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Growth inhibition through activation of peroxisome proliferator-activated receptor γ in human oesophageal squamous cell carcinoma

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Abstract

Peroxisome proliferator-activated receptor γ (PPARγ) heterodimerises with retinoid X receptor α (RXRα) and is thought to be a novel therapeutic target for human malignancies. We evaluated the ability of troglitazone (TRO) alone or in combination with 9-*cis* retinoic acid (9CRA), ligands of PPARγ and RXRα, respectively, to inhibit the growth of oesophageal squamous cell carcinoma (OSCC). All 10 tested OSCC cell lines of a KYSE series expressed PPARγ and RXRα at both the mRNA and protein levels. In four tested cell lines, TRO inhibited growth, and a synergistic effect was observed with simultaneous 9CRA application. In KYSE 270 cells, a luciferase reporter assay showed that the simultaneous application of TRO and 9CRA to the cells increased the relative luciferase activity approximately 20-fold compared with the controls without TRO or 9CRA application. In this cell line, flow cytometry demonstrated that combined treatment with TRO and 9CRA greatly increased the sub-G1 phase, and Hoechst 33342/propidium iodide (PI) staining showed that apoptotic cell death was mainly induced through ligand treatment. In addition, implanted tumours in nude mice showed significant inhibition of tumour growth when treated with TRO. These results suggest that the PPARγ/RXRα heterodimer may be a new therapeutic target for OSCC.

Keywords: Nuclear receptor; PPARγ; RXRα; Troglitazone; 9-*cis* Retinoic acid; Oesophageal squamous cell carcinoma

1. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the subfamily of nuclear hormone receptors that includes receptors for steroids, retinoic acid, vitamin D and thyroid hormone [1]. PPARγ heterodimerises with retinoid X receptor α (RXRα) and binds to a DNA response element to regulate the expression of specific target genes [2,3]. Transcriptional activity of the PPARγ/RXRα heterodimer is maximal in the presence of both PPARγ and RXRα activators [3]. 15-Deoxy-$\Delta^{12,14}$ prostaglandin J_2 and various polyunsaturated fatty acids have been identified as natural receptor ligands of PPARγ [4–6]. As synthesised ligands of PPARγ, a series of thiazolidinedione (TZD) agents are useful for the treatment of type II diabetes [4,7].

PPARγ is predominantly expressed in adipose tissues, and functions as a central regulator of adipocyte differentiation [8]. However, recent studies have shown that PPARγ is also expressed in other cell types, and its role in cell proliferation and cancer has attracted interest. Several studies reveal that PPARγ ligands can induce growth inhibition in human cancer cells of different tissue origin, including liposarcoma [9], breast cancer [10,11], colon cancer [12–14], prostate cancer [15,16], pancreatic cancer [17–19] and gastric cancer [20,21]. Treatments with PPARγ ligands reduce growth rates based on specific mechanisms including induction of terminal differentiation [9,10,12,15,19], cell-cycle arrest [9,13,14,17,19], apoptosis [11,18,20,21], and non-apoptotic cell death [15,16].

There are two classes of retinoid receptors, RARs and RXRs, each with three sub-types [22]. These receptors form RXR/RAR heterodimers or RXR/RXR homodimers [22], and RXR also heterodimerises with PPARs, as mentioned above, or other nuclear receptors. Recent

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reports have indicated that selective RAR or RXR ligands mediate growth inhibitory effects through RXR/RAR heterodimers or RXR/RXR homodimers in some squamous cell carcinoma cells [23,24]. However, the role of PPARγ/RXRα heterodimers in human oesophageal squamous cell carcinoma (OSCC) is still unclear.

In the present study, we focused on the PPARγ/RXRα heterodimeric system and investigated the effects of ligands on this system on the cellular growth of human OSCC cells.

2. Materials and methods

2.1. Reagents

Troglitazone (TRO), a PPARγ ligand, was kindly provided by Sankyo Pharmaceuticals (Tokyo, Japan). 9-*cis* Retinoic acid (9CRA) was purchased from Sigma (St. Louis, MO, USA). Antibodies against PPARγ and RXRα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody against poly (ADP-ribose) polymerase (PARP) was purchased from CALBIOCHEM (Darmstadt, Germany). Enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK), and the protein assay reagents were purchased from Bio-Rad (Hercules, CA, USA). Hoechst 33342 was obtained from Nakalai Tesque, INC. (Kyoto, Japan). All other reagents and compounds were of analytical grades.

2.2. Cell cultures

Human oesophageal squamous carcinoma cell lines, KYSE series, were established in our department and cultured in Ham's F12/Roswell Park Memorial Institute (RPMI) 1640 with 2% (v/v) fetal calf serum (FCS), according to the method previously reported in Ref. [25]. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. TRO and 9CRA were dissolved in dimethyl sulphoxide (DMSO) to a final concentration of 0.1% (w/v) in the culture medium.

2.3. RNA extraction

Total RNA was extracted from cultured cells using the acid guanidinium thiocyanate/phenol/chloroform method [26]. The resultant RNA samples were quantified using a spectrophotometer at a wavelength of 260 nm.

2.4. Reverse transciptase-polymerase chain reaction (RT-PCR)

An aliquot of 5 μg of total RNA from each sample was reverse transcribed to cDNA using the First-Strand cDNA Synthesis Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the manufacturer's instructions. For detection of human *PPARγ* mRNA, human *RXRα* mRNA, and human glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) mRNA, the following combinations of primers were used: 5′-TCTCTCCGTAATGGAAGACC-3′ and 5′-GCATTATGAGACATCCCCAC-3′ for *PPARγ*; 5′-CCGCACCCTGGAAGCACACG-3′ and 5′-GGAGGGGCTCGGGGTCATCT-3′ for *RXRα*; and 5′-TGGTATCGTGGAAGGACTCATGAC-3′ and 5′-ATGCCAGTGAGCTTTCCCGTTCAGC-3′ for *G3PDH*. The reaction conditions were 35 cycles of amplification (95′C for 40 s, 55′C for 50 s and 72′C for 90 s). In all cases, RNA was processed without reverse transcriptase and the non-template control was used to check carry-over contamination. The PCR products were separated electrophoretically on a 2% (w/v) agarose gel and stained with ethidium bromide.

2.5. Western blotting analysis

Cells were lysed in a sample buffer (2% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 50 mM Tris–HCl, pH 6.8) at room temperature. Cell lysates were sonicated and then centrifuged at 12 000*g* for 30 min, and the supernatants were harvested. Protein concentrations were estimated by Bradford method using Biorad Protein Assay Reagent. Then, 20 μg of protein was boiled at 95 °C and separated by 8% (w/v), 10% (w/v) or 14% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA, USA). The membranes were then blocked with TBS (20 mM Tris, 150 mM NaCl, pH 7.6) containing 5% (w/v) skim milk (Difco, Detroit, MI, USA) and 0.1% (v/v) Tween 20 for 16 h and incubated at room temperature for 1 h with the various concentrations of the primary antibodies described above. The membrane was subsequently incubated at room temperature for 1 h with horseradish peroxidase-linked goat anti-rabbit IgG or goat anti-mouse IgG (EY Laboratories, Inc., San Mateo, CA, USA) and analysed using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.6. Cell counts

KYSE cells were seeded at a density of 2×10^4/well in a six-well plate. After a 48-h incubation period, the cells were again incubated with 10 μM of TRO and 5 μM of 9CRA. At days 0, 1, 3, 5 and 7, the cells were harvested by trypsinisation using trypsin/ethylene diamine tetra acetic acid (EDTA) and counted under the microscope after trypan-blue staining. Three independent experiments were carried out.

2.7. Transfections and luciferase assays

The reporter plasmid, PPRE×3-TK-LUC was kindly donated by Kazuhiko Umezono (Institute for Virus Research, Kyoto University, Kyoto, Japan). KYSE 270 cells were seeded at a concentration of 4×10^5 cells/35-mm dish and transfected with the plasmids 24 h after transfer to fresh media. Transfection was performed using Lipofectin reagent (Life Technologies, Inc., Carlsbad, CA, USA) mixed with 2 μg of PPREX3-TK-LUC and 0.2 μg of pRL-TK (Promega, Madison, WA, USA) for 6 h. The transfection mixture was replaced by the complete media and incubated for 24 h. Then the media was exchanged for new media containing DMSO and several doses of ligands and incubated for an additional 16 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) in a luminometer (MiniLumat, Berthold, Widbad, Germany). The enzyme activity was normalised for efficiency of transfection by dividing by the sea pansy luciferase activity, and relative values were determined. Three independent transfection experiments were carried out, and the average values were calculated.

2.8. Fluorescent activated cell sorting (FACS) analysis

After the treatment, floating and adherent KYSE 270 cells were harvested, washed in phosphate buffered solution (PBS) and fixed in ice-cold 70% (v/v) ethanol for at least 1 h. After washing with PBS, cells were treated with PBS containing 100 mg/ml RNase A (Dnase-free) at 37 °C for 30 min. After centrifugation, cells were resuspended in PBS containing 50 mg/ml propidium iodide (PI) and stained at room temperature for 30 min. DNA contents were analysed by FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). Cell-cycle analysis was performed in duplicate.

2.9. Morphological analysis

Nuclear morphology was assessed using Hoechst 33342 and PI staining. KYSE 270 cells were incubated with 10 μM of TRO and/or 5 μM of 9CRA for 24 h. Cells were harvested and labelled with Hoechst 33342 (5 μg/ml) and PI (2.5 μg/ml) at 37 °C for 10 min. The cells were examined by fluorescence microscopy according to the method previously reported in Ref. [27]. Intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and intact pink nuclei were considered viable, early apoptotic, late apoptotic and necrotic cells, respectively. Morphological analysis was performed in duplicate. The percentage of viable, apoptotic, and necrotic cells were represented out of a total of 1000 cells counted.

2.10. In vivo tumour growth assay

Balb/c-nu/nu mice were purchased from Charles River Co. Ltd. (Wilmington, MA, USA). A total of 2×10^6 KYSE cells were injected subcutaneously (s.c.) into each mouse. A week after inoculation, mice were randomly divided into two groups of six. TRO (200 mg/kg/day) suspended in 0.5% (w/v) carboxylmethylcellulose or vehicle was given orally by gavage six times per week for 4 weeks. There were no RXRα ligands suitable for the animal study; therefore only TRO was administrated. Tumour size was measured every week with callipers and calculated by the formula: $A \times B^2/2$, where A = length and B = width of the tumour. The statistical difference between the two groups was determined by the Student's *t*-test on the last day of administration. All the animal experiments were performed in accordance with institutional guidelines.

3. Results

3.1. Expression of PPARγ and RXRα

We first analysed the expression of human *PPARγ* mRNA and *RXRα* mRNA in 10 human OSCC cell lines using RT-PCR. All of the tested cell lines expressed both *PPARγ* mRNA and *RXRα* mRNA (Fig. 1a). Furthermore, PPARγ protein and RXRα protein were

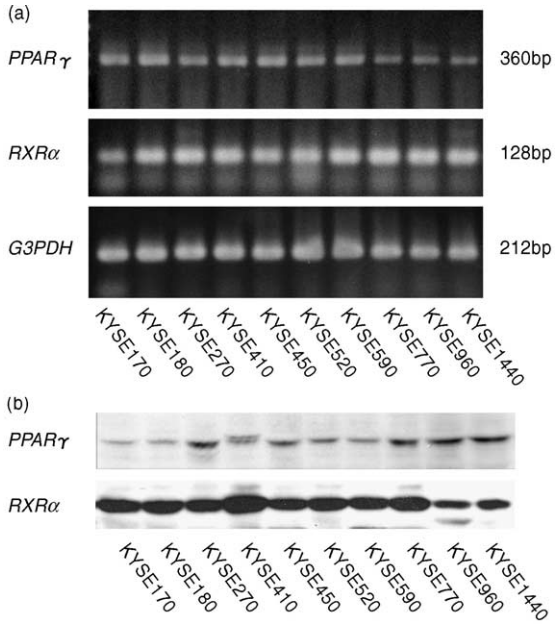

Fig. 1. Expression of peroxisome proliferator-activated receptor γ (*PPARγ*) and retinoid X receptor α (*RXRα*) in 10 oesophageal squamous cell carcinoma (OSCC) cell lines: (a) reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using specific primers for *PPARγ*, *RXRα* and glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*); (b) Western blotting analysis of PPARγ and RXRα. Equal amounts of the proteins were loaded in each lane, which was confirmed by Ponseau S staining. bp, base pairs.

expressed in all of the 10 cell lines (Fig. 1b). Two bands were detected in KYSE 410, demonstrating that PPARγ2 expression was more prominent in KYSE 410 than in the other cell lines.

3.2. Growth inhibition of oesophageal cancer cells by thiazolidinediones

We investigated the effect of TRO alone or in combination with 9CRA on the growth of KYSE cells (Fig. 2). TRO decreased the cell growth of all tested cell lines, and the effect became apparent after several days of the treatment. 9CRA also inhibited cell growth, and the combination of TRO and 9CRA strongly blocked cell growth in all of the cell lines. Especially in KYSE 270, simultaneous treatment of TRO and 9CRA completely blocked cell growth, so we selected this cell line for further examination.

3.3. PPRE transfection in KYSE cells

To determine whether PPARγ was functional in OSCC cells, KYSE 270 cells were transiently co-transfected with PPREX3-TK-LUC and pRL-TK. No significant differences were found in the luciferase activity between untreated cells and cells treated with DMSO as a vehicle (data not shown). Relative luciferase activity was greater in cells treated with both TRO and 9CRA than in those treated with TRO alone. The relative luciferase activity in the cells treated with TRO and 9CRA increased approximately 20-fold compared with vehicle control cells (Fig. 3).

3.4. Cell-cycle analysis

To investigate the mechanism of growth inhibition by the ligands of the PPARγ/RXRα heterodimeric system, we used FACS analysis to reveal the effect of two ligands on the cell-cycle profile. After 24 h of serum starvation and following 36 h of treatment, both attached and floating cells were subjected to FACS analysis. The proportion of sub-G1 cells increased from 6.5% in the controls to 28.6, 9.3, and 70.1% in the TRO, 9CRA and combination-treated cultures, respectively (Fig. 4).

3.5. Induction of both apoptotic and non-apoptotic cell death

To assess the type of cell death induced by activation of the PPARγ/RXRα heterodimeric system, we used

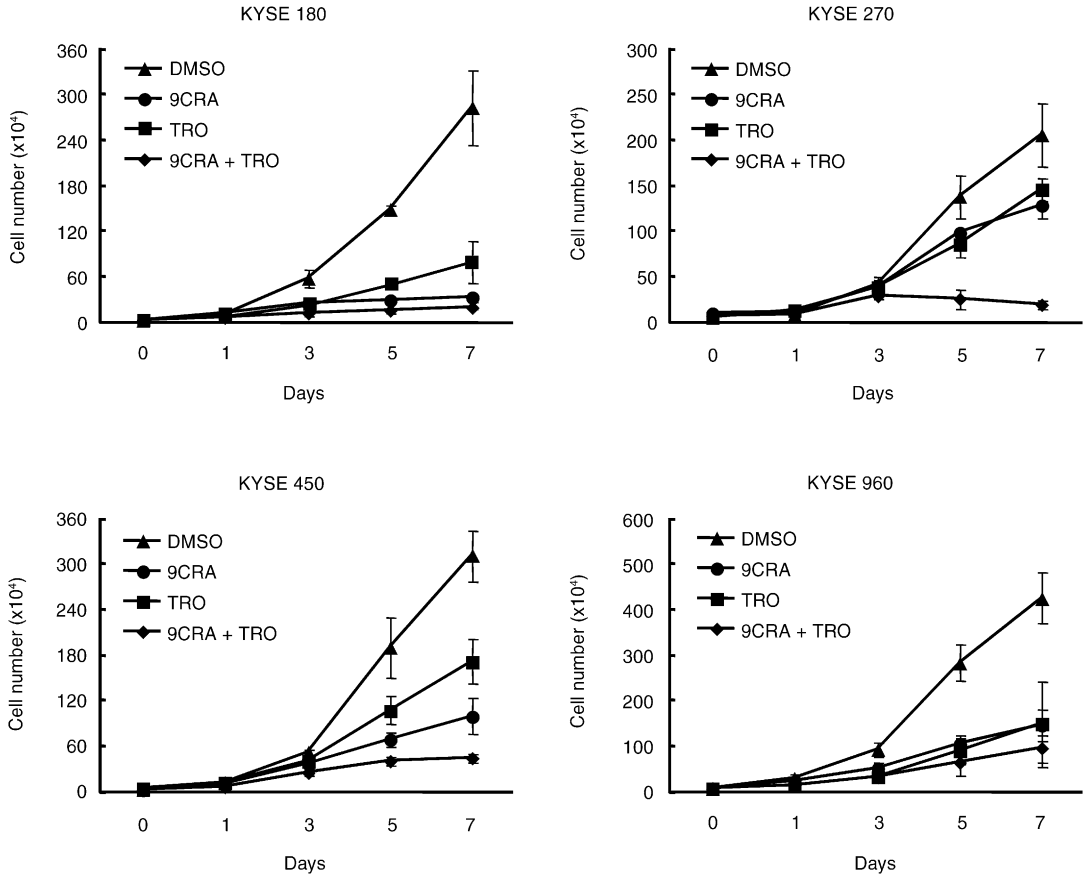

Fig. 2. Effects of troglitazone (TRO) (10 μM), 9-*cis* retinoic acid (9CRA) (5 μM), combination of TRO with 9CRA, and dimethyl sulphoxide (DMSO) in KYSE cells. Cells were seeded 2 days before the treatment, counted at days 0, 1, 3, 5 and 7. Error bars represent the unbiased standard deviations (S.D.).

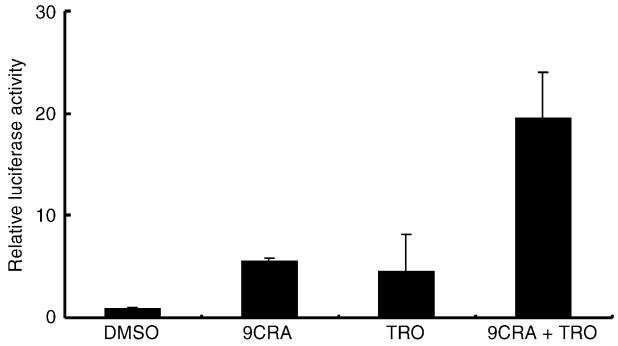

Fig. 3. PPRE transactivation in KYSE 270 cells. Luciferase activity was analysed by the dual luciferase assay after normalising for transfection efficacy by dividing by the sea pansy luciferase activity. Error bars represent the unbiased S.D.

Hoechst 33342/PI staining and analysed the morphological features of KYSE 270 cells. After a 24-h serum starvation period, cells were treated with ligands for 24 h. Combined treatment with TRO and 9CRA induced both apoptotic and non-apoptotic cell death (Fig. 5). However, apoptotic cells were predominantly observed (Table 1).

3.6. Detection of caspase activation

We examined Poly (ADP-ribose) polymerase (PARP) fragmentation by Western blotting analysis to determine whether caspase activation mediated the growth inhibition (Fig. 6). A cleaved fragment of PARP was detected in the extract from the cells treated with TRO or 9CRA alone. Furthermore, the cleaved fragment was observed at an earlier time point in the cells treated with the combination of TRO and 9CRA than in those treated with TRO or 9CRA alone.

3.7. Effect of PPARγ ligand on tumour growth in vivo

Finally, we investigated whether the PPARγ ligand could affect tumour growth *in vivo* using KYSE 270 cells in Balb/c-nu/nu mice. A week after a s.c., TRO or vehicle was given orally by gavage six times per week. Tumour volumes were measured every week (Fig. 7). TRO suppressed the growth of the tumour in a statistically significant manner ($P = 0.0013$).

4. Discussion

In the present study, we found that OSCC cell lines expressed PPARγ and RXRα and the ligands of these nuclear receptors inhibited the cell growth. Interestingly, simultaneous treatment of these two ligands induced an additive growth inhibitory effect. In KYSE 270 cells, treatment with a single ligand resulted in 40%

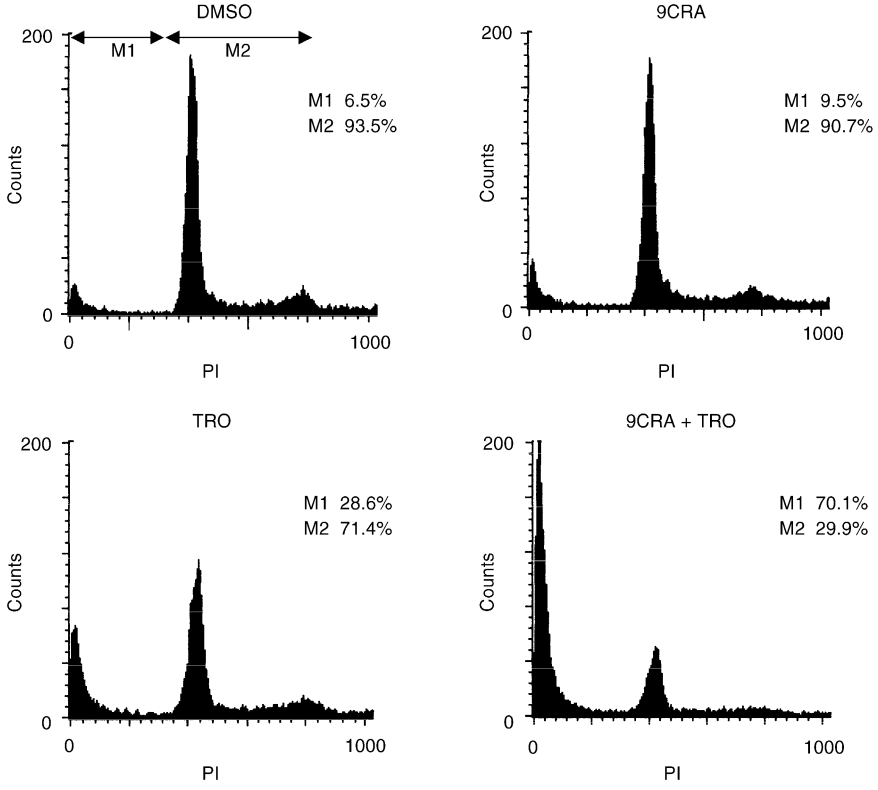

Fig. 4. Effects of ligands on cell cycle profile. KYSE cells were treated with dimethyl sulphoxide (DMSO), troglitazone (TRO) (10 μM), 9-*cis* retinoic acid (9CRA) (5 μM), and the combination of TRO with 9CRA for 36 h. Cells were harvested, stained with propidium iodide (PI) and analysed by FACScan.

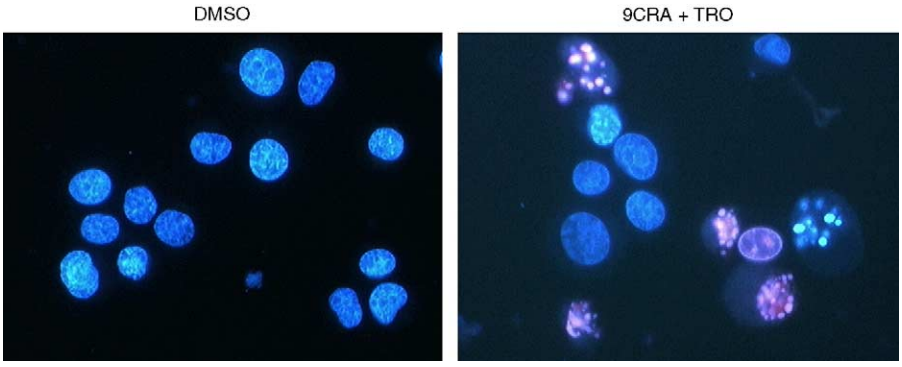

Fig. 5. Morphological changes of cells simultaneously treated with troglitazone (TRO) and 9-*cis* retinoic acid (9CRA). The cells were harvested and stained with Hoechst 33342/PI. Cells with blue intact nuclei were viable, whereas those with blue fragmented nuclei were early apoptotic. Cells with pink intact nuclei were necrotic, whereas cells with pink fragmented nuclei were late apoptotic. The photomicrographs were taken at a ×400 magnification.

growth inhibition, but simultaneous treatment with both ligands caused complete growth inhibition, in a additive effect. Both 9CRA and TRO increased transcriptional activity, and simultaneous treatment with 9CRA and TRO increased relative luciferase activity greater than single treatments with 9CRA or TRO. These results indicate that, as in other previous reports, OSCC cells also have a functional PPARγ/RXRα heterodimeric system [2,3], and we consider that TRO and 9CRA inhibit cell growth partly through the PPARγ/RXRα heterodimeric system.

Thiazolidinediones have been reported to possess activities linked to the induction of cell cycle arrest, terminal differentiation and apoptotic or non-apoptotic cell death in various cancers, although the mechanism of growth inhibition has been different in each report [9–21]. In our present study, with the maximal activation of the PPARγ/RXRα heterodimer in KYSE 270 cells, cell-cycle analysis by flow cytometry demonstrated that the sub-G1 phase greatly increased, and Hoechst 33342/PI staining showed that apoptotic cell death was mainly induced. The activation and action of caspase are believed to be pivotal steps in the mechanism of apoptosis. We assessed the proteolysis of poly (ADP-ribose) polymerase (PARP) because the increase of the cleaved fragment indicated the activation of caspase 3 or other caspase family members. Both TRO and 9CRA induced PARP proteolysis, and these effects appeared at an early time point in the combination treatment. These results suggest, like previous reports [18], that cell death induced by TRO/9CRA in KYSE 270 was caused by activation of the caspase pathway.

Table 1
The percentage of viable, apoptotic and necrotic cells in KYSE 270 cells after treatment for 24 h

Treatment	% Viable	% Apoptotic	% Necrotic
DMSO	95.4	1.8	2.8
9CRA	94.3	3.8	1.9
TRO	92.2	4.0	3.8
9CRA + TRO	75.6	19.5	4.9

DMSO, dimethyl sulphoxide; 9CRA, 9-*cis* retinoic acid; TRO, troglitazone.

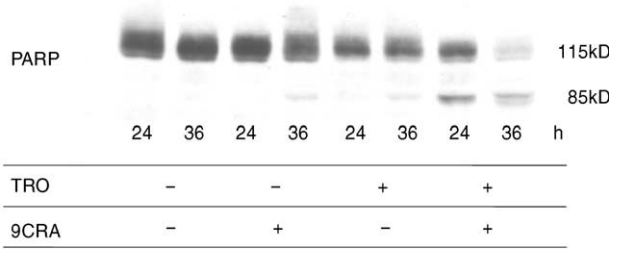

Fig. 6. Proteolysis of poly (ADP-ribose) polymerase (PARP). KYSE 270 cells were treated with dimethyl sulphoxide (DMSO), troglitazone (TRO) (10 μM), 9-*cis* retinoic acid (9CRA) (5 μM) and the combination of TRO with 9CRA. The cleaved PARP appeared as an 85-kD band.

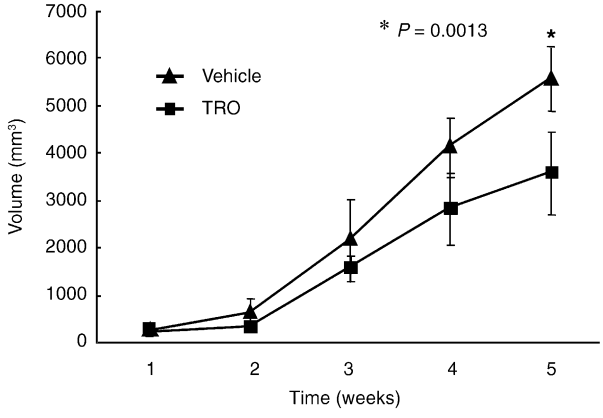

Fig. 7. Effect of troglitazone (TRO) on the growth rate of tumours in nude mice. Mice injected with KYSE 270 cells were given either vehicle or TRO for 4 weeks. On the last day of dosing, the statistical difference between the tumour sizes was determined by the Student's *t*-test ($P = 0.0013$).

Whether a cell has undergone apoptosis or non-apoptotic cell death in response to a given stimulus might be determined by the intracellular milieu. The intracellular level of adenosine triphosphate (ATP) is reported to be a determinant of cell death [28]. In our experimental procedure, TRO-treated cells showed a higher percentage of non-apoptotic cell death compared with 9CRA-treated cells (Table 1). These results indicate that TRO might affect the intracellular ATP level and induce non-apoptotic cell death in KYSE 270 cells. A recent report shows that thiazolidinediones inhibit cell growth by inhibiting translation initiation through a PPAR-independent pathway [29]. Some unknown pathways might be involved in the growth inhibitory effects of combined treatment with TRO/9CRA. Another study shows that all *trans*-retinoic acid (ATRA) and TRO exhibit synergistic growth inhibitory effects in human breast cancer cells [11]. Furthermore, it is reported that selective RAR ligands and paclitaxel express synergistic growth inhibitory effects in cancer [30]. While 9CRA binds to both RAR and RXR, growth of some KYSE cells was inhibited by 9CRA and ATRA in the same manner as that of KYSE 270 cells (data not shown). Thus, it is possible that the synergistic interaction of TRO and 9CRA might stem from a simultaneous activation of RXR/RAR and PPARγ/RXRα heterodimers.

Our animal studies revealed that TRO is also effective *in vivo*. The growth inhibitory action of TRO was statistically superior compared with animals receiving vehicle. Unfortunately, there was no appropriate RXRα ligand for the animal study, so we could not examine whether the synergistic growth inhibitory effect through the maximal activation of PPARγ/RXRα heterodimer exists *in vivo*. Indeed, TRO could only reduce the tumour growth rate. Further study is needed using not only the PPARγ ligand, but also a RXRα ligand that is suitable for clinical use, to determine whether the synergistic effect exists *in vivo* on OSCC.

Oesophageal cancer, one of the most lethal malignant neoplasms, is frequently observed in several countries. The results presented here suggest that therapy targeted at the PPARγ/RXRα heterodimer system might be a promising approach for the future treatment of OSCC.

Acknowledgements

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