Pioglitazone inhibits the growth of human leukemia cell lines and primary leukemia cells while sparing normal hematopoietic stem cells

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Abstract. Peroxisome proliferator-activated receptors (PPARs) compose a subfamily of nuclear hormone receptors functioning as transcriptional regulators. Originally, the PPARγ ligand known as thiazolidinedione (TZD) was used for the treatment of diabetic patients. However, recent studies have shown that TZD also has an antitumor effect that inhibits cell growth in several types of human malignant neoplasms, including leukemia cell lines. Since pioglitazone is the only TZD currently available in clinics in Japan and the role of TZD in normal human hematopoietic cells or primary leukemia cells has not been previously reported, we investigated the effect of pioglitazone on human normal hematopoietic progenitor cells, primary leukemia cells, and leukemia cell lines (HL60, K562, U937, HEL, CEM, Jurkat, and NALM1). Pioglitazone inhibited the proliferation of leukemia cells in a dose-dependent manner. The viable cell numbers of HL60, K562, and Jurkat leukemia cell lines were profoundly reduced when the cells were cocultured with pioglitazone. Colony formation in the leukemia cell lines as well as the primary leukemia cells was significantly inhibited to 20-71% and 1-25% of that in control cultures by the addition of 100 and 300 μM of pioglitazone, respectively. However, the CFU-E and CFU-GM colonies of cells obtained from healthy volunteers were not altered in the presence of 100 μM of pioglitazone. Pioglitazone (300 μM) induced slight decrease of CFU-E and CFU-GM. BFU-E was more sensitive to pioglitazone than CFU-E and CFU-GM. Pioglitazone-induced growth inhibition in HL60 cells was associated with cell cycle arrest at the G1 phase, as has been reported for another TZD, troglitazone. Similar levels of PPARγ protein were observed in both leukemia and normal bone marrow cells by Western blotting, suggesting that the expression of PPARγ protein was not associated with the inhibitory potency of pioglitazone. In conclusion, our results suggest that pioglitazone may offer a new therapeutic approach to aid in the treatment of leukemia.

Introduction

Many leukemia patients achieve complete remission with current treatment protocols, while some of the patients eventually relapse and have shortened survival periods. Therefore, the development of effective and novel therapies for patients with refractory or relapsed leukemia is needed to improve patient outcome.

Peroxisome proliferator-activated receptors (PPARs) belong to a subfamily of nuclear hormone receptors that regulate the transcription of target genes through activation by PPAR ligands. To date, three subtypes of PPARs have been identified; termed α, β, and γ, each subtype is encoded by a separate gene and has a distinct tissue distribution pattern (1). PPARγ was initially noted to be highly expressed in adipose tissue and to have a regulatory function in adipocyte differentiation, insulin sensitization and lipid metabolism (2,3). However, a recent study demonstrated that PPARγ is expressed in a variety of cancer cells and has crucial roles in the suppression of cell growth (4). Thiazolidinedione (TZD) is a high-affinity ligand for PPARγ (5). Troglitazone and pioglitazone are two TZD derivatives that share a common thiazolidine-2,4-dione structure, which is responsible for the majority of the pharmacological actions of these compounds. Although these TZDs are able to normalize elevated plasma glucose levels in obese, diabetic rodents (6), data suggest that TZDs also have antitumor effects against liposarcomas (7,8), glioblastomas (9), colon cancers (10,11), pancreatic cancers (12-15), breast cancers (16,17), prostate cancers (18,19), esophageal cancers (20,21), gastric cancers (22,23), lung cancers (24,25), oral cancers (26), salivary gland cancers (27), liver cancers (28) and thyroid cancers (29).

Fujimura et al reported the cytotoxic effect of troglitazone on leukemia cell lines (30), and Asou et al reported that troglitazone was a moderately potent inhibitor of the clonogenic growth of acute myeloid leukemia cell lines when...
combined with a retinoid (31). However, troglitazone has been withdrawn from clinical use because it causes liver toxicity. Ciglitazone, another TZD, was never used clinically because of several adverse-effects. Therefore, pioglitazone is the only TZD currently in clinical use in Japan for the treatment of type II diabetes without major side-effects. These observations led us to perform a preclinical study of pioglitazone as a novel therapeutic agent for leukemia. Zang et al reported that pioglitazone induced growth inhibition and apoptosis of human B lymphocytic leukemia cell lines (32). However, the effect of pioglitazone upon human myeloid leukemia cell lines, primary leukemia cells, and normal hematopoietic stem cells is unknown. In the present study, we investigated the effect of pioglitazone on various leukemia cell lines and primary leukemia cells as well as on normal hematopoietic stem cells in vitro.

Materials and methods

Reagents. RPMI-1640 medium, fetal calf serum (FCS), and phytohemagglutinin (PHA) were obtained from Sigma Aldrich Co. (St. Louis, MO, USA); anti-human PPARγ antibody (sc-7196, rabbit IgG) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Pioglitazone was kindly provided by Takeda Chemical Industries (Tokyo, Japan) and was dissolved with 100% dimethyl sulfoxide (DMSO) to a concentration of 400 mM, then stored at 4˚C in the dark until use. The stock solution was further diluted with RPMI-1640 medium before use. Control cultures received the same amount of DMSO, which was always <0.1% (v/v) of the culture medium. This concentration of DMSO had no effect on the proliferation of these cells. All other reagents were purchased from Nacalai Tesque Co. (Kyoto, Japan).

Cells. HL60 (myelocytic leukemia), K562 (erythroid blast crisis of chronic myelocytic leukemia), U937 (monocytic leukemia), HEL (erythroleukemia), CEM (T-lymphoblastic leukemia), Jurkat (T-lymphoblastic leukemia), and NALM1 (B-lymphoblastic leukemia) cells were grown in RPMI-1640 medium with 10% FCS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. These cells were incubated at 37˚C in a humidified 5% CO2 atmosphere of 3% CO2 in air. Microscopic colonies were counted seven days later. All experiments were performed at least three times.

Leukemia blast colony assay. Heparinized peripheral blood was obtained from four patients with acute myeloid leukemia (AML). The morphology of the leukemia cells was classified according to the criteria of the French-American-British (FAB) committee. The subtypes and the number of cases were as follows: M3 (n=1), M4 (n=1), and leukemic transformation from myelodysplastic syndrome (MDS; n=2). Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation through a Ficoll-Conray (meglumine iotalamate; Mallinckrodt, St. Louis, MO, USA) density gradient (density, 1.077 g/dl). Subsequently, a specific additional T-lymphocyte depletion of the PBMC fraction was performed by immunomagnetic separation using anti-CD3 Dynabeads (Dynal, Hamburg, Germany). PBMCs were incubated in a culture flask with the beads at a target cell ratio of 20:1. Then, the flask was placed twice onto a 'Magnetic Particle Concentrator (MPC)'. The CD3+ T-lymphocytes rosetted by the beads were attached to the wall of the flask by the MPC, while the CD3-negative supernatant was collected for the assay. A total of 5x10^6 blast cells were plated in 35-mm dishes containing 1 ml of RPMI-1640 medium supplemented with 10% FCS, 1.7% methylcellulose, and either 100 or 300 μM of pioglitazone at the final concentration. As a colony stimulating factor (CSF), 10% PHA-stimulated leukocyte-conditioned medium (PHA-LCM) obtained by culturing normal leukocytes with PHA in a serum-free culture for seven days was used. Triplicate cultures were incubated at 37˚C in a humidified atmosphere of 5% CO2 in air. Microscopic colonies were counted seven days later. The mean ± SD was calculated for samples from four AML patients.

Assay for CFU-GM, CFU-E, and BFU-E. To examine whether pioglitazone affects the proliferation of normal hematopoietic progenitor cells, CFU-GM, CFU-E, and BFU-E were assayed with either 100 or 300 μM of pioglitazone using a MethoCult GF H4434V (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Briefly, 10⁶ bone marrow mononuclear cells (BMMNCs) were plated in 35-mm plates with 1.5 ml of MethoCult GF H4434V methylcellulose medium and an appropriate concentration of pioglitazone per plate; experiments were performed in triplicate. The plates were cultured for 12-14 days in a humidified incubator at 37˚C and 5% CO2. The colonies (>50 cells) were counted using a dissecting microscope.

Western blotting. The cells were collected and lysed in 0.1 ml of solubilizing buffer containing 0.5% NP-40, 10 mM Tris-HCl, 150 mM NaCl, 1 mM PMSF (Calbiochem, La Jolla, CA, USA), 0.111 U/ml aprotinin (Sigma) and 0.02% NaN3 in isopropanol. The cell lysates were mixed with sample buffer (0.0625 M Tris-base, 10% glycerol, 100 mM DTT and 2.3% SDS), and then heated in a boiling water bath for 5 min to fully denature the proteins prior to electrophoresis. Equal
amounts of protein (10 mg/lane) were separated on a 10% SDS gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) by electroelution with a constant current of 200 mA for 90 min at room temperature. After blocking with 0.05% Tween-20 in PBS (T-PBS) containing 10% FCS and 5% skim-milk overnight, the membrane was incubated with anti-human PPARα antibody (1 μg/ml) for 1 h at room temperature. The membrane was then washed with T-PBS three times and incubated with horseradish peroxidase-conjugated secondary antibodies (Lake Placid, NY, and Amersham, UK) for 1 h at room temperature. After washing six times with T-PBS, the membrane was treated with Enhanced Chemiluminescence (ECL) solution (Amersham, RPN 2106) for 1 min according to the manufacturer’s instructions, and exposed to Fuji medical X-ray film (AIF new RX) for 1 min.

Cell cycle analysis. After a quantity of 2x10⁵, HL60 cells were exposed to 100 μM pioglitazone or control medium for 48 h in liquid culture, the total cells were collected and washed with PBS. The cells were resuspended in 500 μl of a hypotonic solution containing 50 μg/ml propidium iodide (PI) in 0.1% sodium citrate plus 0.2% Triton X-100. After incubation for 4 h at 4˚C in the dark, nuclei were acquired using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data for 10,000 events were saved as list-mode files and analyzed with CellFIT software (Becton-Dickinson). Doublets were excluded on an FL2 area/FL2 wide (FL2-A/FL2-W) dot plot. G0/G1, S, and G2/M phases of the cell cycle were analyzed using an FL2-A histogram.

Statistical analysis. The data obtained in these experiments were evaluated using a Student’s t-test. A p<0.05 was considered statistically significant.

Results

Effect of pioglitazone on the cell growth of human leukemia cells. Cloned leukemia cells, HL60, K562, and Jurkat, were cultured in the presence of various concentrations of pioglitazone and the number of variable cells was counted on days 3, 5, and 7. The growth of HL60, K562, and Jurkat cells was significantly inhibited in a dose-dependent manner. Coculture with 200 μM of pioglitazone for seven days completely inhibited the growth of HL60 and Jurkat cells (Figs. 1 and 2). Pioglitazone (300 μM) induced the complete inhibition of the proliferation in K562 cells (Fig. 3). HL60, K562, U937, HEL, CEM, and NALM1 were then incubated in a semi-solid culture in the presence of either 100 or 300 μM of pioglitazone. In semi-solid cultures, pioglitazone also inhibited the in vitro growth of all human leukemia cell lines tested in a dose-dependent manner. In the presence of 100 μM of pioglitazone, colony formation significantly decreased to 20.8-71.8% of that seen in control cultures (p<0.01). In addition, 300 μM of pioglitazone strongly inhibited the growth of clonogenic cells to 1.0-25.2% of that seen in control cultures (p<0.01) (Fig. 4). When primary leukemia cells from AML patients were incubated using a similar method, colony formation was significantly inhibited. The number of leukemia cell colonies in the presence of 100 or 300 μM of pioglitazone was reduced to 54.9±14.8% and 23.1±11.6% of that seen in control cultures, respectively (p<0.01) (Fig. 5).
Effect of pioglitazone on CFU-GM, CFU-E, and BFU-E colony formation. Fig. 6 shows the growth of BFU-E, CFU-E, and CFU-GM incubated with pioglitazone. In the control cultures, the absolute number of CFU-GM, CFU-E, and BFU-E colonies was 118±61, 35±13, and 46±16 per 10^5 cells, respectively. Both CFU-GM and CFU-E showed no significant decrease in colony formation when cultured in the presence of 100 μM of pioglitazone. In the presence of 100 μM of pioglitazone, 86.9±21.8% of CFU-E and 76.6±14.7% of CFU-GM colonies survived. Pioglitazone (300 μM) slightly decreased CFU-E and CFU-GM colonies to 69.8±30.2% (p<0.05) and 81.5±18.2% (p<0.01) of control, respectively. BFU-E was more sensitive to pioglitazone than CFU-E and CFU-GM. Overall, 60.7±22.4% (p<0.01) and 33.3±18.4% (p<0.01) of BFU-E colonies survived in the presence of 100 and 300 μM of pioglitazone, respectively.

Assay for the expression of PPARγ protein. The presence of an ~48-kDa band, conforming to the expected band for the PPARγ protein, was observed in all six leukemia cell lines, in a monitor of primary leukemia cells, and in human normal bone marrow mononuclear cells. The amount of PPARγ protein was similar in all cells (Fig. 7).

Cell cycle analysis. Based on the growth inhibitory effect of pioglitazone observed in HL60 cells, we further examined the effect of pioglitazone on cell cycle progression. After 48 h of culture in the presence of 100 μM of pioglitazone, the percentages of G1, S, and G2/M phase cells were 52.9±10.5%, 40.5±10.5%, and 6.7±2.1%, respectively, whereas the distribution in control cultures was 42.5±12.8%, 45.3±4.7%, and 12.3±3.9%. The decrease in G2/M phase cells and the increase in G1 phase cells in the pioglitazone-treated group, compared with that in the control group, suggested that pioglitazone...
induced cell cycle arrest at the G1 phase in HL60 cells. A representative cell cycle analysis is shown in Fig. 8.

Discussion

TZD has been used as an antidiabetic agent to improve hyperglycemia and hyperlipidemia in patients with type II diabetes mellitus. However, recent studies have shown that TZD not only induces adipocyte differentiation and increase insulin sensitivity, but also exerts growth inhibitory effects on several carcinoma cell lines, both in vitro and in vivo. Three TZDs have been widely studied: troglitazone, ciglitazone, and pioglitazone. The clinical use of troglitazone has been discontinued because it causes fatal hepatic injury. Ciglitazone was never used clinically because of several adverse-effects. Therefore, pioglitazone is the only clinically available TZD in Japan. Pioglitazone (AD-4833)-HCl is freely soluble in DMSO, soluble in methanol, slightly soluble in dehydrated ethanol, very slightly soluble in water, and practically insoluble in ether. In this study, we investigated the cytotoxic effect of pioglitazone on the growth of leukemia and normal hematopoietic progenitor cells. As far as we know, this is the first report to describe the effect of pioglitazone on normal hematopoietic progenitor cells and primary leukemia cells. Pioglitazone inhibited the growth of all leukemia cell lines in a dose-dependent manner. In the presence of 100 or 300 μM of pioglitazone, the proliferation of all the leukemia cell lines that were tested and of the primary leukemia cells was significantly inhibited, while the antiproliferative effect was negligible in normal CFU-E and CFU-GM cells cultured under the same conditions. These findings suggest that pioglitazone may be a useful chemotherapeutic agent for the treatment of leukemia.

Figure 6. Effect of pioglitazone on CFU-GM, CFU-E, and BFU-E colony formation in normal bone marrow cells. CFU-GM, CFU-E, and BFU-E were assayed in the presence of 100 or 300 μM of pioglitazone. The results were expressed as a percentage of the control. Each bar represents the mean ± SD from triplicate experiments.

Figure 7. Expression of the PPARγ protein. Upper lane 1, K562 (chronic myelocytic leukemia, erythroid blast crisis); upper lane 2, HL60 (myelocytic leukemia); upper lane 3, U937 (monocytic leukemia); upper lane 4, CEM (T-lymphoblastic leukemia); upper lane 5, HEL (erythroleukemia); upper lane 6, NALM1 (B-lymphoblastic leukemia); lower lane 1, bone marrow mononuclear cells from healthy donor; lower lane 2, bone marrow blast cells from a patient with acute myelocytic leukemia; lower lane 3, HL60. Arrows indicate the ~48-kDa band, agreeing with the expected band for PPARγ.
The treatment of HL60 cells with pioglitazone resulted in cell cycle arrest, as was reported for troglitazone (30). Many previous studies have demonstrated that the activation of PPARγ induces growth inhibition associated with G1 arrest in PPARγ-expressing neoplasms (10-13,15,16,18,20-23,28,31-33). Alterations in the key regulators of the G1/S transition, such as cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (p21/WAF1 and p27/KIP1), may be responsible for the PPARγ ligand-induced cell cycle arrest in a variety of tumor cells. The down-regulation of cyclin D1, CDK2, and CDK4 in human lymphoblastic leukemia cells (32) and other cancer cells (24,34-36) was identified when these cells were exposed to TZDs. In addition, troglitazone has been reported to induce p21/WAF1 in myeloid leukemia cell lines (37) and p27/KIP1 in pancreatic carcinoma cells (12). However, these observations are controversial (4). Further studies are needed to clarify the mechanism by which pioglitazone inhibits cell growth in leukemia cells.

We also studied the expression of PPARγ protein. All cell lines, blasts from AML patients and normal bone marrow cells expressed a similar amount of PPARγ protein, as demonstrated by Western blotting, suggesting that the effect of pioglitazone seems to be independent of the PPARγ protein. Previously, a number of publications suggested that the effects of TZDs were independent of PPARs (31,38,39). First, TZDs inhibited the growth of PPARγ−/− embryonic stem cells in mice (40). Furthermore, the inhibitory effects of troglitazone on cellular proliferation were also observed in keratinocytes not expressing PPARγ (36). Secondly, another PPARγ ligand, GW7845 (41), has no anti-proliferative effects on leukemia cells (32). Thirdly, several PPARγ antagonists could not reverse the cytotoxicity of pioglitazone (32). Finally, to exert an anti-proliferative activity, TZDs must be used at much higher concentrations than those required to activate PPARs (32). Further studies are needed to clarify the mechanism by which pioglitazone inhibits cell growth in leukemia cells.

In conclusion, we have presented evidence that the PPARγ protein is expressed in leukemia as well as normal bone marrow cells and that pioglitazone inhibits the growth of leukemia cells in vitro. Further, pioglitazone is more cytotoxic to leukemia cells than to normal hematopoietic progenitor cells, suggesting that pioglitazone should be considered as a novel therapeutic agent for the management of leukemia patients. Future studies should clarify the mechanisms of pioglitazone's antileukemia effect.

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References


