Activation of PPARγ inhibits cell growth and induces apoptosis in human gastric cancer cells

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Abstract **We investigated the expression of peroxisome proliferator-activated receptor γ (PPARγ) and the role of PPARγ in cell growth in human gastric cancer cells. Reverse transcription-polymerase chain reaction, Northern blot and Western blot analyses showed that a human gastric cancer cell line, MKN45, expressed PPARγ mRNA and protein. Luciferase assay in MKN45 cells showed that troglitazone, a selective ligand for PPARγ, transactivated the transcription of a peroxisome proliferator response element-driven promoter. Troglitazone or pioglitazone, selective ligands for PPARγ, inhibited the growth of MKN45 cells in a dose-dependent manner. Co-incubation of MKN45 cells with troglitazone induced DNA ladder formation. These results suggest that human gastric cancer cells express PPARγ and that activation of PPARγ inhibits cell growth and induces apoptosis in gastric cancer cells.**

Key words: Peroxisome proliferator-activated receptor γ; Growth inhibition; Apoptosis; Gastric cancer

1. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor, provides a strong link between lipid metabolism and the regulation of gene transcription [1,2]. Recent studies show that PPARγ is expressed at high levels in human colonic mucosa and colon cancer cells [3–5]. Ligand activation of PPARγ in human colon cancer cells causes a reduction of growth [3,4]. In contrast, two independent groups demonstrated that activation of PPARγ promoted the development of colon tumors in mice [6,7]. Thus the roles of PPARγ activation in the growth of colon tumors are controversial. In addition to colon cancer, PPARγ activation induced growth arrest in human liposarcoma, prostate cancer and breast cancer [8–10]. These results suggest that PPARγ activation may be implicated in the growth of malignant tumors.

It has been reported that PPARγ gene expression is observed in the heart, liver, small and large intestine, and kidney in addition to the adipose tissue [5,11]. Braissant et al. have shown, using in situ hybridization, that PPARγ is expressed in the rat gastric mucosa [12]. Little is known, however, about the pathophysiological relevance of PPARγ in the human gastric mucosa. In the present study, we investigated the expression of PPARγ and examined the effects of PPARγ activation on cellular growth in human gastric cancer cells.

2. Materials and methods

2.1. Cell culture

The human gastric cancer cell line MKN45 was obtained from the Japanese Cancer Research Resources Bank (Tsukuba, Japan). MKN45 is a cell line established from a poorly differentiated gastric adenocarcinoma metastasized to liver [13]. MKN45 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin, and 10% fetal bovine serum. Since the expression of PPARγ protein has already been reported in a human colon cancer cell line, HT-29 [3], we used this cell line as a positive control in experiments to detect PPARγ mRNA and protein expression. HT-29 was purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 450 mg/dl glucose and supplemented with 2 mM L-glutamine, MEM non-essential amino acid solution (Sigma-Aldrich, Co., Irvine, UK), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin, and 10% fetal bovine serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. Chemicals

Troglitazone and pioglitazone were kindly provided from Sankyo Pharmaceutical Co. (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan), respectively. These thiazolidinediones were dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 0.1% DMSO in the culture medium.

2.3. RNA extraction

Total RNA was extracted from cultured cells using a modified version of the acid guanidinium thiocyanate/phenol/chloroform method employing a single reagent (RNA-STAT 60, TelTest Inc., Friendswood, TX, USA) [14–16]. Samples were dissolved with diethyl pyrocarbonate-treated water (RNase-free). To remove contaminating genomic DNA, the RNA was treated with 10 μl of RQ1, RNase-free DNase (Promega, Madison, WI, USA), 0.5 μl of RNase inhibitor (Takara Shuzou Co., Otsu, Shiga, Japan) and 10 μl of 10×DNase buffer (400 mM Tris-HCl at pH 7.9, 100 mM NaCl, 60 mM $MgCl_2$ and 100 mM $CaCl_2$) in a final volume of 100 μl for 30 min at 37°C. RNA samples were purified by phenol-chloroform extraction and isopropanol precipitation. The resultant RNA samples were quantified using spectrophotometer at a wavelength of 260 nm. The integrity of the isolated RNA samples was analyzed electrophoretically on an agarose gel, followed by staining in ethidium bromide.

2.4. Reverse transcription PCR (RT-PCR)

An aliquot of 1 μg of total RNA from each sample was reverse transcribed to cDNA using a First-Strand cDNA Synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the manufacturer's instructions with oligo(dT) primer. For detection of the human PPARγ mRNA, a combination of a sense primer, 5′-TCTCTCCGTAATGGAAGACC-3′, and an antisense primer, 5′-GCATTATGAGACATCCCCAC-3′, was used as described previously [17]. For the detection of human β-actin mRNA, a combination of a sense primer, 5′-AGGCCAACCGCGAGAAGATGACC-3′, and an antisense primer, 5′GAAGTCCAGGGCGACGTAGCAC-3′, was used according to a previous publication [18]. The amplification was

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; PPRE, peroxisome proliferator response element

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carried out in a 100 μl mixture containing 1 μl of the above cDNA product (corresponding to cDNA synthesized from 67 ng of total RNA), 0.4 μM each of the sense and antisense primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 2.5 units of Taq DNA polymerase (Takara Shuzou Co., Otsu, Shiga, Japan). The reaction conditions were as follows: initial denaturation at 95°C for 2 min and 40 cycles of amplification (95°C for 40 s, 55°C for 50 s and 72°C for 50 s), followed by a final extension step of 7 min at 72°C. The PCR reaction products were separated electrophoretically in a 2% agarose gel and stained with ethidium bromide.

2.5. Northern analysis

Total RNA (10 μg) denatured in formamide and formaldehyde was electrophoresed through 1% formaldehyde-containing agarose gels as described previously [14]. After electrophoresis, the RNA was transferred to nylon membrane (Hybond N, Amersham International, Buckinghamshire, UK) by the capillary blotting and then fixed by UV cross-linker (FUNA-UV-LINKER, Funakoshi, Tokyo, Japan). Prehybridization was performed at 42°C for 2 h in 50% formamide, 25 mM sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), 5×SSC, 5× Denhardt's solution and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out at the same temperature for 20 h in the same solution with ^{32}P-labeled cDNA probes. Probe for PPARγ was amplified by PCR using MKN45 cell cDNA as template and sequenced. A β-actin cDNA probe (Wako Chemicals Industries, Osaka, Japan) was used as internal control. After washing the membrane under appropriately stringent conditions, the hybridization signals were analyzed with a bioimaging analyzer system (Fuji-BAS, Fuji Photo Film Co., Tokyo, Japan) or autoradiography using XAR film (Eastman Kodak, Rochester, NY).

2.6. Western blot analysis

Total proteins were extracted from MKN45 and HT-29 cells. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad Lab., Richmond, CA, USA) following the manufacturer's suggested procedure. 50 μg of protein was separated by 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Amersham Life Science Inc., Piscataway, NJ, USA), blocked overnight in TBS-Twin (TBS-T) with 10% skim milk at 4°C, subsequently reacted with primarily monoclonal antibody against human PPARγ (Santa Cruz Lab., Santa Cruz, CA) and washed. After reaction with horseradish peroxidase-conjugated anti-mouse IgG, immune complexes were visualized by using the ECL detection reagents (Amersham, Buckinghamshire, UK) following the manufacturer's suggested procedure. Simultaneously, mouse immunoglobulin G_1 kappa monoclonal immunoglobulin (Pharmingen, San Diego, CA, USA) was used for isotype control.

2.7. Transfections and luciferase assays

Transfections and luciferase assays were performed according to a previous report [19]. MKN45 cells were seeded at a concentration of 1×10^5 cells/35 mm dish and transfected with the plasmids 24 h after having been transferred to fresh media. Transfection was done using LipofectAMINE reagent (Gibco BRL) mixed with 2 μg of acyl CoA oxidase promoter-luciferase plasmid (kindly donated by Dr. Osumi) [20] and 0.2 μg of pRL-SV40 (Promega, Madison, WI, USA) for 3 h. The transfection mix was replaced by complete medium with or without 100 μM troglitazone and further incubated for 12 h. The cells were lysed with 1×luciferase lysis buffer (Toyo Ink, Tokyo, Japan). Luciferase activity was measured using the PicaGene reagent kit (Toyo Ink, Tokyo, Japan) in a luminometer (MiniLumat, Berthold, Wildbad, Germany). The enzyme activity was normalized for efficiency of transfection, on the basis of sea pansy luciferase activity, and relative values were determined. Transfection experiments were carried out two times independently, and the average values were calculated.

2.8. Cell counting

First, we examined the dose-response effects of thiazolidinediones on the growth of MKN45 cells. MKN45 cells were seeded at a density of 1×10^6 cells/25 cm^2 cell culture dish. After 24 h of culture, medium was changed with several doses (0, 0.1, 1, 10 or 100 μM) of troglitazone or pioglitazone containing culture medium and incubated for 48 h. After incubation with troglitazone or pioglitazone for 48 h, cells were collected and centrifuged. The supernatant was aspirated and cells were resolved with phosphate-buffer saline. The dead cells were excluded by trypan blue staining. Viable cells were counted using a hemocytometer. Next, the time-course change of cell number of MKN45 was evaluated. After incubation with or without 100 μM of troglitazone for 0, 12, 36 or 48 h, MKN45 cells were collected and centrifuged. Viable cells were counted similarly.

2.9. Detection of DNA fragmentation

Cells were exposed to several doses of troglitazone, washed in cold phosphate buffered saline, and then gently homogenized in extraction buffer (10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton X-100 at pH 7.5) [21]. The samples were incubated for 10 min on ice and then centrifuged at 13 000×g for 10 min. The supernatant containing fragmented DNA but not intact chromatin was extracted with phenol and chloroform precipitated with ethanol. The pellet was rinsed with 70% ethanol, air-dried and dissolved in 1×TE (10 mM Tris-HCl and 10 mM EDTA at pH 8.0). After digestion with 0.1 mg/ml RNase A at 37°C for 1 h, the DNA was fractionated in a 2% agarose gel and stained with 10 μg/ml ethidium bromide.

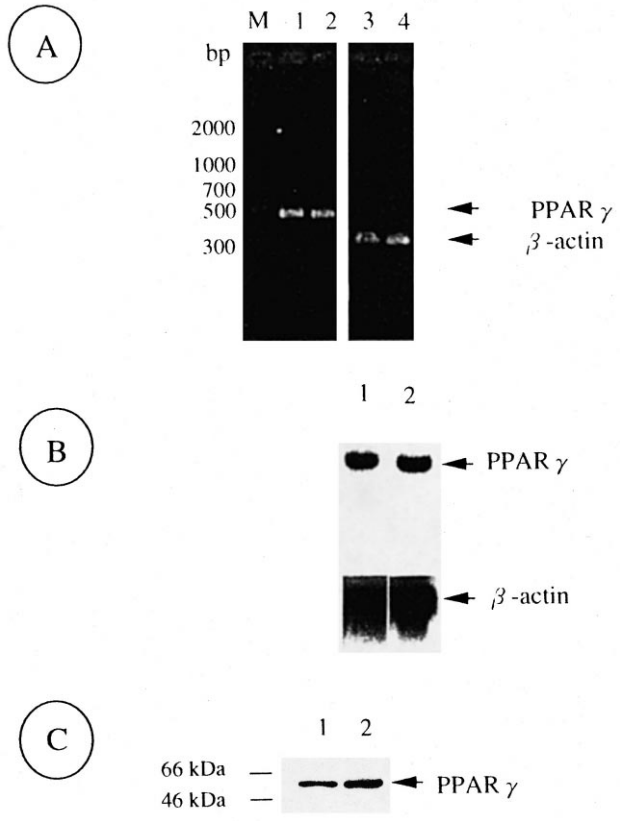

Fig. 1. PPARγ expression in a human gastric cancer cell line, MKN45. A: PPARγ is expressed at the mRNA level in HT-29 and MKN45 cells. Total RNA (1 μg) from either HT-29 or MKN45 cells was subjected to an RT reaction with reverse transcriptase. A 1 μl aliquot from each reaction was subjected to 40 cycles of PCR. Amplification products were electrophoresed in a 2% agarose gel. PPARγ expression (474 bp) is shown in lane 1 (HT-29) and lane 2 (MKN45). The β-actin control (343 bp) is shown in the right lanes (lanes 3 and 4). B: Northern blot for PPARγ in HT-29 (lane 1) and MKN45 (lane 2) cells. 10 μg of each total RNA obtained from HT-29 and MKN45 cells was electrophoresed on 2% agarose gel and transferred to nitrocellulose membranes. Hybridization was performed using a human PPARγ cDNA probe labeled with [^{32}P]dCTP. The β-actin control is shown in the bottom panel. C: PPARγ protein expression in HT-29 (lane 1) and MKN45 (lane 2) cells. 50 μg of each protein obtained from HT-29 and MKN45 cells was separated on SDS-PAGE and probed with an anti-PPARγ antibody.

2.10. Statistical analysis

The results are expressed as mean ± S.E.M. Statistical analysis was performed by one way analysis of variance and subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant.

3. Results

First, we examined whether PPARγ gene and protein are expressed in MKN45 cells. Fig. 1A illustrates the representative result of PPARγ gene expression by RT-PCR. PPARγ mRNA is expressed in both HT-29 and MKN45 cells. The identity of the amplicon was confirmed by DNA sequence analysis. As shown in Fig. 1B, PPARγ mRNA expression was clearly detected by Northern blot analysis in MKN45 cells as in HT-29. The amount of PPARγ mRNA in MKN45 cells was similar to that in HT-29. Fig. 1C demonstrates a representative result of Western blot analysis. PPARγ protein was expressed in both HT-29 and MKN45 cells. The amount of PPARγ protein in MKN45 cells was equal to or even higher than in HT-29 cells. In contrast, control IgG failed to detect a product at the estimated molecular weight (60 kDa).

We transfected MKN45 cells with an acyl-CoA oxidase promoter-luciferase reporter plasmid containing a PPRE. Luciferase activity in the MKN45 cells treated with troglitazone for 12 h was approximately four-fold that in untreated cells (Fig. 2).

Next, we examined the effect of PPARγ activation on cell growth in MKN45 cells that expresses PPARγ as shown in the above study. To activate PPARγ, we used two thiazolidinediones named troglitazone and pioglitazone. Fig. 3A illustrates the effects of troglitazone on the cell number of MKN45 cells 48 h later. Troglitazone inhibited the growth of MKN45 cells in a dose-dependent manner. The mean number of cells ($\times 10^6$) with each dose of troglitazone was 3.71 (vehicle only), 4.15 (troglitazone 0.1 μM), 3.47 (1 μM), 2.63 (10 μM) or 0.67 (100 μM). Statistical significance was obtained at 10 μM and higher doses of troglitazone. Fig. 3B shows the effect of another thiazolidinedione, pioglitazone, on cell growth in MKN45 cells. As with troglitazone, co-incubation of MKN45 cells with pioglitazone dose-dependently inhibited the growth of MKN45. A significant inhibition was seen with pioglitazone at concentrations of 1 μM and higher. Fig. 4 shows the time-course change of MKN45 cell number by troglitazone at a dose of 100 μM. Treatment with troglitazone resulted in a significant inhibition of cell growth even at 12 h and potent inhibition was observed throughout the time period we tested.

Finally, we evaluated whether or not apoptosis is involved in the inhibition of growth of MKN45 cells by troglitazone. To assess the development of apoptosis, we checked DNA fragmentation in MKN45 cells 48 h after application of tro-

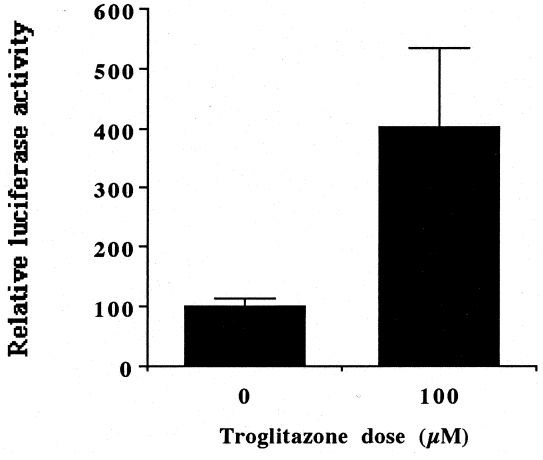

Fig. 2. PPRE transactivation in MKN45 cells. MKN45 cells were transfected with an acyl CoA promoter luciferase construct and pRL-SV40. After treatment with 100 μM troglitazone for 16 h, cells were harvested, and the dual luciferase assay was performed. Each value is expressed as mean ± S.E.M. ($n = 2$).

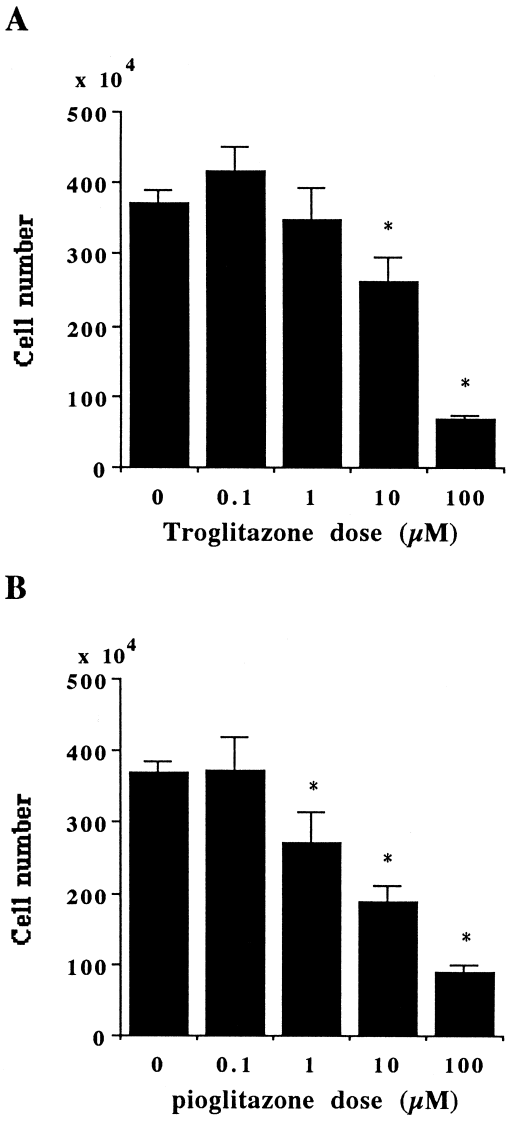

Fig. 3. A: Effect of troglitazone on cell numbers of MKN45. MKN45 cells were treated with either 0, 0.1, 1, 10 or 100 μM of troglitazone and cell numbers were determined at 48 h. Data are expressed as mean ± S.E.M. of five experiments. ANOVA detected a statistical significance ($F(4, 20) = 19.34$, $P < 0.001$). *$P < 0.01$, when compared with vehicle only (troglitazone dose 0). B: Effect of pioglitazone on cell numbers of MKN45. MKN45 cells were treated with either 0, 0.1, 1, 10 or 100 μM of pioglitazone and cell numbers were determined at 48 h. Data are expressed as mean ± S.E.M. of four or five experiments. ANOVA detected a statistical significance ($F(4, 18) = 19.50$, $P < 0.001$). *$P < 0.01$, when compared with vehicle only (pioglitazone dose 0).

glitazone. As demonstrated in Fig. 5, DNA fragmentation was not observed in MKN45 cells when vehicle only or troglitazone at concentrations of 0.1, 1 or 10 μM was co-incubated. In contrast, clear DNA ladder formation was observed in MKN45 cells when troglitazone had been applied at the highest concentration (100 μM).

4. Discussion

Increasing evidence suggests that PPARγ may be implicated in cell differentiation [22,23]. So far, PPARγ expression and effects of PPARγ agonists on cell differentiation have been investigated in colon cancer, breast cancer, prostate cancer and liposarcoma [3,4,8–10]. However, whether or not gastric cancer cells express PPARγ is not known. In addition, little is known about the action of PPARγ activation on cell behavior in gastric cancer cells. In the present study, we tried to clarify the above two questions in a human gastric cancer cell line, MKN45.

The present study has demonstrated that PPARγ gene and protein expression was clearly observed in MKN45 cells. Northern blot analysis revealed that PPARγ mRNA was expressed in the positive control HT-29, a human colon cancer cell line. According to a previous observation [3], HT-29 expresses PPARγ mRNA at a high level. When compared with HT-29, PPARγ mRNA expression in MKN45 was detected equally, suggesting that the gastric cancer cell line, MKN45, expresses PPARγ mRNA at relatively high levels. Western blot analysis also revealed equal or even higher PPARγ protein expression in MKN45 cells when compared with that in positive control HT-29 cells. These results indicate that the human gastric cancer cell line, MKN45, expresses PPARγ gene and product. To determine if the PPARγ in MKN45 cells is functional as a transcriptional activator, we performed transient transfection assays in MKN45 cells using a PPARγ-responsive element cloned upstream of luciferase. Treatment of the transfected cells with a PPARγ-selective agonist, troglitazone, resulted in an approximately four-fold increase in luciferase activity, strongly suggesting that PPARγ expressed in MKN45 is indeed functional as a transcriptional activator.

Since we confirmed that the high PPARγ expression in MKN45 cells is functionally relevant, we next examined the effect of activation of PPARγ on cell growth in these cells. Co-incubation of MKN45 cells with troglitazone potently inhibited cell growth in a dose-related fashion. The dose-dependent suppression of cell growth in MKN45 cells was also seen after another thiazolidinedione, pioglitazone. These results suggest that thiazolidinediones have a growth inhibitory action on MKN45 cells. Since thiazolidinediones are specific and high affinity agonists for PPARγ [24] and troglitazone indeed transactivated the transcription of a PPRE-driven promoter in the MKN45 cells as shown in the present study, we would suggest that PPARγ activation by thiazolidinediones may lead to growth inhibition in gastric cancer cells.

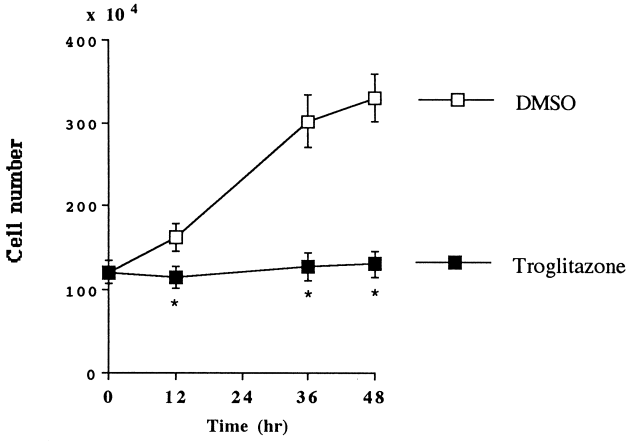

Fig. 4. Time-course change of cell number by troglitazone. MKN45 cells were treated with either vehicle only or 100 μM of troglitazone and cell numbers were determined at 0, 12, 36 or 48 h. Data are expressed as mean ± S.E.M. of six experiments. $*P < 0.01$, when compared with vehicle only (DMSO).

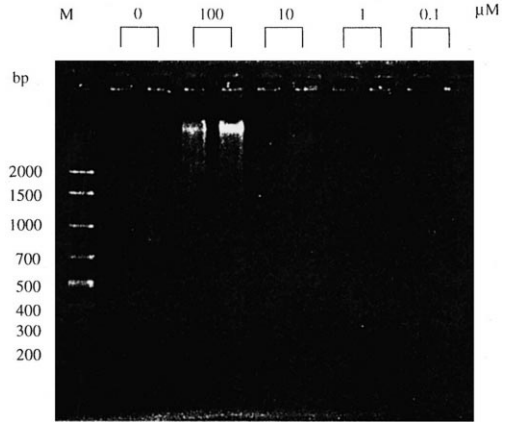

Fig. 5. DNA fragmentation induced by troglitazone in MKN45 cells. MKN45 cells were treated with troglitazone in a dose of 0, 0.1, 1.0, 10 or 100 μM, and 48 h later DNA solution obtained from MKN45 cells was separated on a 2% agarose gel and then stained with 10 μg/ml ethidium bromide. DNA samples applied in this experiment were obtained according to a previous report [21]. In this procedure, the samples contain fragmented DNA but not intact DNA. Every two results in each dose of troglitazone are shown.

The doses used in the present study were chosen because they have been shown to be clinically relevant in studies of peripheral insulin resistance [25]. The effective dose range of troglitazone to inhibit cell growth in MKN45 cells in this study is the same as that needed to induce growth inhibition of human colon cancer cells as shown by Sarraf et al. [3]. Sarraf et al. have demonstrated that troglitazone inhibited colon cancer cell growth in vitro and that the growth of transplanted colon cancer cells was suppressed when mice were treated with troglitazone [3]. The effects of troglitazone occurred at concentrations of ligand that are achieved in the treatment of human type 2 diabetes with troglitazone [25]. This evidence may suggest that troglitazone at doses used clinically may induce inhibition of growth of colon cancer cells. The same dose range of troglitazone that induced inhibition of cell growth in colon cancer and gastric cancer cells in vitro suggests that anti-tumor action of troglitazone for gastric cancer might be given at clinically relevant doses of troglitazone.

The mechanism by which troglitazone induces growth inhibition in MKN45 cells remained to be clarified. To address this problem, we examined the possibility that apoptosis is involved in growth suppression. Neither vehicle only nor low doses of troglitazone induced apoptosis in MKN45 cells. However, troglitazone at the highest concentrations (100 μM) tested in this study induced an apparent DNA fragmentation

in MKN45 cells, suggesting an apoptotic change. Chinetti et al. [26] have recently shown that PPARγ activation induces apoptosis in human monocyte-derived macrophage. The evidence obtained from benign cells might support our speculation that PPARγ activation by troglitazone could induce apoptosis in malignant cells. The present evidence that the dose (100 μM) of troglitazone potently inhibited cell growth and induced apoptosis revealed that apoptosis might be involved in the mechanisms by which troglitazone at a dose of 100 μM prevented cell growth in gastric cancer cells. However, the dose-response effect of troglitazone on the induction of apoptosis was much less potent than that on the inhibition of cell growth. These results may suggest that apoptosis is not a major mechanism by which troglitazone induced the inhibition of cell growth.

A couple of reports show that PPARγ is closely associated with cell cycle to alter cell growth. Altiok et al. [27] reported that the reduction in cell growth is closely associated with a loss of E2F/DP DNA binding through modulation of phosphorylation by PP2A phosphatase in fibroblasts. Brockman et al. [4] demonstrated that activation of PPARγ resulted in G1 cell cycle arrest in colon cancer cells. Thus, cell cycle withdrawal together with induction of apoptosis might be implicated in the troglitazone-induced growth inhibition of gastric cancer cells. Further studies are needed in this matter.

Based on data about the relative time trends of the age-adjusted mortality rate for gastric cancer in Japan in 1955–1993, gastric cancer mortality has shown a declining trend since around 1960 and the mortality rate in 1993 is about 50% or less than in 1955. The reason for the marked change over time in the mortality rate of gastric cancer in Japan is not clear. With regard to this point, Tominaga and Kuroishi [28] examined the relationship between diet/nutrition and incidence of gastric cancer and showed that increased fat intake may be inversely associated with mortality of gastric cancer. This result may indicate that increased fat intake may be one of the factors to decrease the incidence of gastric cancer in Japan. It has been shown that fat activates PPARγ. For example, PPARγ modulates gene expression in response to fatty acids and lipid-derived metabolites [1,2]. We may therefore be allowed to speculate that PPARγ activation by increased intake of fatty food might have a suppressive action on the development of gastric cancers in Japan. In other words, PPARγ activation may provide a molecular link between a high-fat diet and decreased risk of gastric cancer.

In summary, the present study shows that PPARγ is expressed in human gastric cancer cells and that PPARγ activation inhibits cell growth and induces apoptosis.

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