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Troglitazone Induces G1 Arrest by p27^{Kip1} Induction That Is Mediated by Inhibition of Proteasome in Human Gastric Cancer Cells

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We examined in the present study whether human gastric cancer cells express peroxisome proliferator-activated receptor γ (PPARγ), the effect of PPARγ activation by troglitazone, a selective ligand, on cellular growth, and the mechanism of the growth arrest by troglitazone in gastric cancer cells. RT-PCR, northern blot and western blot analysis demonstrated that all four tested human gastric cancer cell lines, MKN-28, MKN-45, MKN-74 and KATO-III, expressed PPARγ mRNA and protein. WST-1 assay and flow cytometric analysis revealed that troglitazone inhibited the growth and induced G1 arrest in all four gastric cancer cell lines. To examine the role of p27^{Kip1}, a cyclin-dependent kinase inhibitor, in the G1 arrest by troglitazone, we determined p27^{Kip1} protein expression by western blot analysis in gastric cancer cells that had been treated with troglitazone. Troglitazone increased p27^{Kip1} in all four gastric cancer cell lines. Since it has been reported that the ubiquitin-proteasome system plays a vital role in the degradation of p27^{Kip1} protein, we evaluated the hypothesis that inhibition of proteasome mediates the troglitazone-induced p27^{Kip1} accumulation. Lactacystin, a proteasome inhibitor, inhibited cell growth and increased p27^{Kip1} expression in MKN-74 cells. It was further demonstrated that troglitazone inhibited proteasome activity in a dose-dependent manner in MKN-74 cells. All these results suggest that troglitazone inhibited proteasome activity, followed by induction of p27^{Kip1}, which arrests cells at the G1 phase of the cell cycle in gastric cancer cells. The troglitazone-mediated inhibition of the proteasome suggests a novel mechanism for the anti-proliferative effect of this agent in cancer cells.

Key words: Troglitazone — PPARγ — p27^{Kip1} — Proteasome — Gastric cancer

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily, which includes receptors for steroids, thyroid hormone, vitamin D and retinoic acid.[1)] PPARγ is expressed at high levels in adipose tissue and functions as a key molecule in adipocyte differentiation.[2, 3)] In addition to adipose tissue, PPARγ expression is detected in a wide variety of tumor cells.[4–15)] In the tumor cells, PPARγ activation by high-affinity ligands could inhibit cell growth. Thus, PPARγ is involved in not only lipid metabolism, but also cellular proliferation in cancer cells. It has therefore been suggested that PPARγ is a possible molecular target for cancer treatment. We have reported that MKN-45 cells, a poorly differentiated gastric cancer cell line, express PPARγ, and PPARγ ligands such as troglitazone and pioglitazone induce growth inhibition.[13)] Little is known, however, about whether other human gastric cancer cells express PPARγ, and whether PPARγ ligands induce growth arrest in them.

Although increasing evidence has established that PPARγ activation induces growth arrest in cancer cells,[4–15)] the molecular mechanism of the growth inhibition by PPARγ ligands is not understood. With regard to this point, we have very recently demonstrated that p27^{Kip1}, a cyclin-dependent kinase inhibitor (CDKI),[16, 17)] may be a key molecule in the cell growth inhibition by troglitazone in human pancreatic cancer cells.[14)] Little is known, however, about whether p27^{Kip1} is up-regulated by PPARγ ligands in cancer cells other than pancreatic cancer cells. In addition, there is no report on the mechanism of p27^{Kip1} protein accumulation by PPARγ ligands.

In the present study, we tried to clarify whether PPARγ is expressed in human gastric cancer cell lines, MKN-28, MKN-45, MKN-74 and KATO-III cells, whether troglitazone can induce growth inhibition in the four gastric cancer cells, whether p27^{Kip1} is also increased by troglitazone in gastric cancer cells, and the mechanism of the increase in p27^{Kip1} by troglitazone.

MATERIALS AND METHODS

Cell culture Human gastric cancer cell lines, MKN-28, MKN-45, MKN-74 and KATO-III were obtained from the Japanese Cancer Research Resources Bank (Tsukuba) and Tohoku University (Sendai). MKN-28 and MKN-74 are moderately differentiated gastric adenocarcinomas, while MKN-45 and KATO-III are cell lines established from a poorly differentiated gastric cancer and a signet ring cell

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gastric cancer, respectively. Gastric cancer 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. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Chemicals and treatments Troglitazone[18] was kindly provided by Sankyo Pharmaceutical Co. (Tokyo) and was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1% in the culture medium.

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).[19] 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-DNase (Promega, Madison, WI), 0.5 μl of RNase inhibitor (TaKaRa Shuzou Co., Otsu) and 10 μl of 10× DNase buffer (400 m*M* Tris-HCl at pH 7.9, 100 m*M* NaCl, 60 m*M* $MgCl_2$ and 100 m*M* $CaCl_2$) 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 a spectrophotometer at a wavelength of 260 nm. The integrity of the isolated RNA samples was analyzed electrophoretically on agarose gel, followed by staining with ethidium bromide.

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 of 5′-TCTCTCCGTAATGGAAGACC-3′ and an antisense primer of 5′-GCATTATGAGACATCCCCAC-3′ was used as described previously.[20] The amplification was 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 μ*M* each of the sense and antisense primers, 10 m*M* Tris HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* dNTPs, and 2.5 units of *Taq* DNA polymerase (TaKaRa Shuzou Co.). 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.

Northern analysis Ten micrograms of total RNA denatured in formamide and formaldehyde was electrophoresed through 1% formaldehyde-containing agarose gels. After electrophoresis, the RNA was transferred to a nylon membrane (Hybond N, Amersham International, Buckinghamshire, UK) by capillary blotting and then fixed with a UV cross linker (FUNA-UV-LINKER, Funakoshi, Tokyo). Prehybridization was performed at 42°C for 2 h in 50% formamide, 25 m*M* 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. The probe for PPARγ was amplified by PCR using MKN-45 cell cDNA as the template and sequenced. β-Actin cDNA probe (Wako Chemicals Industries, Osaka) was used as an internal control. The membrane was washed under appropriately stringent conditions, and the hybridization signals were analyzed with a bioimaging analyzer system (Fuji-BAS, Fuji Photo Film Co., Tokyo) or by autoradiography using XAR film (Eastman Kodak, Rochester, NY).

Western blot analysis Total protein was extracted from each human gastric cancer cell line. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad Lab., Richmond, CA) following the manufacturer's suggested procedure. Fifty micrograms of protein was separated on a 10% Tris-Glycine gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Amersham Life Science, Inc., Piscataway, NJ), blocked overnight in PBS-Tween (PBS-T) with 10% skim milk at 4°C, then reacted with primarily monoclonal antibody against human PPARγ (Santa Cruz Biotechnology, 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 G1 κ monoclonal immunoglobulin (Pharmingen, San Diego, CA) was used as an isotype control.

Cell growth assay To evaluate the effect of PPARγ activation on cell growth, cells were seeded on a 96-well cell culture cluster (Corning, Inc., Corning, NY) at a concentration of 1×10^4/well in a volume of 100 μl. Twenty-four hours later, each well was incubated with troglitazone at several concentrations. Cell numbers were measured colorimetrically using a Cell Counting Kit (Dojindo, Kumamoto) with an ImmunoMini NJ-2300 (NJ InterMed, Tokyo) at a test wavelength of 450 nm. This assay is based on the cleavage of the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) by mitochondrial dehydrogenase in viable cells.[21] In comparison with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, WST-1 is more sensitive and does not require cells to be solubilized.

Cell cycle assay by flow cytometry Gastric cancer cells treated with troglitazone or DMSO for 0 or 48 h were collected by centrifugation, and permeabilized with ice-cold

70% ethanol for at least 1 h. They were washed with PBS, and treated with PBS containing 100 mg/ml RNase A (DNase free) at 37°C for 30 min. Following centrifugation, the cells were resuspended in PBS containing 50 mg/ml propidium iodide and stained at 37°C for 30 min. DNA contents were analyzed by FACScan (Becton Dickinson).

Protein expression of cyclin-dependent kinase inhibitors, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ detected by western blot analysis The effect of troglitazone on the expression of CDKIs, $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ in gastric cancer cells was studied by western blot analysis. The cells were treated with several doses of troglitazone and total proteins were extracted from the cells at several time points. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad Lab.) following the manufacturer's suggested procedure. Fifty micrograms of protein was separated by 5–20% SDS-PAGE (Ready Gels J, Bio-Rad Lab.). After electrophoresis, the proteins were transferred to nitrocellulose membrane (Amersham Life Science, Inc.), blocked in TBS with 10% skim milk at room temperature for 60 min, then reacted overnight with goat polyclonal antibody against $p27^{Kip1}$ or goat anti-$p21^{Cip1/Waf1}$ polyclonal antibody (Santa Cruz Biotechnology) at 4°C, and washed. After reaction with horseradish peroxidase-conjugated anti-goat IgG, immune complexes were visualized by using the ECL detection reagents (Amersham) following the manufacturer's suggested procedure. Simultaneously, normal goat IgG was used as a control.

Proteasome assay The proteasome assays were performed after treatment of MKN-74 cells with troglitazone for 48 h. The medium was removed and the cells were washed several times before they were lysed. To measure the proteasome chymotrypsin peptidase activity, 10 μl of cellular extract (100 μg, prepared by brief sonication of cells and fractionation at 15 000*g*) was diluted in a cuvette containing 2 ml of 20 m*M* Hepes, 0.5 *M* EDTA, pH 8 and 0.035% SDS. The cell extracts contain 26S proteasome. The above mixture was incubated at 37°C before the addition of the fluorogenic substrate, 10 μM succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin. Substrate hydrolysis was measured by continuous monitoring for fluorescence (emission at 460 nm, excitation at 380 nm) of the liberated 7-amido-4-methylcoumarin for 750 s as described.[22)]

Statistical analysis The results are expressed as mean± SEM. Statistical analysis was performed by one-way analysis of variance followed by Fisher's LSD test. $P<0.05$ was considered statistically significant.

RESULTS

First, we examined whether four gastric cancer cell lines, MKN-28, MKN-45, MKN-74 and KATO-III, express PPARγ. As demonstrated in Fig. 1, PPARγ mRNA expression was observed in RT-RCR and northern blots in all the tested cell lines. Western blots revealed that PPARγ protein was also present in all the cell lines.

Fig. 2 illustrates the effect of troglitazone, a specific ligand for PPARγ, on the cell growth of the four gastric

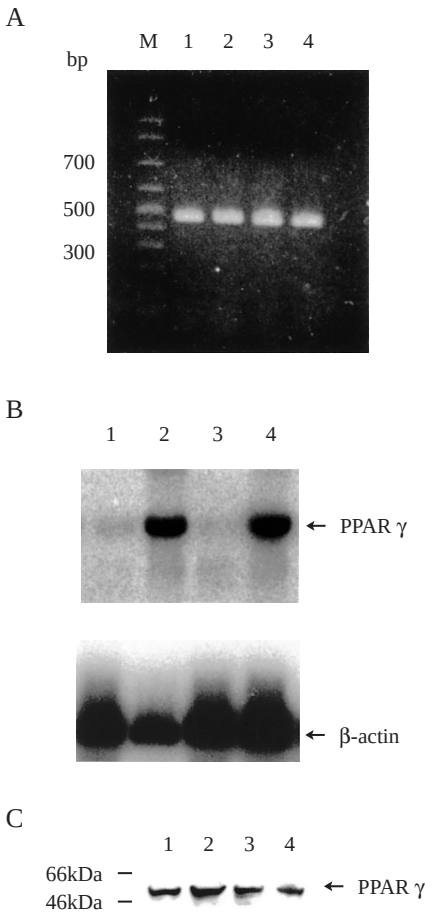

Fig. 1. PPARγ expression in four human gastric cancer cell lines, MKN-28, MKN-45, MKN-74 and KATO-III. (A) PPARγ is expressed at the mRNA level in all the tested cell lines. Total RNA (1 μg) from each gastric cancer cell line was subjected to an RT reaction. A 1 μl aliquot from each reaction mixture 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 (MKN-28), lane 2 (MKN-45), lane 3 (MKN-74) and lane 4 (KATO-III). (B) Northern blot for PPARγ in MKN-28 (lane 1), MKN-45 (lane 2), MKN-74 (lane 3) and KATO-III (lane 4). Ten micrograms of total RNA obtained from each cell line was electrophoresed on 2% agarose gel and transferred to a nitrocellulose membrane. 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 MKN-28 (lane 1), MKN-45 (lane 2), MKN-74 (lane 3) and KATO-III (lane 4). Fifty micrograms of each protein obtained from gastric cancer cells was separated by SDS-PAGE and probed with an anti-PPARγ antibody.

cancer cell lines. Troglitazone significantly inhibited cell growth in all the cell lines. The inhibition of cell proliferation was dose-dependent in MKN-28, MKN-45 and MKN-74. In KATO-III, only the highest dose (100 μM) of troglitazone inhibited cell growth, suggesting that KATO-III may be relatively resistant to troglitazone compared with MKN-28, MKN-45 and MKN-74. The difference between KATO-III and the other three cell lines may depend upon the time-course of cell growth in the control DMSO group. For example, in MKN-28, MKN-45 or MKN-74 cells, the cell number at 48 h in the DMSO control groups was 4-fold higher than that at 0 h, indicating rapid cell growth, while in KATO-III, the cell number at 48 h in the DMSO control was not much higher than that at 0 h.

We next examined the cell cycle profiles in the four cell lines by flow cytometry. Fig. 3 illustrates the effects of troglitazone on the cell cycle profile in the four cell lines and the percentages of cells in G1, S and G2/M phase are shown in Table I. Flow cytometric analysis revealed that the population of G1-phase cells in all the cell lines 36 h after troglitazone at a dose of 100 μM was much larger than that of the DMSO control. It was also demonstrated that the population of S-phase cells in the four cell lines treated with troglitazone was much smaller than that of the

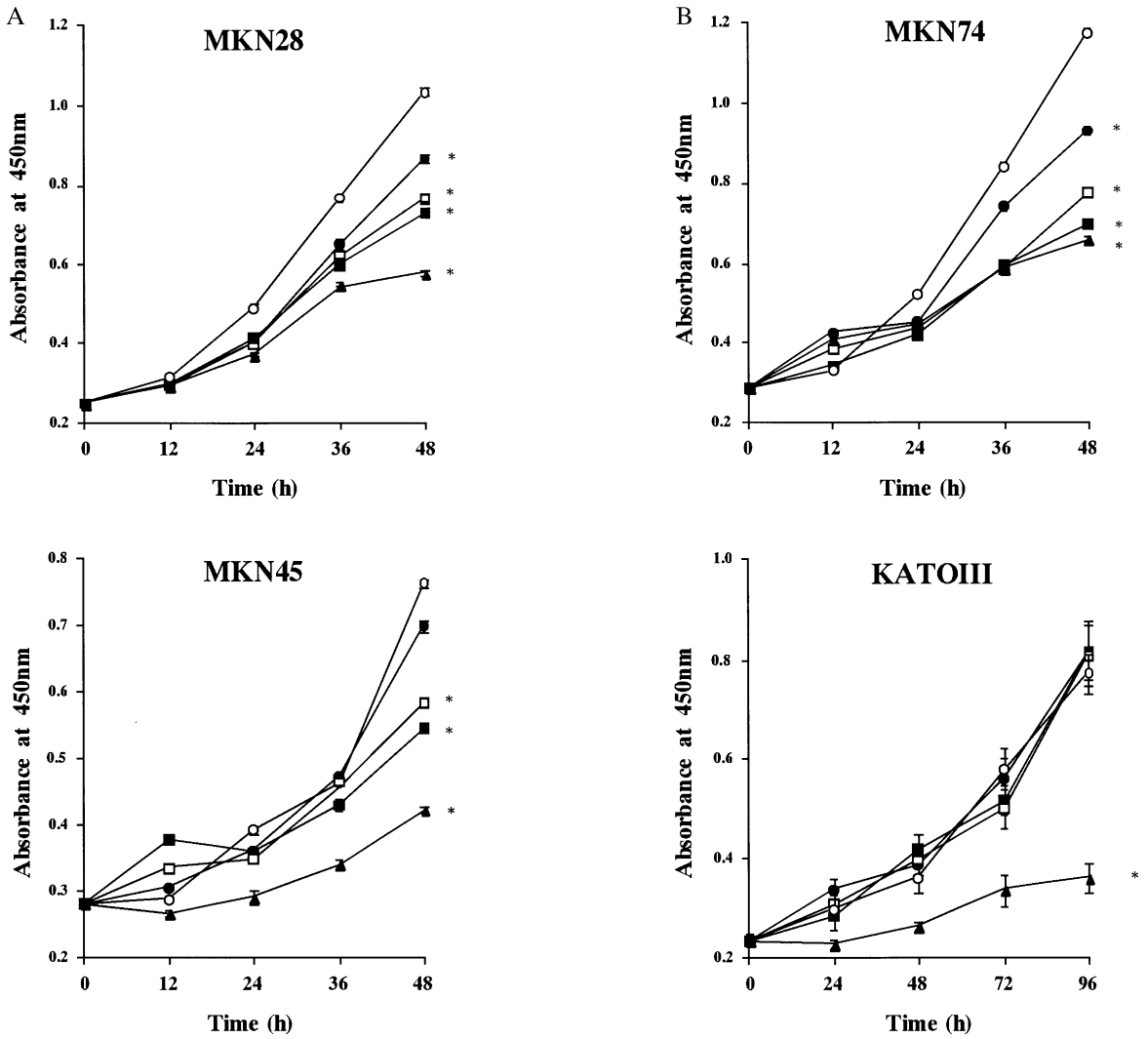

Fig. 2. Effect of troglitazone on cell growth of human gastric cancer cell lines, MKN-28, MKN-45, MKN-74 and KATO-III. Gastric cancer cells were treated with either 0, 0.1, 1, 10 or 100 μM troglitazone and cell numbers were determined by WST-1 assay at several time points. Data are expressed as mean±SEM of 5 experiments. * $P<0.01$, when compared with vehicle (DMSO) alone. ○ DMSO, ● 0.1 μM, □ 1 μM, ■ 10 μM, ▲ 100 μM.

DSMO control. These results suggest that troglitazone arrests cells at the G1 phase.

To evaluate the mechanism of the G1 arrest in gastric cancer cells by troglitazone, we examined the effects of troglitazone on the expression of CDKI, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. Fig. 4 shows the time-course of expression of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ evaluated by western blotting in the four gastric cancer cell lines after troglitazone at a dose of 100 μM. Troglitazone increased the protein expression of $p27^{Kip1}$ in a time-dependent manner in all four cell lines, suggesting that $p27^{Kip1}$ may be involved in the G1 arrest by troglitazone in gastric cancer cells. $p21^{Cip1/Waf1}$ protein expression was also up-regulated by troglitazone in MKN-28, MKN-45 and MKN-74, but not in KATO-III.

Next, we tried to clarify the mechanism of the $p27^{Kip1}$ accumulation by troglitazone in gastric cancer cells. Because a recent report indicated that the ubiquitin-proteasome system plays a role in $p27^{Kip1}$ degradation,[23–25] we hypothesized that inhibition of proteasome is implicated in the $p27^{Kip1}$ accumulation by troglitazone in gastric cancer cells. Fig. 5 illustrates the effect of lactacystin, a selective proteasome inhibitor, on cell growth and $p27^{Kip1}$ protein levels in MKN-74. Lactacystin in a dose of 10 μM significantly inhibited the cell growth and increased the expression of $p27^{Kip1}$, suggesting that inhibition of proteasome may induce growth arrest through an increase of $p27^{Kip1}$, which is very similar to the cell behavior seen after troglitazone in gastric cancer cells. To test whether troglitazone inhibits proteasome activity, we examined the proteasome activity of MKN-74 cells treated with troglitazone. As can be seen in Fig. 6, troglitazone dose-dependently inhibited the proteasome activity in MKN-74 cells.

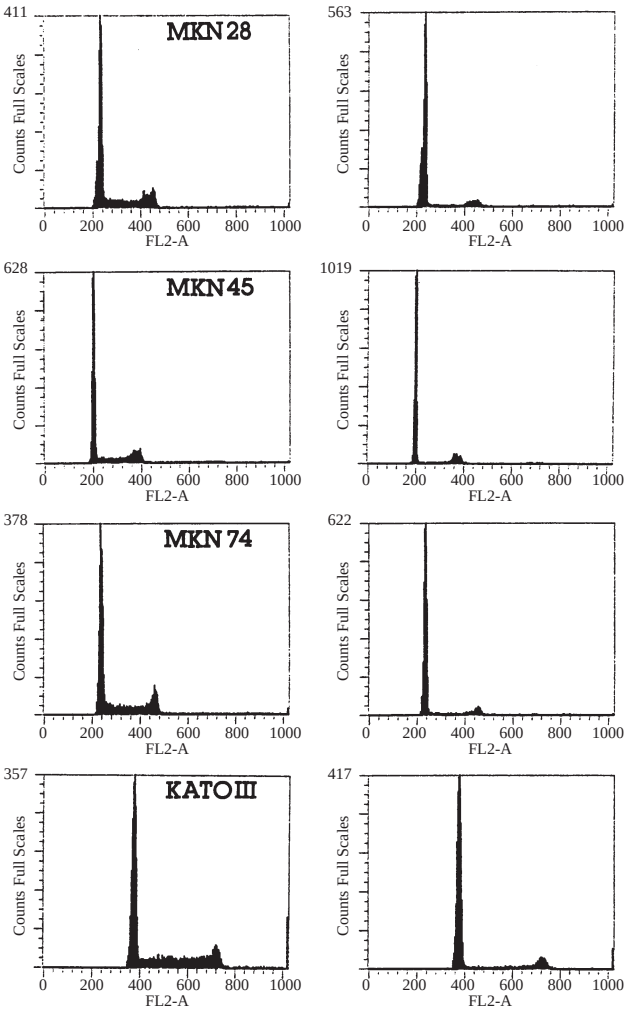

Fig. 3. Effect of PPARγ activation by troglitazone on the cell cycle profile. After treatment with DMSO or troglitazone for 36 h, gastric cancer cells were collected, and cell-cycle analyses with propidium iodide were performed using flow cytometry. Cell-cycle distributions were quantified by Cell-quest software. The population of cell cycle in each cell line is shown in Table I. Each histogram illustrated the results of MKN-28, MKN-45, MKN-74 or KATO-III cells treated with DMSO (each left panel) or troglitazone at a 100 μM dose for 36 h (each right panel).

DISCUSSION

We have demonstrated for the first time that human gastric cancer cells express PPARγ.[13] In that study, we showed that one cancer cell line, MKN-45, expresses PPARγ by RT-PCR, northern blot and western blot analy-

Table I. Cell Cycle Analysis by Flow Cytometry (%)

	G1	S	G2/M
MKN-28			
DMSO	55.6	36.3	8.1
Troglitazone	88.3	6.8	4.9
MKN-45			
DMSO	63.4	31.0	5.6
Troglitazone	87.1	7.2	5.7
MKN-74			
DMSO	56.2	31.5	12.3
Troglitazone	88.0	6.2	5.7
KATO-III			
DMSO	56.8	35.0	8.2
Troglitazone	85.2	6.6	8.2

Four gastric cancer cell lines, MKN-28, MKN-45, MKN-74 and KATO-III, were treated with DMSO or troglitazone in a dose of 100 μM for 36 h. Cells were harvested and subjected to flow cytometry.

sis. In the present study, expression of PPARγ in not only MKN-45, but also MKN-28, MKN-74 and KATO-III was observed. Sato *et al.* have recently demonstrated PPARγ expression in human gastric cancer cells from surgically resected specimens and in human gastric cancer cells, MKN-7, MKN-28, MKN-45 and AGS.[26] These results indicate that the expression of PPARγ in human gastric cancer cells is a common biological character.

With regard to the discrepancy between mRNA and protein data on PPARγ expression in two gastric cancer cell lines (MKN-28 and MKN-74) in Fig. 1, we do not know the reason for the discrepancy. According to the paper by Sato *et al.*,[26] they examined PPARγ expression in four different gastric cancer cell lines by northern and western blotting and obtained similar data to ours. The figure in the paper shows that MKN-28 cells express a little PPARγ mRNA and a large amount of PPARγ protein. This is similar to the results in our study. As to the discrepancy between PPARγ mRNA and protein levels, they did

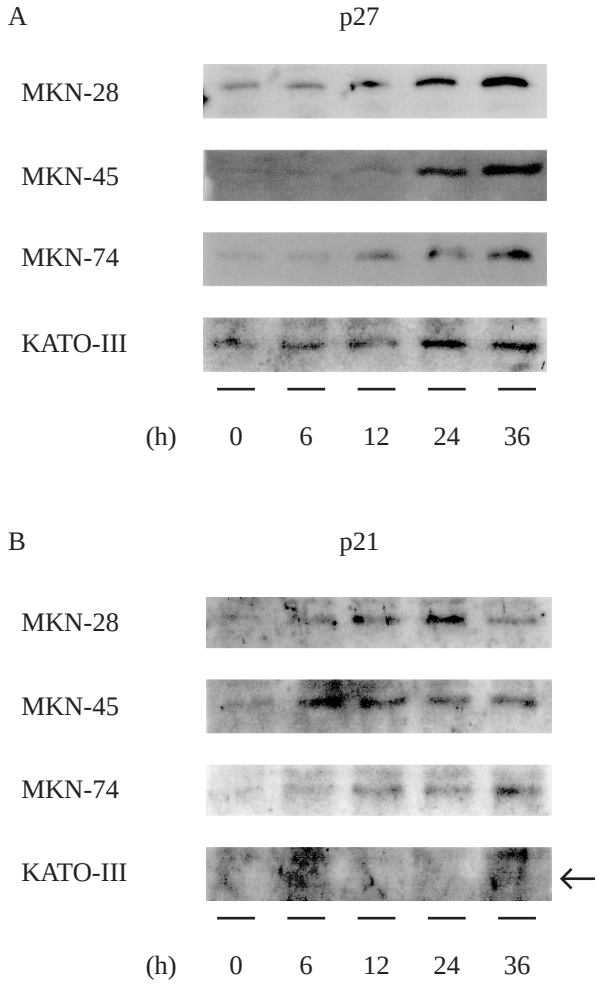

Fig. 4. $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ expression in gastric cancer cells by troglitazone. Western blot analyses were performed using anti-$p27^{Kip1}$ polyclonal antibody. $p27^{Kip1}$ protein expression was detected in MKN-28, MKN-45, MKN-74 and KATO-III cells at several time points after treatment with troglitazone at a dose of 100 μM (A). B shows the effect of troglitazone on $p21^{Cip1/Waf1}$ protein expression in each gastric cancer cell line by troglitazone. Western blot analyses were performed using anti-p21 polyclonal antibody. $p21^{Cip1/Waf1}$ protein expression was detected in gastric cancer cells at several time points after treatment with troglitazone at a dose of 100 μM.

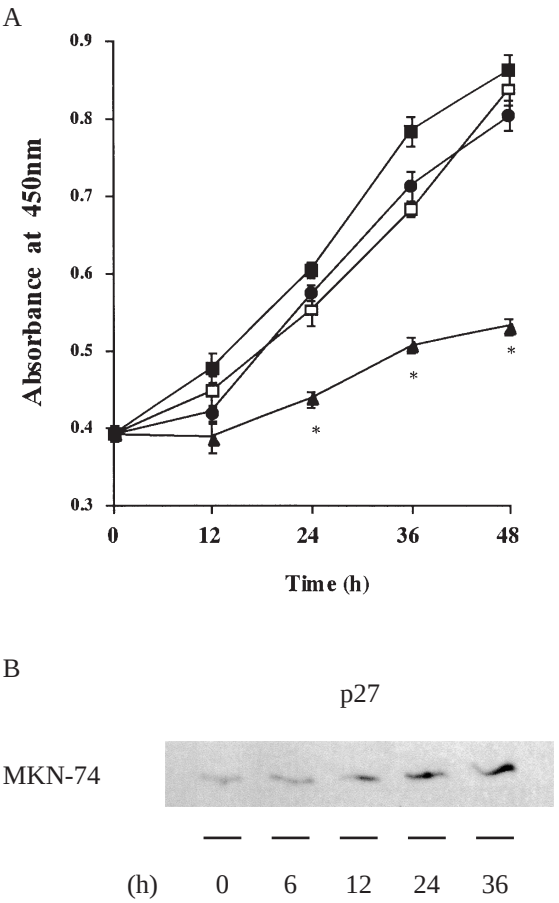

Fig. 5. The effect of lactacystin on cell growth of a human gastric cancer cell line, MKN-74, is shown in A. Gastric cancer cells were treated with either 0, 0.1, 1, or 10 μM lactacystin and cell numbers were determined by WST-1 assay at several time points. Data are expressed as mean±SEM of 5 experiments. $* P<0.01$, when compared with vehicle. B shows the effect of lactacystin on $p27^{Kip1}$ expression in MKN-74 cells. Western blot analysis was performed using anti-$p27^{Kip1}$ polyclonal antibody. $p27^{Kip1}$ protein expression was detected in cells at several time points after treatment with lactacystin at a dose of 10 μM. □ 0 μM, ● 0.1 μM, ■ 1 μM, ▲ 10 μM.

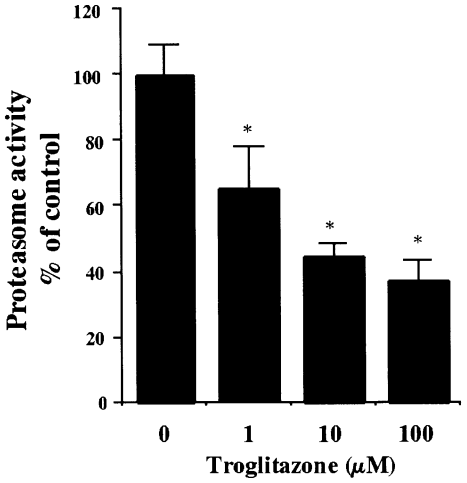

Fig. 6. Effect of troglitazone on proteasome activity in MKN-74 cells. Cell extracts were prepared from MKN-74 cells treated with several doses of troglitazone for 36 h. The extracts were assayed for proteasome activity. Data are expressed as mean±SEM of 4 experiments. * $P<0.01$, when compared with vehicle.

not comment. PPARγ protein level might be regulated by posttranscriptional and/or posttranslational mechanisms. According to the information provided by Santa Cruz Biotechnology, the antibody used in this study reacts with PPARγ1 and PPARγ2 and, to a lesser extent, human PPARα and PPARβ in western blotting. Therefore, we cannot exclude the possibility that the antibody used might have cross-reacted with proteins other than PPARγ.

One may speculate whether PPARγ is expressed in normal gastric epithelial cells or is specific to gastric cancer cells. With regard to this point, Braissant *et al.*[27] reported that rat normal gastric mucosa expressed PPARγ and Sato *et al.* have recently reported an immunohistochemical study of surgically resected gastric specimens showing that normal human gastric mucosa with intestinal metaplasia adjacent to cancer also expresses PPARγ.[26] These results suggest that not only gastric cancer cells, but also normal gastric epithelial cells express PPARγ.

In all the tested cell lines, troglitazone induced cell growth inhibition, suggesting that PPARγ activation by troglitazone caused the inhibition of cell growth. Flow cytometry revealed that troglitazone arrested gastric cancer cells at the G1 phase. It is therefore suggested that troglitazone-induced inhibition of cell growth depends upon the G1 arrest induced by troglitazone in gastric cancer cells. It has been shown that troglitazone evoked G1 arrest in human colon, pancreatic and breast cancer cells.[6, 14, 15] The G1 arrest observed after troglitazone treatment in gastric cancer cell lines further supports the idea that PPARγ activation arrests the cell cycle at G1 in a wide variety of cancer cells.

Little is known about the mechanism by which troglitazone induces G1 arrest. We have very recently demonstrated that troglitazone increased the protein level of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, and antisense oligonucleotide against $p27^{Kip1}$ blocked the troglitazone-induced cell growth arrest in human pancreatic cancer cells, strongly suggesting that $p27^{Kip1}$ is implicated in the cell growth arrest by troglitazone in pancreatic cancer cells.[14] These results suggested for the first time that $p27^{Kip1}$ mediates troglitazone-induced cell growth arrest. In the present study, we have examined whether $p27^{Kip1}$ is similarly involved in the troglitazone-induced G1 arrest in human gastric cancer cells and we clearly demonstrated that troglitazone increased the protein level of $p27^{Kip1}$ in all the tested gastric cancer cells. It was also shown that the protein level of $p21^{Cip1/Waf1}$ as well as $p27^{Kip1}$ was increased by treatment with troglitazone in MKN-28, MKN-45 and MKN-74, but not in KATO-III. In KATO-III, troglitazone at a dose of 100 μM inhibited cell proliferation, but failed to increase $p21^{Cip1/Waf1}$, suggesting that $p21^{Cip1/Waf1}$ is not the cause of the cell growth inhibition by troglitazone. These results suggest that troglitazone induced G1 arrest via an increase of $p27^{Kip1}$ in gastric cancer cells.

Akama *et al.*[28] have examined the expression of cyclins and CDKI in human gastric cancer cell lines and reported that $p21^{Cip1/Waf1}$ protein was detected by western blot in MKN-45 and MKN-74, whereas $p21^{Cip1/Waf1}$ protein was not detectable in MKN-28 and KATO-III. In the present study, $p21^{Cip1/Waf1}$ protein was detected in MKN-28 as well as MKN-45 and MKN-74, especially in the cells treated with troglitazone. The detection of $p21^{Cip1/Waf1}$ protein in MKN-28 may be a result of induction by troglitazone. In KATO-III cells, $p21^{Cip1/Waf1}$ was not detected in the cells even after troglitazone treatment. It has been demonstrated that the *p53* gene is completely deleted in KATO-III, while MKN-28, MKN-45 and MKN-74 express p53.[29, 30] The deletion of the *p53* gene may be the reason for the undetectable $p21^{Cip1/Waf1}$ in KATO-III, because $p21^{Cip1/Waf1}$ is a p53-inducible gene.[31, 32]

Although we do not know in detail the mechanism of accumulation of $p27^{Kip1}$ by troglitazone, it has been reported that a ubiquitin-proteasome pathway is implicated in one of the posttranslational mechanisms of $p27^{Kip1}$ regulation.[23–25] Based upon this evidence, we hypothesized that troglitazone-mediated G1 arrest occurs through inhibition of the proteasome. To test this idea, we examined 1) if inhibition of the proteasome induces growth arrest, 2) if inhibition of the proteasome up-regulates $p27^{Kip1}$ and 3) if troglitazone inhibits proteasome activity.

We found that lactacystin, a specific proteasome inhibitor, inhibited cell proliferation and up-regulated $p27^{Kip1}$

protein expression in MKN-74 cells. It was demonstrated that lactacystin increased the $p27^{Kip1}$ protein level in a breast cancer cell line, MDA-MB-157,[24] supporting the present finding that proteasome inhibition by lactacystin increased $p27^{Kip1}$ also in gastric cancer cells. The increase in $p27^{Kip1}$ protein may lead to the inhibition of cell growth in gastric cancer cells.

Next, we examined whether troglitazone inhibits proteasome activity and found that proteasome activity was significantly inhibited by troglitazone in a dose-dependent manner. The proteasome inhibition by troglitazone was observed at doses that caused cell growth inhibition and $p27^{Kip1}$ up-regulation in gastric cancer cells. These results suggest for the first time that troglitazone inhibits proteasome activity. We speculate that the inhibition of proteasome by troglitazone could lead to inhibition of the degradation process of $p27^{Kip1}$, thereby resulting in the accumulation of $p27^{Kip1}$ protein. Thus, inhibition of proteasome may mediate troglitazone-induced growth arrest through $p27^{Kip1}$ accumulation.

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