Expression of survivin and bax/bcl-2 in peroxisome proliferator activated receptor-γ ligands induces apoptosis on human myeloid leukemia cells in vitro

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The present study was undertaken to investigate the mechanisms of peroxisome proliferator activated receptor-γ (PPAR-γ) ligand-induced apoptosis on human myeloid leukemia K562 and HL-60 cell lines. The results revealed that both 15-deoxy-delta(12,14)-prostaglandin J2 (15d-PGJ2) and troglitazone (TGZ) have significant anti-proliferation- and apoptosis-inducing effects on these two kinds of leukemia cells. Marked morphological changes of cell apoptosis including condensation of chromatin and nuclear fragmentation were observed clearly using Wright’s and Hoechst 33258 staining. Reverse transcription–PCR and western blot analyses demonstrated that both survivin and bcl-2 expression were downregulated markedly, while bax expression was upregulated concurrently when apoptosis occurred. We therefore conclude that 15d-PGJ2 and TGZ have significant apoptosis effects on K562 and HL-60 cells in vitro, and that upregulation of bax as well as downregulation of survivin and bcl-2 expression may be the important apoptosis-inducing mechanisms. The results suggest that PPAR-γ ligands may serve as potential therapeutic agents for both acute and chronic myeloid leukemia.

Key words: apoptosis, bax, bcl-2, PPAR-γ, survivin

Introduction

Peroxisome proliferator activated receptor-γ (PPAR-γ) ligands have recently been demonstrated to affect proliferation, differentiation and apoptosis of different cancer cell types [1]. In this study, we investigated the expression of PPAR-γ in human leukemia K562 and HL-60 cell lines and detected the expressions of survivin and bax/bcl-2 in PPAR-γ ligands troglitazone (TGZ)- and 15-deoxy-delta(12,14)-prostaglandin J2 (15d-PGJ2)-induced apoptosis on the two cell lines.

Materials and methods

Main reagents

TGZ and 15d-PGJ2 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The following antibodies were purchased from Santa Cruz Company (Heidelberg, Germany): anti-PPAR-γ, anti-survivin, anti-bcl-2 and anti-bax. TRIZOL reagent was from Gibco (Life Technologies, Rockville, MD, USA), and the reverse transcriptional kit was from MBI (San Francisco, CA, USA). The PCR primers were purchased from Shanghai Shenggong Company (Shanghai, China).

Cell culture and cell viability assay

K562 and HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified 5% CO₂ incubator at 37°C. Cells in logarithmic growth phase were used for further experiments.

Cell viability was assessed by MTT assay. Briefly, cells at 1×10⁵/ml were treated with various concentrations of PPAR-γ ligands (TGZ: 5, 10, 20, 40, 60, and 100 μmol/l; 15d-PGJ2: 1, 5, 10, 20, 30, 40, and 50 μmol/l) in 96-well plates for 72 h. Then, MTT working solution was added to each well and cells were incubated for 4h. The water-insoluble formazan was formed during incubation and was solubilized by adding solubilization agent to each well. The amount of formazan was determined measuring the absorbance at 540 nm using an ELISA plate reader.

Flow cytometry detection

For flow cytometry (FCM) analysis, cells treated with TGZ and 15d-PGJ2 for 72 h were collected, pelleted, washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 20 mg/l propidium iodide (PI) and 1 g/l ribonuclease A (RNase A). Fixed cells (1×10⁶) were examined per experimental condition by flow cytometry. The percentage of degraded
DNA was determined by the number of cells displaying subdiploid (sub-G<sub>1</sub>) DNA divided by the total number of cells examined.

**Detection of mRNA expressions using reverse transcription–PCR**

Reverse transcription (RT)–PCR was used to detect the expression of PPAR-γ mRNA as well as survivin and bcl-2/bax before and after the cells were treated with TGZ and 15d-PGJ2 for 72 h. The total RNA was extracted using TRIZOL reagent. First-stranded cDNA was synthesized using 5 μg total RNA by RT–PCR kit. The related PCR primers listed in Table 1 were used to produce the respective correlated products.

The PCRs for PPAR-γ and β-actin cDNAs were performed with 30 amplification cycles and the reaction conditions were: denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. The PCRs for both survivin and bcl-2/bax cDNAs were performed with 40 amplification cycles each as follows: (i) survivin and bcl-2: denaturation at 94°C for 45 s, annealing at 61°C for 1 min and extension at 72°C for 1 min; (ii) bax: denaturation at 94°C for 45 s, annealing at 58°C for 1 min and extension at 72°C for 1 min. All of the above PCRs were incubated in an automatic heat-block (Model PJ 2000 DNA Thermal Cycler; Perkin-Elmer, NJ, USA). The PCR products were then run on 1.5% agarose gel and visualized by ethidium bomide staining.

**Statistical analysis**

All experiments were performed in triplicate and the results are expressed as mean ± SD. Statistical analyses were performed by Student’s t-test using SAS 6.12 software. Statistical significance was accepted at the level of P <0.05.

**Hoechst 33258 staining**

After the cells were treated with 15d-PGJ2 (40 μmol/l) and TGZ (80 μmol/l) for 72 h, cells were harvested and fixed with 4% formaldehyde in PBS for 10 min, stained with Hoechst 33258, and then subjected to fluorescence microscopy.

**Wright’s staining**

The morphological variations such as apoptosis, as well as maturation and differentiation, were observed after the cells were treated with 40 μmol/l 15d-PGJ2 for 72 h.

**DNA fragmentation assay**

Cells (2 x 10<sup>6</sup>) were immersed in cytolysis buffer and incubated for 3 h at 4°C. DNA was extracted with phenol–chloroform, precipitated in 1/10 volume of 2 M NaAc and two volumes of ethanol overnight, recovered by centrifugation, and resuspended in TE buffer. RNase A was then added (200 mg/l) and treated for 30 min, and then electrophoresed on a 1.2% agarose gel.

**Western blot analysis**

Cells (2 x 10<sup>6</sup>) were washed twice with ice-cold PBS and lysed for 30 min at 4°C; debris was then removed by centrifugation and equivalent amounts of protein were separated by 10% SDS–PAGE and transferred onto nitrocellulose filter. The filters were first stained to confirm uniform transfer of all samples and then incubated in blocking solution for 2 h at room temperature. The filters were reacted first with the following antibodies: anti-PPAR-γ, anti-survivin, anti-bcl and anti-bax at a dilution of 1:1000 for 2 h, followed by two extensive washes with PBS twice and Tris-buffered saline, pH 8.0, with 0.1% Tween (TBST). Filters were then incubated with horseradish peroxidase-conjugated secondary antibodies of 1:1000 for 1 h, washed with TBST and developed using the Super Signal West Pico Kit (Super Signal West Pico, Pierce, Rockford, IL, USA). As an internal control, β-actin was detected with anti-β-actin antibodies.

**Table 1. PCR primers used in this study**

<table>
<thead>
<tr>
<th>PPAR-γ mRNA (474 bp)</th>
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<tr>
<td>Sense primer: 5'-TCTCCTCAAGATGACAGTGCTG-3'</td>
</tr>
<tr>
<td>Anti-sense primer: 5'-GCATTATGAGACATCCCA-3'</td>
</tr>
<tr>
<td>β-actin mRNA (243 bp)</td>
</tr>
<tr>
<td>Sense primer: 5'-TTCTCTCAAGGACCCG-3'</td>
</tr>
<tr>
<td>Anti-sense primer: 5'-ACACCCGAGGTCGAG-3'</td>
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**Figure 1. Peroxisome proliferator activated receptor-γ (PPAR-γ) expression in HL-60 and K562 cells detected by reverse transcription (RT)–PCR and western blot: (A) PPAR-γ mRNA expression. Lane 1, HL-60 cells; lane 2, K562 cells; lanes 3 and 4, β-actin; lane 5, marker; (B) western blot analysis of PPAR-γ expression in HL-60 and K562 cells.**
Results

PPAR-γ expression in the two cell lines

In the two myeloid leukemia cell lines, PPAR-γ expression was detected by RT–PCR and western blot. As shown in Figure 1A (agarose), the fragment of PPAR-γ mRNA was 474 bp in length, and the internal control, β-actin mRNA, was detected (length 243 bp). As shown in Figure 1B, PPAR-γ protein expression was observed very clearly in the two kinds of cells using western blot analysis (molecular mass of PPAR-γ protein was ~55 kDa).

Cell viability and FCM analysis

As shown in Figure 2, both TGZ and 15d-PGJ2 had significant growth inhibition effects on the two kinds of cells in a dose-dependent manner. The two kinds of leukemia cells showed different sensitivity to TGZ and 15d-PGJ2, both K562 and HL-60 cells were more sensitive to 15d-PGJ2 than to TGZ. To show whether the cell growth inhibition induced by 15d-PGJ2 and TGZ was caused by induction of apoptosis, the cells were stained with PI and analysed by FCM. As shown in Figure 3, along with the enhancement of cell growth inhibition, apoptotic cells gradually increased, and the percentage of sub-G1 cells of both K562 and HL-60 cells was >50% when treated with 40 μmol/l 15d-PGJ2 for 72 h.

Expression of apoptosis related genes

The mRNA expressions of both survivin and bcl-2 were downregulated while mRNA expression of bax was upregulated. The protein levels of survivin and bcl-2 were downregulated and bax protein expression was upregulated concurrently, as examined by western blotting when apoptosis occurred (Figure 4).
Hoechst 33258 and Wright’s staining, and DNA fragmentation analysis

Marked morphological changes of cell apoptosis such as condensation of chromatin and nuclear fragmentations were seen clearly using Hoechst 33258 staining (Figure 5). Cell apoptosis was also observed using Wright’s staining (Figure 6); there was no cell differentiation or maturation in cell morphology during the whole culture period as observed using Wright’s staining. Cell apoptosis was confirmed using DNA fragmentation analysis. Incubation of K562 and HL-60 cells with both 40 \( \mu \text{mol/l} \) 15d-PGJ2 and 80 \( \mu \text{mol/l} \) TGZ for 72 h...
elicited a characteristic ‘ladder’ of DNA fragments representing integer multiples of the internucleosomal DNA length (∼180–200 bp) (Figure 7).

Discussion

In this study, we found that the PPAR-γ ligands 15d-PGJ2 and TGZ could induce apoptosis in myeloid leukemia K562 and HL-60 cells. RT–PCR and western blot analysis revealed that both survivin and bcl-2 expressions were downregulated markedly, while bax expression was concurrently upregulated. We concluded that 15d-PGJ2 and TGZ have significant apoptosis effects on myeloid leukemia cells in vitro. The upregulation of bax and downregulation of survivin and bcl-2 expression may be important apoptotic-inducing mechanisms. To our knowledge, this is the first report to clarify the mechanisms of PPAR-γ ligand-induced apoptosis on HL-60 and K562 cells.

The regulation of apoptotic cell death has a profound effect on the pathogenesis and progression of cancer cells, and antia apoptotic signals are important in the development and prognosis of hematological malignancies [2, 3]. Survivin, one member of inhibitor of apoptosis family, has recently been reported to play an important role in both cell proliferation and cell death [4]. Downregulation of survivin expression may cause a cell-cycle defect that leads to programmed cell death [4]. bcl-2 and bax are two members of the bcl-2 family, which play different roles in programmed cell death [5]. When bax was overexpressed in cells, apoptotic death in response to death signals was accelerated, leading to its designation as a death agonist [6], and when bcl-2 was overexpressed it heterodimerized with bax and death was repressed. Therefore, the ratio of bcl-2 to bax is important in determining susceptibility to apoptosis [5].

In summary, the results of this study suggest that PPAR-γ ligands have significant apoptosis effects on K562 and HL-60 cells in vitro. Upregulation of bax and downregulation of survivin and bcl-2 expression may be the important apoptotic-inducing mechanisms.

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References