Inhibition of human chondrosarcoma cell growth via apoptosis by peroxisome proliferator-activated receptor-γ

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A rare immunohistochemical study using 28 surgical sections of human chondrosarcoma revealed that 67.9% of tumour cells had weak (10-40%) or strong (>40%) positive immunoreaction for peroxisome proliferator-activated receptor-γ. The expression of peroxisome proliferator-activated receptor-γ mRNA and protein in human chondrosarcoma cell line OUMS-27 was also determined by reverse transcription-polymerase chain reaction and immunocytochemistry, respectively. Furthermore, the effects of peroxisome proliferator-activated receptor-γ ligands on cell proliferation and survival were investigated in OUMS-27 cells. Pioglitazone, a selective ligand for peroxisome proliferator-activated receptor-γ, and 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), a putative endogenous ligand for peroxisome proliferator-activated receptor-γ, inhibited the proliferation of OUMS-27 cells in a dose-dependent manner. The mechanism of cytotoxic effects of 15d-PGJ2 was via apoptosis as shown by DNA fragmentation using TUNEL stain and DNA ladder formation, and by ultrastructural analysis using transmission electron microscopy. Flow-cytometric analysis using annexin-V-fluorescein and propidium iodide detected the early change of apoptosis, as well as necrosis of OUMS-27 cells at 4 h after co-incubation with 15d-PGJ2. These results suggest that peroxisome proliferator-activated receptor-γ may play a significant role in the pathogenesis of chondrosarcoma, and peroxisome proliferator-activated receptor-γ ligands, especially 15d-PGJ2, may be of therapeutic value in the treatment of human chondrosarcoma.

Keywords: chondrosarcoma; apoptosis; PPARγ; 15d-PGJ2

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors. To date, three mammalian PPAR subtypes have been isolated, and termed PPARα, PPARβ and PPARγ, which heterodimerise with the retinoid X receptors (Kliwer et al., 1992). PPARα is predominantly expressed in the liver, and studies have focused on its role in lipid metabolism (Latruffe and Vamecq, 2000). PPARγ is ubiquitously expressed, but its function is not well understood. Although PPARγ was first found to be expressed at high levels in adipose tissue, and functions as a critical regulator of adipocyte differentiation and fat metabolism (Chawla et al., 1994), the expression of PPARγ is not restricted to the cells of the adipocytic lineage, and has been demonstrated in many other human cell types including chondrocytes (Bordj et al., 2000).

Induction of differentiation and apoptosis in cancer cells by ligands of PPARγ is a novel therapeutic approach against malignant tumours. Recent studies reported that 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), the most potent endogenous ligand for PPARα, and the synthetic PPARγ ligand thiazolidinedione (TZD) can induce terminal differentiation and growth inhibition of human liposarcoma (Tontonoz et al., 1997), breast (Elstner et al., 1998; Yee et al., 1999), prostate (Kubota et al., 1998), colon (Sarraf et al., 1998; Kitamura et al., 1999), gastric (Takahashi et al., 1999; Sato et al., 2000), lung (Chang and Szabo, 2000; Tsubouchi et al., 2000), and pancreas (Motomura et al., 2000; Ellemr et al., 2000) cancer cells, as well as hepatoma cells (Koga et al., 2001) in vitro and in vivo. The in vivo induction of histological and biochemical differentiation of liposarcomas by clinical trial of troglitazone administration was reported by Demetri et al. (1999). Among these patients the immunohistochemical expression of Ki-67, a marker of cell proliferation was markedly reduced. The authors of a similar in vivo trial have suggested the clinical efficacy of troglitazone in prostate cancer (Mueller et al., 2000). Although the precise mechanism is still unknown, the interference in tumour cell growth by PPARγ ligands may have a favourable clinical effect.

Chondrosarcoma is a relatively rare malignant bone tumour accounting for only 3 – 6% of primary bone tumours (Mirra, 1989; Schajowics, 1994; Unni, 1996). The histologic grade of chondrosarcoma which indicates the differentiation status of tumour cells, is one of the most important factors for the prognosis of the disease (Evans et al., 1977). The surgical treatment of chondrosarcoma with adequate marginal resection is effective for low-grade chondrosarcoma with better clinical outcomes (Ozaki et al., 1996). However, for high-grade chondrosarcoma, the prognosis is poor even after adequate surgery (Sheth et al., 1996), the efficacy of systemic chemotherapy or irradiation is still unclear. Thus, the development of clinically useful agents which inhibit cell growth and induce cell death may be useful in the treatment of chondrosarcoma.

In the current study, the first of its kind, the authors examined the expression of PPARγ both in surgically resected sections of human chondrosarcoma and OUMS-27 cells (Kunisada et al., 1998), a novel cell line established from a grade III human chondrosarcoma of a 65-year-old male. The examination of the effects of PPARγ ligands on OUMS-27 cells demonstrated that PPARγ ligands inhibited cell proliferation in a dose-dependent manner, and induced apoptosis of OUMS-27. The findings of the current...
study might contribute to the development of novel therapies for human chondrosarcoma.

MATERIALS AND METHODS

Immunohistochemistry on patient specimens and semiquantitative analysis

Immunohistochemistry was performed as previously described (Nishida et al., 2001) on formalin-fixed, paraffin-embedded tissues of human chondrosarcoma surgically resected from 28 patients. Briefly, sections were treated by polyclonal anti-human PPARγ antibody (Affinity BioReagents, Inc, Golden, CO, USA) diluted into 10 μg ml⁻¹ with PBS containing 0.1% NaN3 and 0.1% bovine serum albumin (British Biocell International Co, UK). After incubation for 30 min at room temperature with 7.5 μg ml⁻¹ of biotinylated goat anti-rabbit IgG (Vector Laboratories Inc, CA, USA), tissues were then treated by ABC method for 30 min, visualised with diaminobenzidine (DAB) (Sigma) in H2O2 (0.01%) for 5 min, and counterstained with methyl-green. Negative control was obtained in the same fashion but excluding the primary antibody. They were then examined under a light microscope.

Each specimen showed pathologically different grades of chondrosarcoma; grade I (n=20), grade II (n=6) and grade III (n=2). For the semiquantitative analysis of PPARγ positive tumour cells in the human tissue, the amount of the staining was evaluated in each of the three grades of chondrosarcoma.

Cells and cell culture

The human chondrosarcoma cell line OUMS-27 was a generous gift from Dr Masayosi Namba (Okayama University Medical School, Japan). OUMS-27 is a cell line established from grade III chondrosarcoma of a 65-year-old patient. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 450 mg dl⁻¹ glucose, supplemented with 10% foetal bovine serum (FBS) (Life Technologies, Rockville, MD, USA), 100 μg ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin (Life Technologies). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were sub-cultured at a split ratio of 1:3 every 7–10 days with 0.1% trypsin (Life Technologies), 5 mM EDTA solution. The medium was regularly changed twice a week.

RNA extraction and RT–PCR

Total cellular RNA was extracted from confluent cells using the RNA plus kit (Bioprobe System, Montreuil, France). For reverse transcriptase-polymerase chain reaction (RT–PCR), RNA samples (5 μg) were reverse-transcribed to cDNA using reverse transcriptase (RevertAid, Toyobo Co. Ltd., Osaka, Japan) and subsequently amplified by PCR using as a sense primer, 5’-TCTCTCGGATGGAAACC-3’ and an antisense primer, 5’-GCATTAGGACTCCCC-3’ as previously reported (Takahashi et al., 1999). Human adult normal adipose total RNA was purchased from BioChain Institute, Inc, CA, USA (Catalogue Number 061005). Samples were reverse-transcribed to cDNA and used for the positive control of PPARγ mRNA expression. The reaction conditions were as follows: initial denaturation at 94°C for 2 min and 30 cycles of amplification (30 s at 94°C, 30 s at 58°C, and 40 s at 72°C) in an automated thermal cycler (Perkin Elmer Applied Biosystems, Foster, CA, USA), followed by a final extension step of 4 min at 72°C. PCR products were run on 1% agarose gels.

Immunocytochemistry

The expression of PPARγ at the protein level was determined by immunocytochemistry. Briefly, cells were cultured at a density of 1 × 10⁵ cells per well in 6-well plastic culture dishes (Primaria, France), and fixed in 2% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) for 30 min at room temperature, washed in PBS. After permeabilisation in 0.1% Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan) for 5 min at room temperature, non-specific staining was blocked by 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA). Immunocytochemistry for PPARγ was performed in the same fashion as described above.

Reagents for activation of PPARγ

Pioglitazone, a selective ligand for PPARγ, was kindly provided from Takeda Chemical Industries (Osaka, Japan). 15d-PGJ2, a potent natural ligand for PPARγ, was purchased (from Biomol Research Laboratories, Inc, MI, USA). These PPARγ ligands were dissolved in dimethyl sulphoxide (DMSO) with a final concentration of 0.1% DMSO in the culture medium.

Cell proliferation and viability assay

Cells were seeded at 1 × 10⁴ cells per well in a 96-well culture plate or 8-well culture plate (Coster, Cambridge, MA, USA) in 100 ml 10% FCS-DMEM and incubated for 24 h at 27°C. For the activation of PPARγ, cells were treated with increasing concentrations of pioglitazone and 15d-PGJ2 for 24 h.

To know the effects of pioglitazone and 15d-PGJ2 on the proliferation grade of cell growth, an immunocytochemical study was performed in the same manner as described above on the cells cultured in 8-well culture plate, using monoclonal anti-Ki-67 antibody (Zymed Laboratories Inc, CA, USA) as a primary antibody and anti-mouse IgG (Vector Laboratories Inc, CA, USA) diluted to a final concentration of 10 μg ml⁻¹ as a secondary antibody. PBS without primary antibody was used as the negative control. Semi-quantitative analysis on the Ki-67-immunostained cells were performed by counting the positive cell number per total cells in four fields at a magnification of ×200 under light microscopy.

Cell viability in a 96-well culture plate was evaluated using the colorimetric MTT assay (Chemicon International, Inc, Temecula, CA, USA) according to the manufacturer’s instructions. Quantitation was then conducted using Model 550 microplate reader (BioRad, Hercules, CA, USA) at 595-655 nm. Results of optimal density units per 1 × 10⁶ adherent cells were expressed as % MTT.

DNA fragmentation analysis

Cells were cultured for 24 h in the presence or absence of 15d-PGJ2 (10 μg ml⁻¹) at a density of 1 × 10⁵ cells per well in 6-well plastic culture dishes. Cells were fixed in 2% paraformaldehyde in PBS (30 min at room temperature). Cells were then doubly stained by nick end-labelling method to detect the fragmented DNA, and Hoechst 33258 (Wako, Osaka, Japan) to observe the nuclear morphology of the cells. Briefly, cells were first incubated with TdT reaction solution (2 μg terminal deoxynucleotidyl transferase (TdT) and 5 μg FITC-conjugated deoxyuridine triphosphate (dUTP) in 1200 μl TdT buffer). The cells were then stained by Hoechst 33258 (10 μg ml⁻¹) for 5 min at room temperature. After thorough washing in PBS, cells were observed by fluorescent microscopy.

The Apoptotic DNA Ladder Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for DNA ladder assay according to the manufacturer’s instructions. Briefly, cells were cultured in serum-free DMEM at a density of 2.5 × 10⁴ cells in 75 mm² tissue culture flask (Greiner, Frickenhawen, Germany), stimulated with 15d-PGJ2 (10 μg ml⁻¹) for 24 h. Cells were homogenised in lysis buffer, and DNA was isolated. The samples were separated on a 2% agarose gel and stained with 10 μg ml⁻¹ ethidium bromide.

FACS analysis using annexin-V-FITC

The expression of phosphatidylserine on the outer leaflet of apoptotic cell membranes was examined by fluorescence-activated cell
Transmission electron microscopy

Ultrastructural appearances of apoptotic cells were confirmed by electron microscopic study. Cells were first incubated with or without 15d-PGJ$_2$ (10 μg ml$^{-1}$) for 4, 8 and 24 h, washed with PBS and centrifuged at 200 g for 5 min. Cell pellets were then re-suspended in 100 μl of 2% Annexin-V-fluorescein labelling reagent in Hepes buffer containing 2% propidium iodide (PI) and incubated for 15 min at 15 to 25°C. After washing extensively, stained cells were analysed using a FACScan (Becton Dickinson, San Jose, CA, USA) and 1000 events in the live gate were recorded (a 515 nm bandpass filter for fluorescein and a filter >600 nm for PI detection).

Expression of PPAR$_{gamma}$ in human chondrosarcoma and OUMS-27 cells

Immunohistochemical examination revealed that human chondrosarcoma cells frequently express PPAR$_{gamma}$ at protein level (Figure 1). In the nuclei of differentiated chondrocytes showed strong positive reaction for PPAR$_{gamma}$, but not in the well differentiated hypertrophic chondrocytes nor undifferentiated mesenchymal cells. The results of the semiquantitative analysis for PPAR$_{gamma}$-positive cells were summarised in Table 1. The positivity (the cutoff positivity of 10%) of chondrosarcoma cells were 65.0% in grade I, 83.3% in grade II, and 50.0% in grade III, and overall positivity was 67.9%. RT–PCR analysis was done to examine the expression of PPAR$_{gamma}$ mRNA in OUMS-27 cells. The predicted band size (474 bp) of PPAR$_{gamma}$ mRNA was clearly detected in OUMS-27 cells (Figure 2A). Immunocytochemistry using polyclonal anti-human PPAR$_{gamma}$ antibody showed the nuclear localisation of PPAR$_{gamma}$ in OUMS-27 cells. Negative controls treated with non-immune serum or without primary antibodies showed no positive reaction (Figure 2B a,b). These data indicated that PPAR$_{gamma}$ is frequently expressed both in primary chondrosarcomas as well as cancer cell line OUMS-27.

**Table 1** Summary of immunohistochemical study for PPAR$_{gamma}$ in human chondrosarcoma tissues

| Pathological grade of chondrosarcoma | (n=20) | (n=6) | (n=2) |
|-------------------------------------|--------|-------|-------|
| None                                | 7      | 1     | 1     |
| Weak                                | 8      | 2     | 1     |
| Strong                              | 5      | 3     | 0     |

Positive cell ratios labelled by polyclonal antibodies for PPAR$_{gamma}$ were expressed: None; <10%, Weak; 10–40%, Strong; >40% of cells positive.
Inhibition of OUMS-27 cell proliferation by PPAR\(\gamma\) ligands

After the treatment with two different kinds of PPAR\(\gamma\) ligands, the morphologic features of OUMS-27 cells were observed under phase contrast microscopy. Cells treated with vehicle only (0.1% DMSO) had a polygonal shape and adhered to the plastic surface. Cells treated with 15d-PGJ\(_2\) at 1 \(\mu\)g ml\(^{-1}\) appeared less active, but still had a polygonal appearance. When cells were treated with doses of 15d-PGJ\(_2\) at 5 \(\mu\)g ml\(^{-1}\) or higher, they showed relatively round shapes with cell shrinkage and apoptotic body-like structure and some cells could no longer adhere to the dish. The treatment with pioglitazone did not induce so much morphological change when compared with 15d-PGJ\(_2\) treatment (Figure 3A). The results of immunostain for Ki-67 and MTT assay indicated that both pioglitazone and 15d-PGJ\(_2\) for 24 h inhibited the cell growth and reduced cell viability in a dose-dependent manner (Figure 3B,C).

Interestingly, cell growth was markedly reduced when treated with 15d-PGJ\(_2\).

Induction of apoptosis of OUMS-27 cells by PPAR\(\gamma\) ligands

In order to confirm the apoptosis induced by PPAR\(\gamma\) activation, DNA fragmentation was analysed both biochemically and morphologically. Hoechst staining showed condensed DNA chromatin morphology in OUMS-27 cells treated by 15d-PGJ\(_2\) for 24 h. The appearance of these cells was consistent with that of the cells positive for TUNEL staining which indicate DNA fragmentation (Figure 4A). Treatment of OUMS-27 cells with 15d-PGJ\(_2\) (10 \(\mu\)g ml\(^{-1}\)) for 24 h induced DNA ladder formation (Figure 4B). Semi-thin sections of LR-White embedded cells stained by toluidine blue showed a large number of OUMS-27 cells treated with 15d-PGJ\(_2\) with characteristic apoptotic features; cell shrinkage and nuclear condensation (Figure 5A,B). TEM study revealed that untreated OUMS-27 cells have extensive rough-surfaced endoplasmic reticulum, thickened mitochondria and numerous cytoplasmic vesicles (Figure 5C). In contrast, when treated with 15d-PGJ\(_2\), the sections contained many apoptotic cells with condensed chromatin, many vacuoles in the cytoplasm and membrane budding (Figure 5D). FACS analysis indicated the population of apoptotic cells with PS at the outer membrane of the cells (annexin-V positive, PI positive).
DISCUSSION

There are few human chondrosarcoma cell lines with chondrocytic properties, OUMS-27 is a novel cell line established from grade III human chondrosarcoma by Kunisada et al (1998). OUMS-27 cells do not show contact inhibition after reaching confluence and grow rapidly in multiple layers. The cells express proteoglycan, as well as type I, II, III, IX and XI collagen after 120 passages, showing stable maintenance of the differentiated chondroctic properties. The transplantation of OUMS-27 cells into athymic mice resulted in formation of grade II (of III) chondrosarcoma at injected site (Kunisada et al, 1998). Thus, the OUMS-27 cell line appears to be a useful model for studies for chondrocyte function as well as the aetiology and treatment of human chondrosarcoma.

Recent studies strongly suggest the attractive concept that PPARγ ligands might be useful agents in the induction of cell differentiation or apoptosis in cancer cells. In the current study, the authors demonstrated for the first time that PPARγ is expressed in both surgically resected human chondrosarcoma and OUMS-27 cells. The frequent expression of PPARγ in chondrosarcoma cells indicated the involvement of PPARγ in the pathogenesis of chondrosarcoma and suggested the possible inhibitory effect on cancer cell growth and proliferation by PPARγ activation.

TUNEL staining, DNA ladder formation and TEM revealed that the type of cell death induced by 15d-PGJ2 was apoptosis. Interestingly, early apoptotic change, and the translocation of phosphatidylserine on the outer leaflet of the cell membrane were demonstrated only 4 h after co-incubation with 15d-PGJ2. To date, the exact intracellular signaling pathways leading to apoptotic cell death following specific activation of PPARγ is not fully understood, several factors have been suggested to be involved in PPARγ ligand-induced apoptosis. The activation and actin of caspases is believed to be a pivotal step in the mechanism of apoptosis. In JEG3 choriocarcinoma cells, 15d-PGJ2-induced apoptosis measured by a decline in MTT activity was significantly inhibited by the caspase inhibitor ZVAD-fmk, but the inhibitory effect was up to 50% (Keelan et al, 1999). Previous reports suggested the involvement of a caspase-3-independent mechanism in 15d-PGJ2-induced loss of MTT activity (Miyashita et al, 1998). We found that the inhibitors for caspase-1, 3, 8, 9, and ZVAD-fmk (2 μM) did not have significant effect on the 15d-PGJ2-induced loss of MTT activity in OUMS-27 (data not shown). It is speculated that...
the caspase-independent mechanism might be partly involved in the 15d-PGJ2-induced apoptosis in OUMS-27.

We also demonstrated that the ligands of PPARγ, both pioglitazone and 15d-PGJ2, inhibited cell growth and induced cell death in a dose dependent manner. The 15d-PGJ2 had more noticeable effects on OUMS-27 cell growth than pioglitazone. Thus, it is still unclear whether the effects of ligands on OUMS-27 cells were strictly due to PPARγ activation. The detailed analysis of the effects of ligands on the cells which do not express PPARγ should provide important clues to understand this phenomenon. Okura et al. (2000) demonstrated that troglitazone can induce vascular smooth muscle cell apoptosis via the tumour suppressor p53, but not by PPARγ activation. We have previously shown by functional analysis of separated alleles in yeast (FASAY) assay that the p53-gene status of OUMS-27 is a mutant-type (Kunisada et al., 1998). The mutant p53 is known to inhibit the tumour-suppressor activity of wild-type p53 protein (Levine, 1997). In this line, mutant status of p53 might be involved in the pathway of the inhibition of OUMS-27 cell growth by PPARγ ligands. Other studies indicated that inhibition of transcriptional activation by nuclear receptors can be effected by competition for limited amounts of co-activators such as cAMP response element binding protein (CREB)-binding protein (CBP)/p300, Src-1 or TIF1 (Kamei et al., 1998; Göttlicher et al., 1998; Sheppard et al., 1998). It may be speculated that the difference in the recruitment of co-activators might influence the effectiveness of each ligand.

It has been shown that PPARγ has regulatory functions in the cell cycle by altering cell growth (Motonou et al., 2000; Okura et al., 2000; Morrison and Farmer, 1999). Recently, Motomura et al. (2000) reported that activation of PPARγ by troglitazone inhibited cell growth and G1 arrest through the increase of cyclin-dependent kinase inhibitor p27Kip1 in human pancreatic carcinoma cells. They found that the effect of troglitazone on the proliferation of cancer cells was inhibited by antisense for p27Kip1. We have also found by immunohistochemistry that OUMS-27 cells express p27Kip1 at the protein level after incubation with 15d-PGJ2 (10 μg ml−1) for 4 h, but failed to show its expression by Western blot analysis (data not shown). Further cell cycle analysis on p27Kip1 is under investigation to elucidate the mechanism of PPARγ ligands-induced cell growth inhibition in OUMS-27 cells.

In conclusion, the current study showed that PPARγ activators induce apoptosis of human chondrosarcoma cells, suggesting that PPARγ activators might have potential therapeutic benefit in the treatment of chondrosarcoma. The signal transduction pathway for the induction of apoptosis is still unclear. Whether 15d-PGJ2 can also induce tumor cell death in experimentally transplanted chondrosarcoma models remains to be examined.

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