Troglitazone Inhibits HCT15 Human Colorectal Cancer Cell Proliferation Through the ERK Pathway Dependent Activation of $p21^{Cip/WAF1}$

Thesis by

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Brain Korea 21 Project for Medical Sciences

The Graduate School of Yonsei University

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Directed by Professor Yong Ho Ahn

A Dissertation Submitted to the Faculty of

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December, 2001

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A Dissertation for the Master of Science in Medical Sciences by Jin-Ah Kim has been approved by

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(Supervisory committee, Chairman)

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(Supervisory committee)

The Graduate School of Yonsei University

December, 2001

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2001 년 12 월

김진아

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Jin-Ah Kim

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(Directed by Professor Yong Ho Ahn)

In this study, we identified a new mechanism for the anti-proliferation of HCT15 colorectal cancer cells by troglitazone (TGZ). Treating HCT15 cells with 20 μM of TGZ transiently increased ERK activity within 15 min and this subsequently induced $p21^{Cip/WAF1}$ cell cycle regulator and localized in the nucleus. Raf-1 modification and MEK activation also occurred after TGZ treatment, and Elk-1 dependent trans-reporter gene expression was concomitantly induced. The induction and nuclear localization of $p21^{Cip/WAF1}$ by TGZ were blocked by PD98059 pre-treatment, which suggested a role for the ERK pathway in $p21^{Cip/WAF1}$ activation. TGZ inhibited BrdU incorporation and no BrdU incorporation was observed in most $p21^{Cip/WAF1}$ activated cells. Therefore, TGZ regulates the proliferation of HCT15 cells at least partly by a mechanism involving the

ERK pathway dependent activation of $p21^{Cip/WAF1}$.

Keywords: Troglitazone; ERK; $p21^{Cip/WAF1}$; cell cycle; anti-proliferation

Troglitazone Inhibits HCT15 Human Colorectal Cancer Cell Proliferation Through the ERK Pathway Dependent Activation of $p21^{Cip/WAF1}$

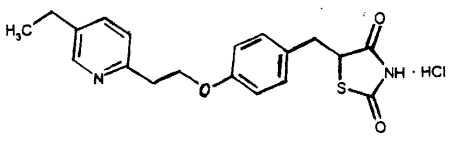
<Directed by Professor **Yong Ho Ahn**>

Brain Korea 21 Project for Medical Sciences
The Graduate School of Yonsei University

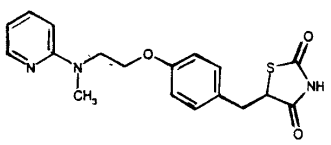
Jin-Ah Kim

Ⅰ. Introduction

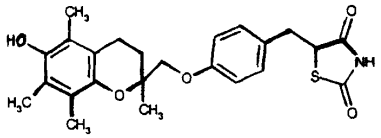
Troglitazone (TGZ) is a member of the family of thiazolidinediones (TZDs) including pioglitazone and rosiglitazone (Figure 1)[1], which are orally active drugs that improve insulin sensitivity in both animals and humans with insulin resistance.[2,3] TGZ binds to and activates peroxisome proliferator-activated receptor γ (PPARγ), a member of the ligand-activated nuclear hormon receptor family.[4,5] Peroxisome proliferator activated receptor-γ (PPAR-γ), a nuclear hormon receptor, provides a strong link between lipid metabolism and the regulation of gene transcription.[6] PPAR-γ acts in the adipose tissue and promotes lipogenesis under anabolic conditions. Recently, the receptor has also been implicated in inflammation and tumorgenesis. Significant evidences from many experimental system suggest that PPAR-γ is important carcinogenesis. PPAR-γ is up-regulated in malignant tissue, and



Pioglitazone HCl



Rosiglitazone



Troglitazone

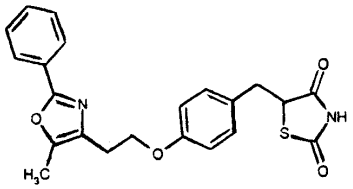


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Figure 1. Structures of thiazolidinedione derivatives.

PPAR-γ ligands induce terminal differentiation in breast, colon and lung cancer cells,[7,8] and inhibit the growth of human breast, prostate, gastric and lung cancer cells .[9-12] Recent studies found that TGZ also inhibits growth of human breast and prostate cancers, myeloid leukemia, and vascular smooth muscle cells.[13-16] A role of TGZ in the growth regulation of colorectal cancer cells was also identified and related with the activation of the PPARγ.[17] However, any regulatory mechanism of cell growth by TGZ independent of PPARγ has not been identified. A recent study also found that TGZ induced cyclin-dependent kinase inhibitor $p21^{Cip/WAF1}$ and this resulted in the suppression of the growth of a myeloid leukemia cell line.[15] However, the $p21^{Cip/WAF1}$ induction by TGZ has not been reported in solid tumors, and the mechanism of $p21^{Cip/WAF1}$ induction has not been determined. The $p21^{Cip/WAF1}$ induction is closely related with the growth arrest of many different cell types, including colorectal cancer cells[18,19] and this is dependent upon the activation of either p53 tumor suppressor[20,21] or the extracellular signal regulated kinase (ERK) pathway.[22-24] The mitogen-activated protein kinase (MAPK) pathway (also known as extra-cellular signal regulated kinase, ERK, pathway) is a major network of for the proliferation and differentiation of cells.[26-28] The role of activation of MAPK pathway in the proliferation of cells has been well elucidated.[25,27,29] However, the role of activation of the MAPK pathway in growth inhibition has also been reported.[30-32] Therefore, the ERK pathway is likely to be involved in the growth

arrest, as well as the proliferation of cells.[22-24]

In this study, we found that TGZ induced $p21^{Cip/WAF1}$ cell cycle regulator in HCT15 human colorectal cancer cells, and further identified a mechanism for the induction and nuclear localization of $p21^{Cip/WAF1}$ by TGZ. The $p21^{Cip/WAF1}$ activation is related with the anti-proliferation of colorectal cancer cells, and that provide an alternative mechanism for the growth regulation of HCT15 colorectal cancer cells by TGZ, which is independent of PPARγ.

Ⅱ. Materials and methods

1. Materials

HCT15 (CCL-225), HT-29 (HTB-38), and HCT116 (CCL-247) human colorectal cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM) and McCoy's 5A media, fetal bovine serum (FBS), antibiotics and Lipofectamin plus reagent were purchased from Life Technologies, Inc. (Grand Island, NY). Phospho-ERK and phospho-MEK antibodies were obtained from New England Biolabs Inc. (Beverly, MA), and ERK antibody from Stratagene (La Jolla, CA). The $p21^{Cip/WAF1}$ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Raf-1 antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG antibodies were from Transduction Laboratories (Lexington, KY). The BlotMate enhanced chemiluminescence (ECL) system was purchased from Genepia (Seoul, Korea). The Elk-1-dependent pathdetect plasmids (pFR-Luc and pFA2-Elk1) were from Stratagene (La Jolla, CA), the Luciferase assay kit and U0126 were from Promega Co. (Madison, WI), PD98059 from Calbiochem (La Jolla, CA), the protein assay solution from Bio-Rad Laboratories (Hercules, CA), 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI) from Boehringer Mannheim (Mannheim, Germany), the goat anti-mouse-Cy™2- or goat anti-rabbit Rhodamin Red™-X-conjugated secondary antibodies from Jackson Immuno Research Laboratories Inc.

(West Grove, PA), the anti-BrdU monoclonal antibody was purchased from DAKO Co. (Carpinteria, CA), and finally TGZ was a gift from Sankyo, Japan. All other chemicals described in this study were purchased from Sigma (St. Louis, MO).

2. Cell culture

The HCT15[33] cell line was maintained in DMEM, and the HT29[34] and HCT116[35] cells were maintained in McCoy's 5A media. The media were supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml of penicillin and 250 ng/ml of streptomycin in 5% CO_2 at 37°C. Experiments were performed on cells at 70% confluence, unless otherwise stated. To observe the effects of TGZ, cells were serum starved by being grown in a medium containing 1% FBS for 18 to 20 h and treated with TGZ to 20 μM. In cases requiring, an MEK inhibitor, 30 μM PD98059 or 0-2 μM U0126, was added at 1 h prior to TGZ treatment.

3. Extract preparation and Western blot analysis

Cells were rinsed twice with ice-cold phosphate buffered saline (PBS) and harvested by scraping in 500 μl of ice-cold PBS. They were then centrifuged and resuspended in 200 μl of lysis buffer (70 mM β-glycerophosphate pH 7.2, and 0.1 mM each of meta- and ortho-vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.5% Triton X-100, 0.2 mM phenylmethylsulphonyl fluoride, and 5

μg/ml each of pepstatin A, chymostatin, leupeptin and peptin). After incubation on ice for 30 min, the lysate was sonicated for 20 s on ice and unbroken cell debris was removed by centrifugation at 23,000 g for 15 min. Samples were immediately aliquoted and stored frozen at −70°C. Protein concentrations were determined using a Bio-Rad protein assay kit.

The lysates containing 40-50 μg of protein were separated by 8-12% sodium dodecyl sulfate (SDS) polyacrylamide gel (acrylamide: bis-acrylamide at a ratio of 29:1). Western blot analysis was performed, as described previously[36] using anti-ERK rabbit polyclonal antibody, phospho- MAPK, phospho- MEK antibody, anti-c-Raf-1 antibody or anti-p21 antibody followed by HRP-conjugated secondary antibodies at a dilution of 1 : 3000. The blots were developed using a BlotMate ECL kit.

4. PathDetect trans-reporting system and luciferase assay

To determine the activation status of the MAPK pathway *in vivo*, we adapted the Elk-1[37] dependent Trans-reporting system. For transfection, HCT15 cells were plated into 6-well plates at 5×10^5 cells per well using DMEM supplemented with 10% FBS, 100 units of penicillin and 250 ng/ml of streptomycin. After cultivating for 24 h, the cells were transiently transfected with 0.5 μg of pFR-Luc trans-reporter vector together with 25 ng of pFA2-Elk-1 trans-activator plasmids using Lipofectamin plus

reagent according to the manufacturer's instructions. When required, 0.5 μg of pCMV-MEK-2A (gift from Dr. G. Johnson at National Jewish Medical and Research Center, Denver, CO, U.S.A.), pSV-Sport1-Raf-1-dn (gift from Dr., J. H. Kim at Kwangju Institute of Science and Technology, Kwang-Ju, Korea) or the empty vector was co-transfected. For normalization purposes, the cells were co-transfected with 0.1 μg of plasmid containing the gene for β-galactosidase under the control of a CMV promoter. TGZ treatment was performed after 18-20 h of serum starvation in DMEM containing 1% FBS. One hour prior to the TGZ treatment, PD98059 was added to 30 μM in the required cases. The cell extracts were prepared 7 h after TGZ treatment. Luciferase activity was measured using a Luciferase assay kit, and normalized using the β–galactosidase level as an internal control.

5. Cell counting

HCT15 cells were seeded into 12-well plates at 0.8×10^5 cells/well. After growing for 24 h in DMEM, the cells were placed into the same medium containing 1% FBS for 18-20 h. TGZ was then added to a concentration of 20 μM. The cells were incubated in a CO_2 incubator for the indicated time at 37°C, and the attached cells washed with PBS and harvested using trypsin (0.05% trypsin, 0.5 mM EDTA in PBS). Cell numbers were counted by mounting 10 μl of the cell mixture (1:1

mixture of 0.4% trypan blue and cell suspension) onto a Tiefe Depth Profondeur 0.0025 mm^2 cell counting plate (Superior Co., Germany) and observed under the microscope.

6. Immunocytochemistry and BrdU incorporation

For immunocytochemistry, HCT15 cells were plated onto coverslips at a density of 2 x 10^5 cells/coverslip into 6-well plates, grown in DMEM containing 1% FBS for 18-20 h and treated with 20 μM TGZ for 9 h before being subjected to immunocytochemistry. If required 30 μM PD98059 was added 1 h before TGZ treatment. The cells were then washed twice with PBS, fixed in methanol/formaldehyde (99:1) mixture at –20°C for 15 min, permeabilized with PBS containing 0.2% Triton-X-100, and finally gently washed 5 times with PBS for 5 min. Cells were then treated for 30 min with blocking solution (PBS containing 1% BSA, 0.1% gelatin, and 5% goat serum), the cover slips were further incubated with anti-rabbit-p21 antibody for 2 h, and washed 5 times with PBS containing 1% BSA and 0.1% gelatin for 5 min. They were then further incubated with goat anti-rabbit-Rhodamin Red™-X-conjugated secondary antibody at a dilution of 1: 100 for 1 h, and washed 5 times with PBS for 5 min.

For the BrdU incorporation study, HCT15 cells were grown in DMEM containing 20 μM of BrdU for 5 h before performing the immunocytochemistry,

which was described as above. The cells were then fixed in 3.7% formaldehyde for 30 min at room temperature and rinsed once with PBS for 5 min before being incubated for 10 min in 2 N HCl. They were then washed three times with PBS for 5 min. After blocking, the cells were incubated with anti-BrdU moncoclonal antibody at a dilution of 1: 20 for 2 h and washed 5 times with PBS containing 1% BSA and 0.1% gelatin for 5 min. The goat anti-mouse-Cy™2-conjugated secondary antibody at a dilution of 1:100 was incubated for 1 h and the cells washed 5 times with PBS for 5 min. Each experiment was performed at least three times. DAPI was then treated at a final concentration of 1μM in PBS for 10 min, and the cells extensively washed 5 times with PBS and 3 times with distilled water. The samples were mounted for photography, which was conducted using a Radiance 2000/MP, multi-photon imaging system (Bio-Rad, UK).

Ⅲ. Results

1. TGZ transiently increases ERK activity and subsequently induces $p21^{Cip/WAF1}$ cell cycle regulator in HCT15 colorectal cancer cells

The ERK pathway, which is also often called the mitogen-activated protein kinase (MAPK) pathway is an important signaling route in cell proliferation, and aberrant activation of the MAPK pathway by genetic alterations of the signaling components often results in the development of cancers.[38-40] However, recent studies have also shown that the activation of the MAPK pathway plays a role in the growth arrest of the cells, and that this is related with the activation of the cell cycle regulator, $p21^{Cip/WAF1}$.[22,33,41] Because TGZ is known to regulate colorectal cancer cell growth,[17] we were interested to determine whether the TGZ is also involved in the activation of the ERK pathway.

When minimal serum starved HCT15 human colorectal cancer cells were stimulated by 20 μM of TGZ, ERK activities transiently increased within 15 min, peaked at 30 min and thereafter decreased (Figure 2A). Treatment with TGZ, also significantly increased the cell cycle regulator $p21^{Cip/WAF1}$ from 2 h after treatment (Figure 2A). The increased ERK activity and the subsequent $p21^{Cip/WAF1}$ induction suggested that the $p21^{Cip/WAF1}$ levels could be increased by activating ERKs.[22,23,41,42]

Therefore, we investigated whether $p21^{Cip/WAF1}$ induction is dependent on ERK

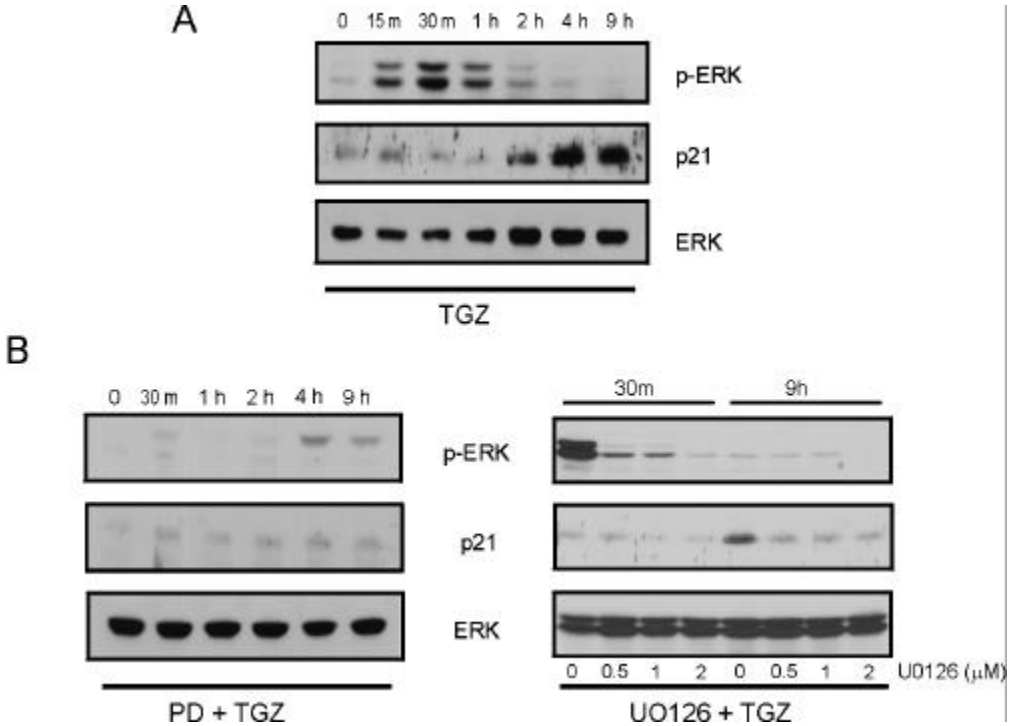

Figure 2. Transient activation of ERKs and induction of p21$^{Cip/WAF1}$ by TGZ. **(**A) HCT15 colorectal cells were grown in DMEM, as described in Materials and Methods, and then serum starved for 18 h by growing them in identical medium containing 1% FBS before treatment with 20 μM TGZ. (B) The HCT15 cells were grown as shown in Figure 2A except that 30 μM of PD98059 or 0-2 μM of U0126 was added 1 h before TGZ treatment. The cells were harvested at different time points for extract preparation. The activities of ERKs were measured by Western blot analysis using anti-phospho-ERK antibody, and the levels of p21$^{Cip/WAF1}$ and ERK proteins were detected by Western blot analysis using anti-p21$^{Cip/WAF1}$ or anti-ERK antibody.

activation by checking the effect of a MEK inhibitor, PD98059[43] or U0126[44]. Both transient activation of ERKs and subsequent induction of $p21^{Cip/WAF1}$ were mostly abolished by PD98059 or U0126 pre-treatment (Figure 2B). Therefore, the induction of $p21^{Cip/WAF1}$ by TGZ is dependent on the activation of ERKs.

2. TGZ transiently activates Raf-1-MEK-ERK module kinases and activates trans-reporter gene expression

To determine mechanism for the activation of ERKs by TGZ, we measured a modification of Raf-1 and the activation status of MEK kinases after stimulation by TGZ. Specifically, we checked changes in the kinases 30 min and 9 h after TGZ stimulation, to monitor both transient ERK activation and subsequent $p21^{Cip/WAF1}$ induction. In cells with activated ERKs by TGZ, both an increase of phospho-MEK and a modification of Raf-1, to a higher molecular weight form, were also observed within 30 min of TGZ treatment (Figure 3). Therefore, all the MAPK module kinases (Raf-1, MEK, and ERK) were concomitantly activated by TGZ.

To determine whether the signal for activating the MAPK cascade is further transmitted into nucleus or not, we used Elk-1[37] dependent trans-reporter to determine whether TGZ activates Elk-1 trans-reporter transcription. As shown in Figure 4A, TGZ did increase Elk-1 trans-reporter gene expression by around 25%, and this was inhibited by PD98059 pretreatment. However, the level of Elk-1 trans-

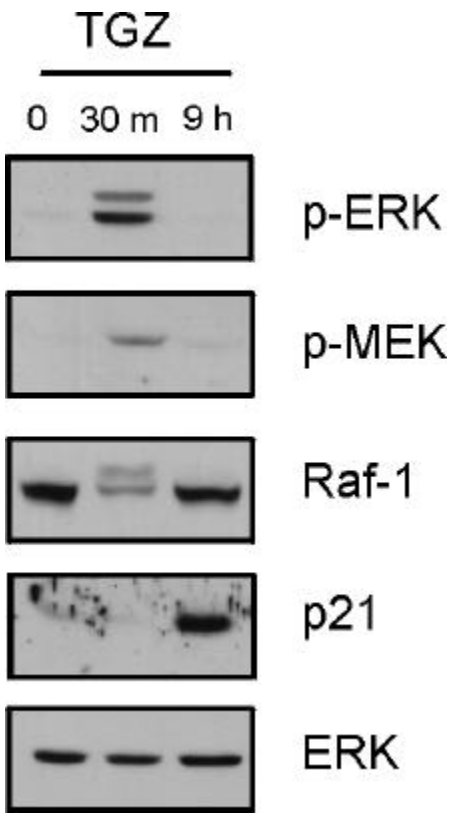

Figure 3. Activation of the ERK pathway by TGZ. HCT15 cells were grown in DMEM as described in Figure 2A, and the cells harvested at 0, 30 min, and 9 h after 20 μM TGZ treatment. ERK and MEK activity were detected by Western blot using anti-phospho-ERK and anti-phospho–MEK antibodies, respectively. Raf-1, $p21^{Cip/WAF1}$, and ERK proteins were also detected by Western blot analysis using anti-Raf-1, - $p21^{Cip/WAF1}$, and -ERK antibodies.

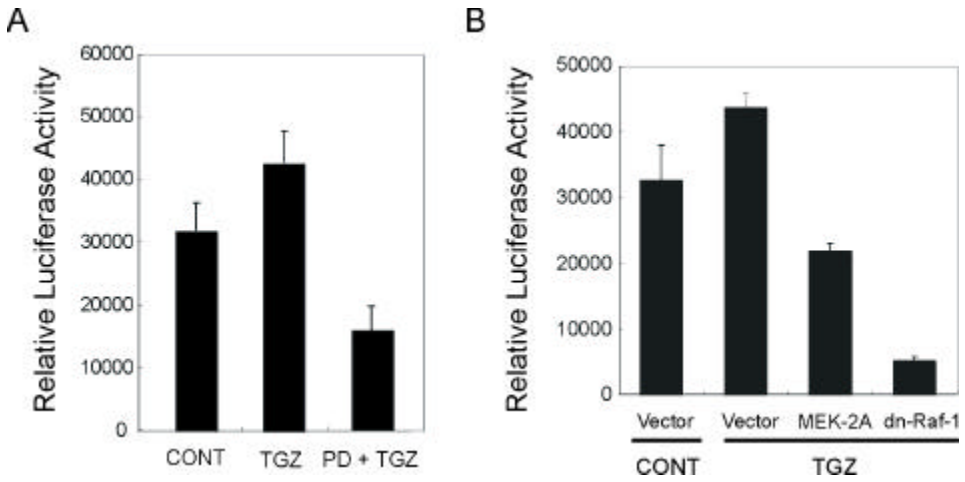

Figure 4. The activation of transcription factor through ERK pathway.

(A) HCT15 cells were grown in DMEM, and the cells were co-transfected with reporter plasmid pFR-Luc and trans-activator pFA2-Elk-1. For normalization, the cells were co-transfected with a plasmid containing the gene for β-galactosidase under the control of CMV promoter. The cells were transferred into medium containing 1% FBS for an additional 18 h and replaced with fresh medium containing 1% FBS containing 20 μM of TGZ. When required, 30 μM of PD98059 was also treated for 1 h before TGZ treatment. Extracts were made **7** h after TGZ treatment. Lucifierase activities were measured and normalized using β-galactosidase levels as a control. (B) HCT15 cells were grown and transfected, as described in Figure 4A with pFR-Luc and pFA-2-Elk-1 plasmids together with a vector, or an expression vector for kinase inactive MEK (MEK-2A), or dnRaf-1. TGZ was treated as described in Figure 4A. Each data point represents the average value of three independent experiments. Error bars indicate standard deviations.

reporter gene expression observed after PD98059 pre-treatment was much lower than the level of that was observed in resting cells (Figure 4A) suggesting high basal ERK activity in our growth condition of cells. Significantly, Elk-1 dependent trans-reporter gene expression induced by TGZ was also significantly decreased by the expression of a catalytically inactive MEK (MEK-2A) or dominant negative Raf-1 (dn-Raf-1) (Figure 4B). In this case, the levels of expression, which were lowered by either the expression of kinase inactive MEK-2A or dn-Raf-1, were also significantly lower than the levels of Elk-1 trans-reporter gene expression in resting cells.

3. Induction of $p21^{Cip/WAF1}$ by TGZ varies by colorectal cancer cell types.

To understand the relationship between cell type and the induction of $p21^{Cip/WAF1}$ by TGZ, two other colorectal cancer cell lines (HT29 and HCT116), were also examined to determine the activation status of ERKs and the induction of $p21^{Cip/WAF1}$ by TGZ. Transient ERK activation was observed in both HT29 and HCT116 (Figure 5A and 5B). However, the $p21^{Cip/WAF1}$ induction was only observed in HT29 and not in HCT116 (Figure 5). The $p21^{Cip/WAF1}$ protein level in HCT116 cells was high in the unstimulated state and was not further increased by TGZ (Figure 5B). The observed TGZ induced effects (i.e., ERK activation in HT29 and HCT116 cells, and the induction of $p21^{Cip/WAF1}$ in HT29 cells) were also inhibited by PD98059 pre-

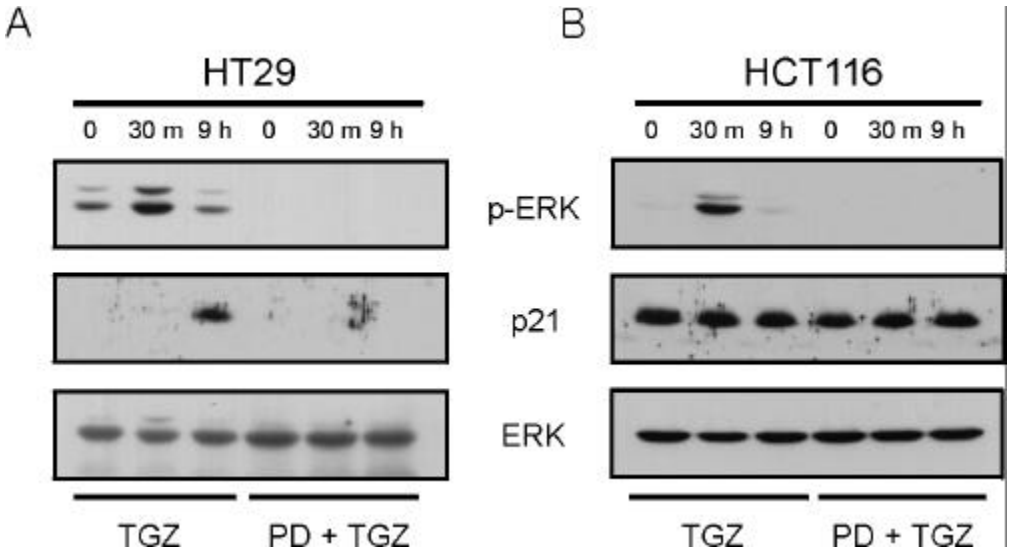

Figure 5. Induction of p21$^{Cip/WAF1}$ by TGZ varies by colorectal cancer cell types. HT29 and HCT116 cells were grown in McCoy' s 5A medium, and serum starved for 18 h by growing them in a medium containing 1% FBS before treatment with 20 μM of TGZ. Cells were harvested at 0, 30 min, and 9 h after TGZ treatment. When required, 30 μM of PD98059 was treated before TGZ treatment. The p-ERK, p21$^{Cip/WAF1}$, ERK protein levels were detected by Western blot analysis, as described in Figure 2.

treatment (Figure 5A and 5B).

4. TGZ stimulates induction and nuclear localization of $p21^{Cip/WAF1}$

To further confirm the role of TGZ in the induction of $p21^{Cip/WAF1}$, HCT15 cells were treated with TGZ and the numbers of cells inducing $p21^{Cip/WAF1}$ were determined by immunocytochemistry. When HCT15 cells were grown without stimulating media containing 1% FBS, only 3% of the cells retained high $p21^{Cip/WAF1}$ level in its nuclei (Figure 6; representative data are shown in Figure 8B). The percentage of cells inducing $p21^{Cip/WAF1}$ protein was increased to around 12% by TGZ treatment, and this was significantly reduced by PD98059 pre-treatment (Figure 6).

5. TGZ inhibits HCT15 cell growth

To determine the role of TGZ in the regulation of HCT15 colorectal cancer cell growth, we measured cell numbers after TGZ treatment. Cell numbers significantly declined over time after TGZ treatment compared to untreated control (Figure 7). Therefore, TGZ is involved in the growth inhibition of HCT15 colorectal cancer cells.

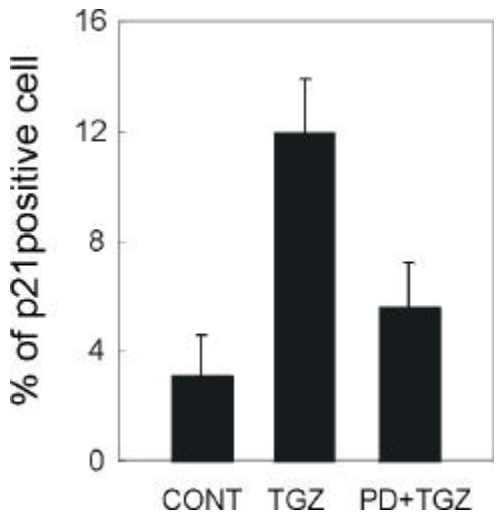

Figure 6. Activation $p21^{Cip/WAF1}$ by TGZ, and its inhibition by PD98059. HCT15 cells were grown in DMEM and treated with 20 μM TGZ for 9 h. When required, 30 μM of PD98059 was treated before TGZ treatment. Cells containing induced and nuclear localized $p21^{Cip/WAF1}$ proteins were recorded as a $p21^{Cip/WAF1}$ positive cells.

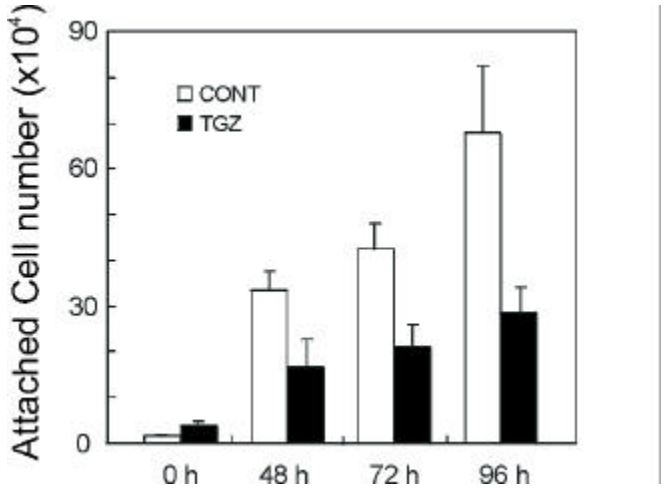

Figure 7. Inhibition of HCT15 cell growth by TGZ. HCT15 cells were seeded at a density of 0.8 x 10^5 cells per well in 12-well plates. The cells were grown in the medium containing 1% FBS for 18 h, and either treated or left untreated with 20 μM TGZ. The numbers of attached cells were counted at 0, 48, 72, and 96 h after TGZ treatment, as described in Materials and Methods. Each data point represents the average value of three independent experiments. Error bars indicate the standard deviations of three independent experiments.

6. TGZ inhibits anti-proliferation of HCT15 colorectal cancer cells through the activation of $p21^{Cip/WAF1}$

To identify a role for the TGZ in anti-proliferation of HCT15 cells, we monitored DNA synthesis at the single cell level by measuring BrdU incorporation into TGZ treated cells. In the resting state, around 53% of the HCT15 cells incorporated BrdU, but this percentage reduced to 31% after TGZ treatment (Figure 8A and 8B). Therefore, TGZ inhibits the proliferation of colorectal cancer cells by inhibiting the G1 to S phase cell cycle transition.

To understand the role of $p21^{Cip/WAF1}$, which was induced by TGZ in colorectal cancer cells, we also counted percentages of those cells that incorporated BrdU among those that induced $p21^{Cip/WAF1}$. Although around 53% cells incorporated BrdU in the total cell population, almost no cells that induced $p21^{Cip/WAF1}$ incorporated BrdU (Figure 8A and 8B). Therefore, $p21^{Cip/WAF1}$ which were induced by activation of the ERK pathway may play a role in anti-proliferation of HCT15 cells.

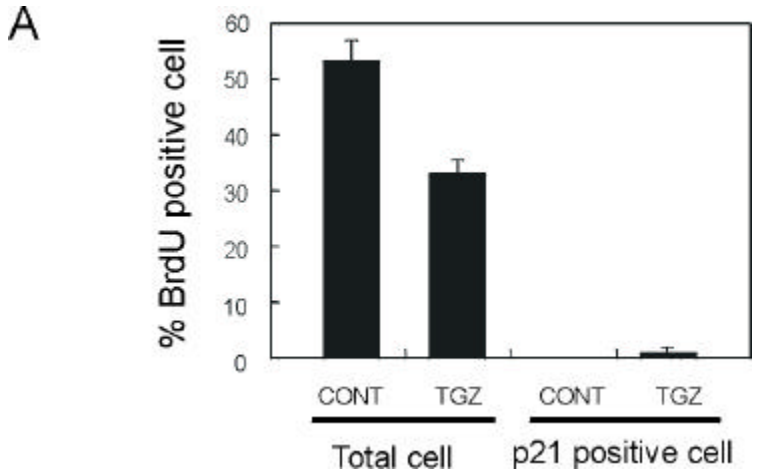

Figure 8. Inhibition of BrdU incorporation by TGZ and an effect of $p21^{Cip/WAF1}$ activation. HCT15 cells were grown in DMEM, and starved for 18 h by growing the cells in medium containing 1% FBS before treatment with 20 μM TGZ. The cells were labeled with 20 μM of BrdU for last 5 h before assay, and $p21^{Cip/WAF1}$ was visualized using anti- $p21^{Cip/WAF1}$ antibody followed by incubation with Rhodamin-Red-conjugated goat anti-rabbit IgG, and BrdU incorporated into nuclei was observed as a green color by using anti-BrdU antibody followed by Cy2-conjugated goat anti-mouse IgG. Cell nuclei were stained with DAPI. The cells containing induced and nuclear localized $p21^{Cip/WAF1}$ were scored as $p21^{Cip/WAF1}$ positive cells. The experiments were repeated at least three times. (A) Inhibition of BrdU incorporation by TGZ among total or $p21^{Cip/WAF1}$ positive cells.

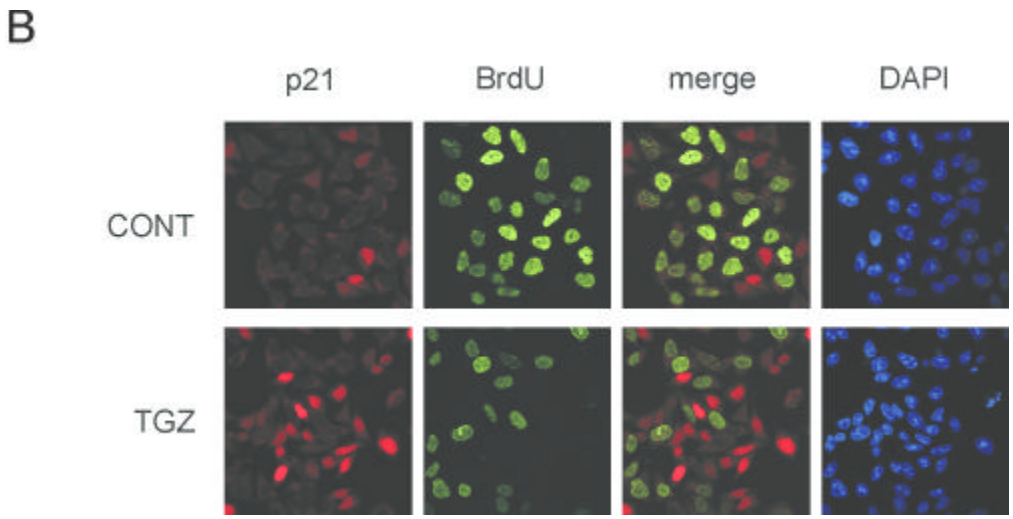

Figure 8. Inhibition of BrdU incorporation by TGZ and an effect of $p21^{Cip/WAF1}$ activation. (B) Representative data for the inhibition of BrdU incorporation by TGZ, and the inhibition of BrdU incorporation by $p21^{Cip/WAF1}$ activation.

Ⅳ. Discussion

The $p21^{Cip/WAF1}$ is a cell cycle regulator, which is involved in the anti-proliferation of cells by inhibiting cell cycle progression at the G1 to S phase, moreover, the induction of $p21^{Cip/WAF1}$ is a good indicator of growth arrest in colorectal cancer cells.[18-19] In the present study, we identified a mechanism for the activation (induction and nuclear localization) of $p21^{Cip/WAF1}$ by TGZ in HCT15 cells, and this related with cell growth. Transient activation of the ERK pathway appeared to be a route for the induction of $p21^{Cip/WAF1}$, with respect to the inhibition of colorectal cancer cell proliferation. TGZ activated ERKs by direct signal transduction, which occurred within 15 min of treatment, and might be acquired by the Raf-1→MEK→ERK module. TGZ further induced Elk-1 dependent trans-reporter gene expression, these results suggest that the signal that activates the MAPK pathway is further transmitted into the nucleus to induce the target gene(s). The levels of Elk-1 dependent trans-reporter gene expression were decreased by PD98059 or by the expression of catalytically inactive MEK/dn-Raf-1. These results suggest that TGZ increases expression of the Elk-1 trans-reporter through the activation Raf-1, MEK, and ERK module kinases.

Together with the activation of the ERK pathway, TGZ subsequently activated $p21^{Cip/WAF1}$, and this was inhibited by PD98059 or U0126 pre-

treatment, suggesting that the activation of $p21^{Cip/WAF1}$ was caused by the transient activation of ERK by TGZ. Induction time of $p21^{Cip/WAF1}$ after TGZ treatment in HCT15 cells was similar to that found in a myeloid leukemia cell line.[15] The induction of $p21^{Cip/WAF1}$ in HT29 cells, which have a p53 mutation, supports that the induction of $p21^{Cip/WAF1}$ by TGZ occurs independently on p53, via the ERK pathway.[19,22] In HCT116 cells, the $p21^{Cip/WAF1}$ level was not increased by TGZ, and the basal $p21^{Cip/WAF1}$ level was high. The $p21^{Cip/WAF1}$ protein level might be increased to a maximum inducible status in HCT116 cells, and this level could be acquired by ERK pathway activation by stimuli such as TGZ in HCT15 and HT29. It is also possible that a regulatory mechanism of $p21^{Cip/WAF1}$ effect by TGZ may be defective in HCT116 cells, and this may have resulted in the high $p21^{Cip/WAF1}$ protein level in the HCT116 cells compared to its levels in HCT15 or HT29.

The percentage of cells incorporating BrdU were decreased by around 20% by TGZ among total cell population, suggesting a role for TGZ in anti-proliferation of cells. The $p21^{Cip/WAF1}$ induced cells incorporated BrdU less than 1%, otherwise total cells including no $p21^{Cip/WAF1}$ induced cells incorporated BrdU up to 50%. Less than 1% of the cells inducing $p21^{Cip/WAF1}$ incorporated BrdU compared to 50% of the total cells were incorporated BrdU. These results show that the $p21^{Cip/WAF1}$ protein, which is induced by

the ERK pathway, is involved in the anti-proliferation of HCT15 colorectal cancer cells.

We noticed that many cells blocked BrdU incorporation independent on the $p21^{Cip/WAF1}$ induction (Figure 8). Although $p21^{Cip/WAF}$ positive cells were increased by around 9%, BrdU positive cells were decreased by around 20% by TGZ (compare Figure 6 and Figure 8A). These results suggest that TGZ also inhibits the proliferation of colorectal cancer cells via mechanism(s) independent on the induction of $p21^{Cip/WAF1}$. It is known that TGZ regulates the growth of colorectal cancer cells via a PPARγ dependent mechanism[17] and this maybe provide an alternative route for the growth regulation of colorectal cancer cells by TGZ.

In conclusion, we identified a new mechanism for the anti-proliferation of HCT15 cells by TGZ, which involved the regulation of the G1 to S phase cell cycle progression by $p21^{Cip/WAF}$ induction, and which in turn was dependent on the activation ERK pathway.

Ⅴ. Conclusion

1. In minimal serum starved HCT15 human colorectal cancer cells, TGZ activates ERKs within 15 min and the ERK activity was peaked at 30 min. The $p21^{Cip/WAF1}$ cell cycle regulator was induced from 2 h after TGZ treatment and that was blocked by pre-treatment of MEK inhibitors, PD98059 and U0126.

2. TGZ transiently activates Raf-1-MEK-ERK module kinases and Elk-1 dependent trans-reporter gene expression. The trans -reporter gene expression induced by TGZ was significantly decreaced by PD98059 pre-treatement and by a catalytically inactive MEK (MEK-2A) or dominant negative Raf-1

3. In two other colorectal cancer cell lines (HT29 and HCT116), ERK was also transiently activated by TGZ. However the $p21^{Cip/WAF1}$ induction was only observed in HT29 and not in HCT116. The $p21^{Cip/WAF1}$ protein level in HCT116 cells was high without stimulation.

4. When HCT15 cells were treated with TGZ, the induction and nuclear localiazation of $p21^{Cip/WAF1}$ was observed by immunocytochemistry. The percentage of cells inducing $p21^{Cip/WAF1}$ protein was increased from 3 % to 12 % by TGZ treatment and this was significantly reduced by PD98059 pre-treatment.

5. TGZ inhibited HCT15 colorectal cancer cell growth throughout the time course.

6. TGZ inhibited BrdU incorporation and no BrdU incorporation was

observed in most $p21^{Cip/WAF1}$ activated cells.

In conclusion, the mechanism for the anti-proliferation of HCT15 cells by TGZ involves the activation of G1→S cell cycle regulator $p21^{Cip/WAF1}$ through the activation ERK pathway.

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국문요약

Troglitazone 이 대장암 세포주인 HCT15 세포의 성장을 억제하는데 있어서 ERK pathway 의존적인 $p21^{Cip/WAF}$ 의 발현이 미치는 영향

Thiazolidinedione 계열인 Troglitazone (TGZ) 은 peroxisome proliferator activated recetor γ (PPAP γ) 의 기질로 작용하여 지방세포내에서 지방산과 지방의 대사에 관여하고, 지방세포의 분화를 일으킨다. 하지만 최근의 연구들에 의해 TGZ 이 사람의 유방암세포, 전립선암세포 그리고 대장암세포 등의 성장억제에도 관여한다는 보고가 되고 있다. 본 연구에서는 TGZ 에 의한 HCT15 대장암 세포주의 성장이 억제되는 기전을 밝히고자 하였다. HCT15 에 20 μl 의 TGZ 를 처리하면 15 분에서 1 시간 사이에 ERK (extracellular signal regulated kinase) 의 활성이 일시적으로 증가하였다. 이러한 ERK 의 활성화는 세포주기 조절인자인 $p21^{Cip/WAF}$ 의 발현증가와 핵 속으로의 이동을 유도하였다. TGZ 를 처리하였을 때 ERK 상위단계의 신호전달 물질들인 Raf-1 의 변화와 MEK 의 활성화를 관찰하였으며, mitogen activated protein kinase (MAPK) 의 활성화에 의존하는 Elk-1 trans-reporter 유전자의 발현이 증가되었다. TGZ 에 의해 일어나

는 $p21^{Cip/WAF}$ 의 발현증가와 핵 속으로의 이동은 MEK 의 활성저해제인 PD98059 를 전처리 했을 때 방해되었는데 이것은 ERK 가 $p21^{Cip/WAF}$ 의 활성에 관여함을 보여준다. TGZ 은 세포가 BrdU 를 핵 DNA 에 삽입 하는 것을 방해하였으며 $p21^{Cip/WAF}$ 이 활성화된 세포에서는 BrdU 가 거의 삽입되지 못하였다. 이러한 결과는 TGZ 가 대장암 세포주인 HCT15 세포의 성장을 억제하는데 있어서 ERK 에 의해 유도된 $p21^{Cip/WAF}$ 의 발현이 관여한다는 것을 시사한다.

핵심되는 말: Troglitazone; ERK; $p21^{Cip/WAF1}$; 세포주기; 성장억제