow cytometer (Becton Dickinson and Company, New York, USA). Excitation was carried out with the 488-nm line of an argon ion laser operating at a continuous output of 200 mW. The resulting DNA histograms were interpreted using the Cell Quest Pro software (Becton Dickinson and Company, New York, USA).

### Statistical analysis

All experimental data are presented as means ± standard deviations (SD). Statistical analyses were performed using ANOVA and Student's $t$ tests, and $P < 0.05$ was considered to be significant.

## Results

### PTEN induced $G_1$ cell cycle arrest in leukemia cells

To investigate PTEN effects on cell cycle arrest in the $G_1$ phase in leukemia cells, HL-60 cells were infected with vector alone (Ad/LacZ) or PTEN viral vector (Ad/PTEN) for 72 hours. After treatment with LacZ adenovirus or PTEN adenovirus, levels of $G_1$ phase DNA in the PTEN adenovirus increased (Fig. 1). Thus, PTEN expression induced $G_1$ cell cycle arrest in these leukemia cells.

### ATRA and ciglitazone restrained the proliferation of leukemia cells

In previous reports, ATRA (1 μM) and PPAR-γ agonist induced growth arrest.[27,38] To investigate the cell proliferation effects of ATRA (1 μM) and the PPAR-γ agonist, ciglitazone, (10 μM) in leukemia cells, HL-60 cells were exposed to PPAR-γ activators (ciglitazone and ATRA) for 3 days. Both compounds inhibited growth in the cells. Moreover, the combination of both compounds showed synergic effects on growth inhibition (Fig 2). Trypan blue dye exclusion confirmed that there was no toxicity attributable to these agents (data not shown).

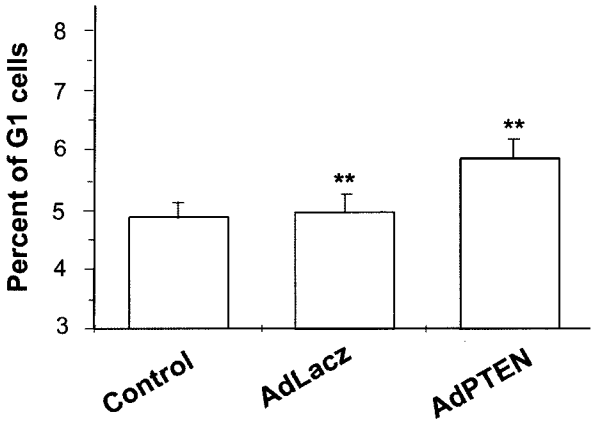


**Fig. 1. PTEN induced $G_1$ cell cycle arrest in leukemic cells. HL-60 cells were cultured and infected with vector alone (Ad/LacZ) or PTEN viral vector (Ad/PTEN). The cells were harvested and then stained with propidium iodide solution. Cell cycle analysis of DNA was performed using a FACScan flow cytometer.**

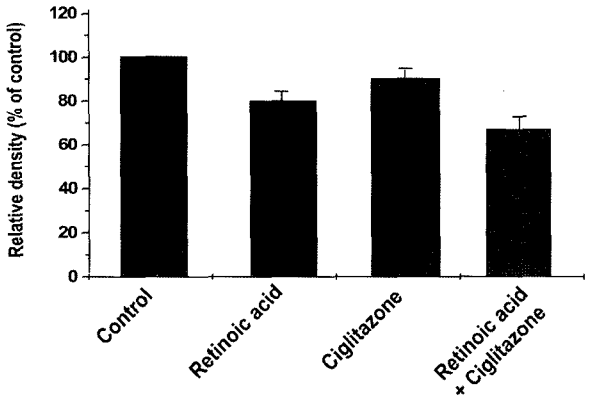


**Fig. 2. Retinoic acid and ciglitazone restrained the proliferation of leukemic cells. HL-60 cells were incubated and exposed to PPAR activators. Cell proliferation assay was performed using Quick Cell Proliferation Assay Kits based on the cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenase.**

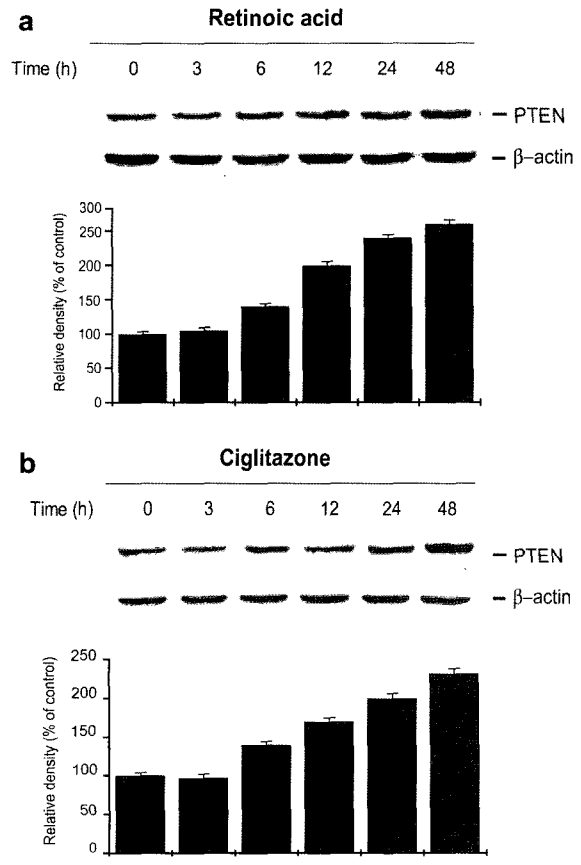


**Fig. 3. Retinoic acid and ciglitazone increased the expression of PTEN in leukemic cells. (A) The time-course study of PTEN expression stimulated with retinoic acid in HL-60 cells. (B) The time-course study of PTEN expression stimulated with ciglitazone in HL-60 cells. Western blotting for PTEN level was performed.**

## ATRA and ciglitazone increases the expression of PTEN in leukemia cells

A recent study found that ATRA and PPAR-γ agonists are mediated via upregulation of PTEN,[32,33] and PTEN is involved in the normal regulation of cell growth, the cell cycle, and apoptosis. To address such a possibility, HL60 cells were treated with ATRA (1 μM) and PPARγ agonist, ciglitazone (10 μM), and then western blot analyses were performed. As shown in Fig. 3, ATRA (1 μM) and ciglitazone (10 μM), increased the protein of *PTEN*, in time-dependent manners.

## Synergic effects of PTEN expression by ATRA and ciglitazone in leukemia cells

To assess the synergic effects of retinoic acid (1 μM)

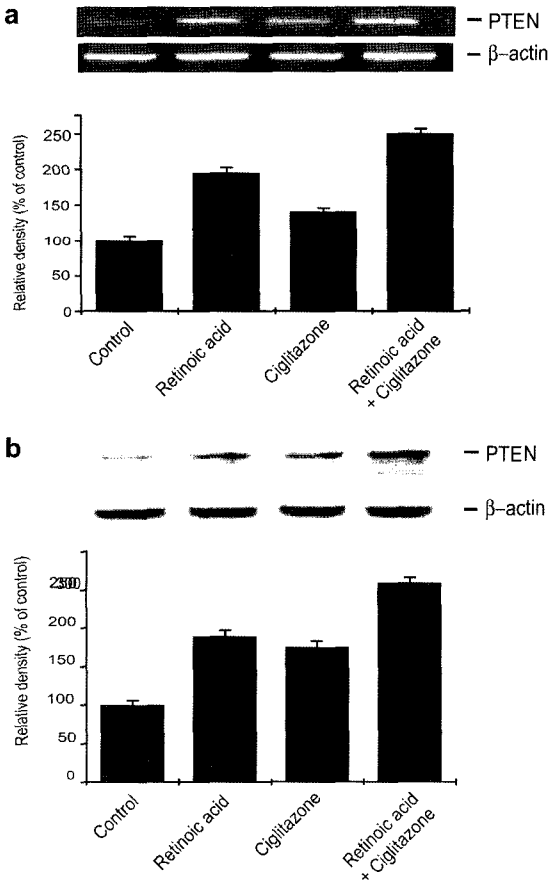


**Fig. 4. Synergic effect of PTEN expression by retinoic acid and ciglitazone in leukemia cells. (A) PTEN protein levels in HL-60 cells were analyzed by western blotting. (B) PTEN mRNA expression in HL-60 cells was analyzed by Reverse Transcription-Polymerase Chain Reaction. HL-60 cells were cultured and stimulated with retinoic acid (1 mM) and ciglitazone (10 mM).**

and ciglitazone (10 μM), PTEN expression levels in HL60 cells were analyzed by western blot analysis and RT–PCR. Both compounds increased PTEN mRNA and protein in leukemia cells. The combination of both compounds showed synergic effects of the protein of *PTEN* and its mRNA (Fig. 4A,B).

### ATRA and ciglitazone induce $G_1$ cell cycle arrest in leukemia cells

We show here that ATRA (1 μM) and ciglitazone (10 μM) increased PTEN expression and restrained cell proliferation. We expected to observe changes to the cell cycle. As shown in Fig. 5, both compounds increased $G_1$ phase arrest in leukemia cells. The combination of both compounds showed the synergic effect of $G_1$ phase arrest. The present results suggest that PTEN expression by ATRA and the PPARγ agonist, ciglitazone, induce $G_1$ cell cycle arrest in leukemia cells.

## DISCUSSION

Here, we found that co-stimulation with the PPARγ

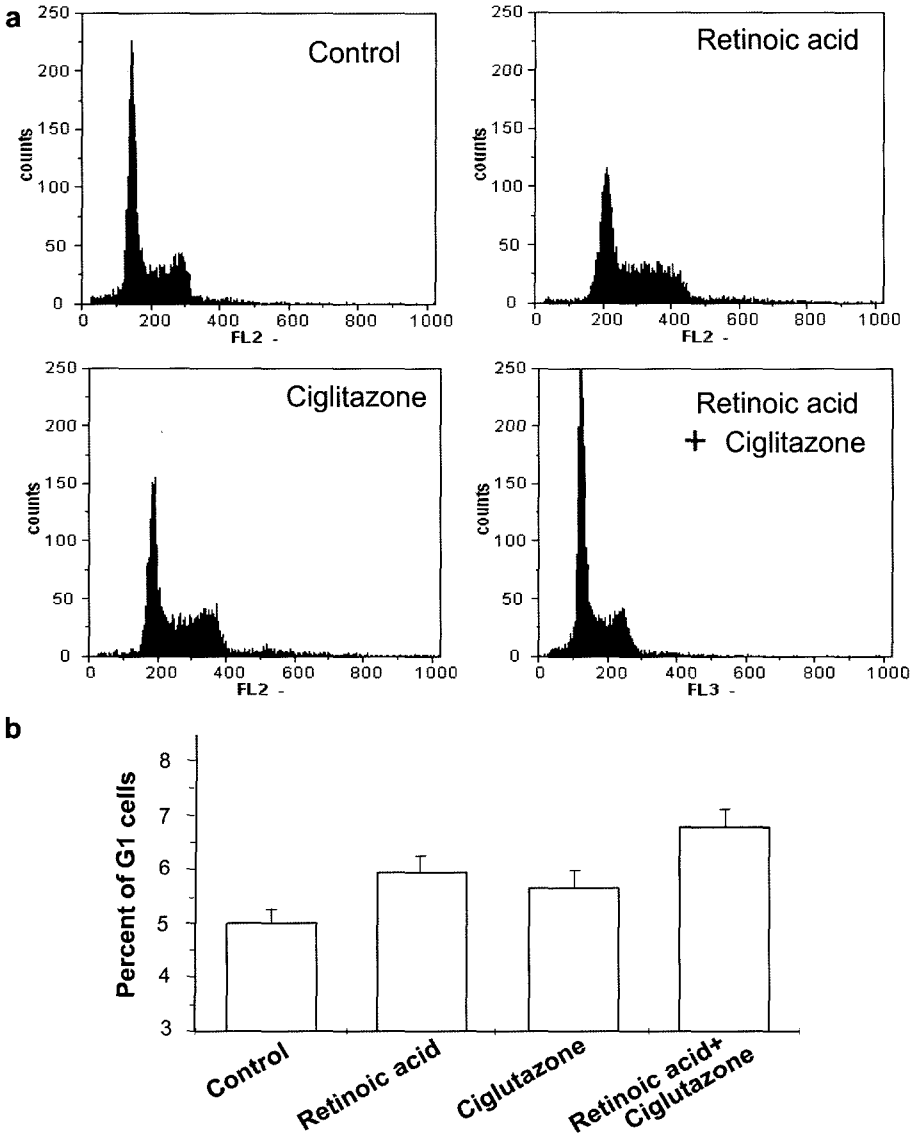


**Fig. 5. Retinoic acid and ciglitazone induce $G_1$ cell cycle arrest in leukemic cells. (A) HL-60 cells were cultured and stimulated with retinoic acid (1 mM) and ciglitazone (10 mM); cells were harvested and stained with propidium iodide solution. (B) Relative activity for Figure (A). Cell cycle analysis of DNA was performed using a FACScan flow cytometer.**

ligand, ciglitazone, and RXR ligands can synergistically upregulate PTEN in human APL cells and consequently potentiate the inhibition of cell growth and cell cycle progression of these cells.

Induction of terminal differentiation of cancer cells is an evolving therapeutic approach and accordingly, PPARγ, a ligand-stimulated transcription factor with differentiation promoting activity and over expressed in a variety of cancers, has emerged as one of the promising therapeutic targets.[39]

Differentiation therapy may play a role in treating AMLs, analogous to ATRA-induced differentiation in APL. PPARγ is known to be induced or expressed in cells of the myeloid or monocytic lineage.[26,40] Moreover, the PPARγ ligands, such as rosiglitazone and the synthetic triterpenoid CDDO, can induce differentiation in various human myeloid and monocytic leukemia cell lines.[26,41,42] Denzlinger *et al.* reported that PPARγ ligands significantly inhibited the proliferation of leukemic cells isolated from patients with primary chronic myelomonocytic leukemia (CMML) and exhibited modest antileukemic activity when administered to patients who were not eligible for intensive chemotherapy.[43] These results suggest that these compounds may have therapeutic potential in treating patients with AML and CMML.

As previously mentioned, PPARγ functions as an obligate heterodimer with the RXR and optimal activation of the heterodimer requires both ligands. Therefore, one possible way to increase the effects of PPARγ ligands would be to combine them with ligands specific for RXR. Of relevance, combinations of PPARγ ligands with the RXR-specific ligand markedly decreased cell growth and induced monocytic differentiation in HL-60 cells, suggesting that activation of the PPARγ-RXR heterodimer represents a novel regulatory pathway for HL-60 maturation.[26]

Recently published data suggest that PPARγ ligand may affect tumor growth indirectly, through regulation of the tumor suppressor PTEN.[33] PTEN blocks the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, resulting in cell death or inhibition of growth or both.[44,45] HL-60 cells control the progression of its cell cycle through the activation of the PI3K/Akt signaling pathway.[46,47] Therefore, there is a possibility that HL-60 cells could be differentiated by the differentiation agent, PPARγ, by affecting the PI3K/Akt signaling pathway. In our study, adenovirus-delivered PTEN overexpression resulted in arrests of both cell growth and the $G_1$ phase of the cell cycle in HL-60 cells, suggesting that the growth-suppressive effect of PTEN is associated with its ability to induce cell cycle arrest.

Recently, we reported evidence for the involvement of tumor suppressor PTEN in the DMSO-induced differentiation of HL60 cells.[48] The upregulation of PTEN by DMSO leads to the decrease of Akt phosphorylation, a downstream event of PI3K.[48,49] We also show that the PPARγ ligand, ciglitazone, and the RXR ligand, RA, increased PTEN expression of HL-60 cells in time- and dose-dependent manners, respectively. Moreover, upregulation of PTEN was significantly enhanced by a combination of both ciglitazone and RA. This is the first report to show that PPARγ ligand, ciglitazone, and the RXR ligand, RA, synergistically enhance the upregulation of the tumor suppressor PTEN and inhibition of cell growth in human promyeloid leukemia cells, even though it need to be confirmed in the patients with APL.

In conclusion, the PPARγ ligand, ciglitazone, and the RXR ligand, RA, can synergistically upregulate PTEN expression in human APL cells, resulting in inhibition of cell growth and cell cycle progression of acute leukemic cells associated with its ability to induce cell cycle arrest. Thus, the activation of the PPARγ: RXR heterodimer might represent a regulatory pathway for HL-60 cells and there may be a possible role for PPARγ and RXR ligand in prophylactic and therapeutic approaches for controlling leukemia through the upregulation of PTEN, particularly to overcome RA-resistant APL, but also for other more common leukemias and types of cancers.

## Acknowledgements

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## 국문요약

Peroxisome proliferator-activated receptor-gamma(PPARγ)는 DNA와 결합하기 위해 retinoid-X receptor(RXR)와 heterodimer를 형성해야만 한다. 그리고 전사에 대한 최대활성은 수용체에 대한 리간드 특이성에 의하는 것으로 생각되고 있다. 활성화된 PPARγ와 PPARγ 리간드는 종양억제 PTEN의 조절을 통해 종양세포의 성장에 영향을 끼치게 된다. 본 연구의 목적은 PPARγ ligand, ciglitazone 그리고 RXR ligand로 동시에 자극하였을 때 급성전골수성백혈병(APL)세포에 대해 이들이 함께 PTEN upregulate를 조절할 수 있는지를 결정하기 위함이다. 그리고 이들 세포의 성장과 분화주기에 대해 강력한 억제 능이 있는지를 결정하고자 하였다. 즉, 사람의 백혈병세포주인 HL-60세포에 all-trans-retinol과 ciglutazone을 노출시킨 뒤 PTEN 발현에 대한 측정을 위해 RT-PCR법으로 PTEN mRNA 발현 정도를 확인하고 western blot으로 분석하였다. 세포주기의 분석은 propidium iodide(PI) 염색법과 FACScan으로 분석하였고, HL-60 cells에서 PPARγ ligand, ciglitazone, 그리고 RXR ligand, retinoic acid 그리고 upregulated PTEN 발현에 대한 time- and dose-dependent 방법으로 각각 확인하였던 바 ciglitazone과 retinoic acid를 동시 조합하여 처치하였을 때 유의적인 효과를 인정할 수 있었다. 더욱이 이들 혼합 물질은 세포의 성장과 $G_1$ phase를 동시 억제하는 능력이 있었다. 그러므로 PPARγ의 활성에 있어 RXR heterodimer가 사람의 백혈병세포에 대한 조절 경로로서 존재하며, PTEN의 upregulation을 통해 백혈병을 조절하기 때문에 백혈병의 예방 및 치료 접근에 PPARγ와 RXR ligands가 중요한 역할을 할 것이다.


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