Osteosarcoma (OS) is the most common primary malignant tumor of the bone and this disease primarily afflicts individuals in the second and third decade of life. Many helpful chemotherapeutic regimens are currently being used for the treatment of OS, and they have improved the survival rates in recent years. Yet there still patients who don't respond to chemotherapy. Moreover, the toxic and adverse effects associated with chemotherapy can significantly reduce the quality of a patient's life. Therefore, more efficacious therapeutic drugs are needed to improve the quality of a patient's life and the long-term OS survival rate.

Recent studies have proposed using Peroxisome Proliferator-Activated Receptor (PPAR) ligands as a new chemotherapeutic agents to treat human malignant tumors. PPAR is expressed mainly in adipose tissue, and it is also found in osteoblasts, cartilage, myocytes, hepatocytes, fibroblasts, endothelial cells, epithelial cells, and many types of cancer cells. Recent studies have showed that PPARγ ligands, such as troglitazone, pioglitazone and ciglitazone exhibit growth inhibitory effects by inducing the differentiation and apoptosis, or by inhibiting angiogenesis in a variety of human malignant cells.

This study investigated the relationship between the effects of TRO on the cellular growth in two human osteosarcoma cell lines (U2OS and HOS).

**Materials and Methods**

1. **Ligands and cell culture**

Troglitazone (TRO) was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The stock solution was prepared at a concentration of 100 mmol/L in ethanol.

The human osteosarcoma cell lines HOS and U2OS were obtained from the Korean Cell Line Bank (KCLB) and they were cultured in RPMI 1640 (HOS) or McCoy’s 5A medium (U2OS) that contained 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL) in a water-jacketed incubator with a humidified atmosphere (5% CO₂, 95% air) at 37°C. In all the experiments, the cells were left to recover from trypsinization and they were treated 24 hours after being plated. The amount of TRO used was 0.1, 1 and 10 μM.

**Purpose:** We wanted to investigate the effects of Troglitazone (TRO) on the cellular growth in two human osteosarcoma cell lines (U2OS and HOS).

**Results:** TRO inhibited the growth of both the osteosarcoma cell lines. TRO induced G0/G1 arrest in the cell cycle progression for both osteosarcoma cell lines. TRO induced the apoptosis of HOS cells, but it decreased apoptosis of the U2OS cells. TRO induced Rb dephosphorylation and the increased expression of p21<sup>WAF1</sup>. TRO increased the PTEN and Bcl-2 expressions and it decreased the level of pAkt, pRb and Bax.

**Conclusion:** The present study suggested that TRO may be used as a chemotherapeutic agent for the treatment of human OS. Yet further study is required for uncovering the precise the mechanism of TRO.

**Key Words:** Osteosarcoma, Troglitazone (TRO)
of ethanol added as the vehicle never exceeded 0.1% of the total volume.

2. Effects of troglitazone on the growth of osteosarcoma cell lines

Each cell line was seeded at $1 \times 10^5$ cells/mL per 100 mm on a culture plate. The culture medium was replaced with fresh medium and then TRO or ethanol was added after 24 hours. After 48 hours, the adherent cells were detached with trypsin/EDTA, and the cell number was determined with using a hemocytometer. Cell viability was assessed by trypan blue exclusion assay.

3. FACScan analysis of cell cycle and apoptosis

For the cell cycle analysis, the cells were plated at an initial density of $1 \times 10^6$ cells/mL per 100 mm onto culture plates. The culture medium was replaced with fresh medium and then TRO or ethanol was added after 24 hours. After washing with PBS, the cells were incubated with propidium iodide (Sigma Chemical Corporation, St. Louis, MO, USA) staining solution at room temperature for 1 hour in the dark. The stained cells were analyzed by using a FACStar flow cytometer (Becton-Dickinson, San Jose, CA). The percentages of the cells in the sub-G1, G0/G1, S and G2/M phases were determined by cell Quest software (Becton-Dickinson).

4. TUNEL assay

To study the apoptosis of the cultured cells, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling of DNA fragmentation sites) assay was performed. $5 \times 10^5$ cells/slide were incubated in the presence or absence of 20 μM TRO as an immunohistochemical probe on the slides for 48 hours. Then counted the total cells and the TUNEL-positive cell in ten randomly chosen high power fields. The cells containing condensed chromatin were counted as cells experiencing apoptosis. Positive and negative controls were performed using the cells treated with 1 lg/mL DNase I and using the cells without terminal deoxynucleotidyl transferase treatment.

5. Western blot analysis

The concentrations of the total proteins in the extracts were determined by using the Bradford protein assay, and bovine serum albumin (BSA) was used as a protein standard. Cell extracts (30 g of the protein/lane) were separated on 6-12% SDS-polyacrylamide gels with the Tri-glycine buffer system, and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Boehringer Mannheim, Germany) with using a semi-dry transfer system as recommended by the manufacturer (Bio-Rad, Hercules, CA). The following primary antibodies were used in this study: antibodies against PTEN (Upstate, IL, USA), tAkt, pAKt (Cell Signaling, MA, USA), Bcl-2, Bax, pRB, p21$^{cyc}(\text{Santa Cruz, CA, USA}), \text{and } \beta$-actin (Sigma, USA).

6. Statistical analysis

The $t$ test was used to determine the statistical significance of the obtained data and to compare the means of the two groups. One-way ANOVA and the Student-Newman-Keuls test were used for multiple comparisons. $p$ values<0.05 were considered statistically significant.

![Fig. 1. The TRO-mediated growth effects in human osteosarcoma cells. U2OS and HOS osteosarcoma cells were treated with either ethanol alone or with different concentrations of TRO, as indicated.](image-url)
RESULTS

1. Troglitazone inhibits the growth of osteosarcoma cell lines

As shown in Fig. 1, inhibition of cell growth was observed in the U2OS and HOS cells treated with TRO. Inhibition of cell growth was evident at the concentration of 10 μM of TRO in the U2OS cells and at the concentration of 20 μM of TRO in HOS cells.

2. Troglitazone induces G0/G1 arrest in the cell cycle progression for the osteosarcoma cell lines

When the OS cells were treated with TRO at the concentration of 5 μM, 10 μM, 20 μM, and 50 μM during the 48 hours, the proportion of cells in the G1 phase was increased (Fig. 2, 3). These results indicate that the growth inhibition of the OS cells following TRO treatment can be attributed to arrest in the G1 phase.

3. Troglitazone induced apoptosis of the HOS cells, but it decreased apoptosis of the U2OS cells

As shown in Fig. 4, the TRO effects on apoptosis showed different results according to the cell lines. The apoptosis of the U2OS cells that naturally occurred in the culture was decreased by treatment of TRO. On the other hand, the apoptosis of the HOS cells were increased by TRO treatment at the concentrations of 10 μM, 20 μM, and 50 μM. To further examine this, we performed TUNEL analysis to evaluate the apoptotic effects of TRO. The results of the TUNEL analysis were identical with the flow cytometric analysis (Fig. 5). These two independent methods of measuring apoptosis provided evidence that the effects of TRO on the apoptosis were different depending on the cell lines. As shown in Fig. 4, U2OS cells treated with 10 μM and 20 μM of TRO during 48 hours showed a gradually increased expression of Bcl-2 and a mildly decreased expression of Bax. On the other hand, the expression of Bcl-2 was significantly decreased in the HOS cells treated with 10 μM and 20 μM of TRO in a dose-dependent manner. These results indicate that the decreased apoptosis of the TRO treated U2OS cells was mediated by the increased expression of Bcl-2 and the decreased expression of Bax. On the contrary, TRO acts as an apoptosis inducing factor to the HOS cells by decreasing

Fig. 2. TRO induced G1 cell cycle arrest and it decrease of apoptosis in the U2OS cells.

Fig. 3. TRO induced G1 cell cycle arrest and increase of apoptosis in the HOS cells.

Fig. 4. The TRO effects of apoptosis of TRO showed different results according to the cell lines. (A) Apoptosis of the U2OS cells was decreased by TRO treatment. (B) U2OS cells showed a gradually increased expression of Bcl-2 and a mildly decreased expression of Bax by western blot analysis. (C) On the other hand, apoptosis of the HOS cells were increased by TRO treatment. (D) The expression of Bcl-2 was significantly decreased in the HOS cells.
4. Troglitazone induces Rb dephosphorylation and it increases the expression of p21Cip1

Hyperphosphorylation of Rb is a critical event in the G1 phase which allows cells to pass through a cell cycle check point and then enter the S phase. Rb exists in an active hyperphosphorylated state in quiescent cells and in an inactive hyperphosphorylated state in the G1/S cell-cycle transition. As shown in Fig. 6, TRO treatment decrease the expression of phosphorylated Rb in both the U2OS and HOS cells. The expression of another cell cycle regulator, p21Cip1 was increased in both the U2OS and HOS cells after treatment with 10 μM and 20 μM of TRO for 48 hours. When combined, these data suggest that the induction of G1 arrest in OS cell lines by TRO was caused by hypophosphorylation of Rb and the increased expression of p21Cip1.

5. Troglitazone increases PTEN expression

PTEN activity causes cell cycle arrest and apoptosis. It has been proposed that PTEN blocks the cell cycle by increasing the transcription of the p27 cell cycle inhibitor. With the loss of PTEN, therefore, cells are released into the cell cycle. As shown in Fig. 7, TRO increased the PTEN expression and decreased the level of phosphorylated Akt.

**DISCUSSION**

Recent studies have shown PPARγ activation act as a tumor suppressive agent by inducing cell cycle arrest, apoptosis or differentiation in several human malignant tumor cells. However, conflicting evidence exists on the role of PPARγ.

Fig. 5. Effects of TRO on the induction of apoptosis in U2OS and HOS cells. (A) The cells were stained for apoptotic cells and these were photographed under a light microscopy. The cells containing light brown nuclei were positive for TUNEL assay. (B) The apoptosis of U2OS cells was decreased by a TRO treatment. On the other hand, the apoptosis of HOS cells was increased by TRO treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TUNEL-positive cells/1,000 tumor cells</th>
<th>U2OS</th>
<th>HOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.08 ± 0.78</td>
<td>3.44 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>TRO 20 μM</td>
<td>0.68 ± 0.27*</td>
<td>9.88 ± 1.01*</td>
<td></td>
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</tbody>
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Fig. 6. TRO treatment induces the increased expression of p21 and decreased expression of phosphorylated Rb (pRb) protein.

Fig. 7. TRO treatment induces the increased expression of PTEN and the decreased expression of phosphorylated Akt (pAkt) protein in both the U2OS and HOS cells.
activation in OS, where different studies have shown that PPARγ activation affected the OS survival or, in contrast, it induced apoptosis and differentiation of the OS.

In this study, TRO induced growth inhibition and G1 phase arrest in both the U2OS and HOS cells by upregulating the expression of p21 and decreasing the phosphorylated Rb. The physiologic role of p21 has been linked to the inhibition of cyclin E/CDK2.

In addition to the upregulation of p21 and the downregulation of phosphorylated Rb, TRO treatment also increased the expression of PTEN. The loss of PTEN activity has been suggested to cause increased cell proliferation. A previous study showed that inhibition of PTEN activity resulted in increased levels of Akt phosphorylation and enhanced osteosarcoma cell proliferation. Another study also demonstrated that PPARγ ligands increase the PTEN expression in pancreatic cancer cells. The present study also supports the previous studies that TRO induced the upregulation of PTEN and this has a role in the growth inhibition of OS cells.

Many previous reports have also shown that TRO induced the growth inhibition and G1 cell cycle arrest of human cancer. However, there are different mechanisms of cell cycle arrest, depending on the target cell type, the particular PPAR agonist used, the duration of treatment, and the applied dosage.

The improper balance between death and growth in tumor cells can involve several signals, so we can ask this question: what signaling pathways are critical for OS growth and death? This study showed that although apoptosis was decreased in the U2OS cells by treatment with TRO, the overall tumor cell growth was inhibited in a dose dependent fashion. The growth inhibitory effect of TRO was not evident at treatment concentration of 0.1, 1, and 5 µM TRO, but 10, 20, and 50 µM of TRO significantly inhibited cell growth in a dose dependent fashion. It has been suggested that the other signals inducing cell cycle arrest such as pRb, p21, and the PTEN/Akt pathway may act more potently for inducing growth inhibition of U2OS cells. In the study using melanoma cell lines, the growth inhibitory effect of the PPAR ligands was independent of apoptosis and they seemed to occur primarily through the induction of cell cycle arrest.

In the present study, TRO treatment on the U2OS cell increased the expression of p21 and PTEN. Among these, p21 is controlled by p53 and the increased expression of p53 induces upregulation of p21; this resulted in increased apoptosis. Yet the increased expression of p21 was not in accordance with the reduced apoptosis of U2OS cells. This result suggested that p53 the mediated effects on the p21 pathway were not important in the apoptosis of U2OS cells. A recent study supported this assumption. Kim et al. have suggested that p53 overexpression is required but it is not sufficient enough for inducing apoptosis of the U2OS cells. Therefore, there are other mechanisms other than p53, and TRO act in a different manner according to cell type. Further research is necessary to define the molecular mechanisms of the on the TRO growth inhibitory effects, and especially for the mechanism of apoptosis.

TRO inhibits osteoclast-like cell formation and bone resorption. The inhibitory effects of TRO on osteoclastic bone resorption were not osteotropic factor specific and they did not appear to be related to their adipogenic effects. Thus, TRO may suppress bone resorption and prevent bone loss. PPAR activators modulate the osteoblastic maturation of MC3T3-E1 preosteoblasts. PPAR activators may affect BMD by modulating the maturation of osteoblasts.

Unfortunately, TRO has side effects, including edema and severe hepatotoxicity when it is administered at the high doses. The processing of the quinone metabolite of TRO which is found predominantly in the liver to a sulfate conjugate and the activation of PPAR gamma and PXR (pregnane X receptor) by TRO are supposed to be factors of the hepatotoxic mechanism. It has been recommended to examine every month the liver function in patients treated with the this drug in order detect drug-induced hepatitis at an early stage.

CONCLUSION

The present study suggest that TRO may be used as an efficacious adjuvant chemotherapeutic agent for primary osteosarcoma, as well as that TRO may be used as a potential chemopreventive agent for preventing the recurrence and metastasis of osteosarcoma after the surgical removal of primary tumors. But further study is required for elucidating the precise the mechanism of TRO.

REFERENCES

1. Bae MA, Rhee H and Song BJ: Troglitazone but not rosiglitazone...


목적: U2OS와 HOS 골육종 세포주에 대한 Troglitazone (TRO)의 영향을 알아보고자 하였다.
대상 및 방법: TRO를 처리한 골육종 세포의 생활력을 trypan blue exclusion을 이용하여 측정하였고, 세포주기 분포는 흐름세포측정을 이용하여 판정하였다. 세포자멸사는 흐름세포측정과 TUNEL 분석법을 이용하여 측정하였다. 세포자멸사와 세포 주기에 관련된 유전자 발현은 PTEN, tAkt, pAkt, Bcl-2, Bax, pRB, p21, and β-actin을 이용한 웨스턴 블롯으로 평가하였다.
결과: U2OS와 HOS 세포주 모두에서 세포 성장의 억제를 보였고, 흐름세포측정에서도 모두에서 G1기에서 세포주기 정지가 관찰되었다. 또한 p21 발현이 증가하였고 pRB와 pAkt의 발현은 감소하였다. 그러나 흐름세포측정과 TUNEL 분석법을 이용하여 세포자멸사를 측정한 결과 서로 다른 결과를 보였다. HOS 세포주의 세포자멸사는 증가한 반면 U2OS 세포주의 세포자멸사는 감소하였다. U2OS 세포주에서 세포자멸사의 감소는 Bcl-2 발현의 증가 및 Bax 발현의 감소와 함께 관찰되었다.
결론: TRO가 작용하는 기전이 세포주에 따라 차이가 있을 수 있으나 적절하게 입증된 용량으로 사용한다면 골육종 치료에 이용될 수 있는 가능성을 보여주는 것으로 생각된다.
색인 단어: 골육종, Troglitazone (TRO)