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Research Article

All Trans Retinoic Acid activates the RAR-βand p14 tumor suppressor genes in OVCAR-3 cell line

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Running title: ATRA activates RAR-βand p14

ABSTRACTION
In this study, we investigated the effect of All Trans Retinoic Acid (ATRA) on the expression of the Retinoic Acid Receptor-β(RAR-β) and p14, two important tumor suppressor genes, in human ovarian cancer cells. OVCAR-3 cells were treated by ATRA and the mRNA levels of RAR-βand p14 were measured by real time PCR. The percentage of apoptotic cells also evaluated by flow cytometry assay and cell viability determined by MTT method. Our real time results showed that the expression of RAR-β and p14 gene in 48h treated groups was dramatically up-regulated by ATRA, while in 24 hours treated groups,there was no significant differences between the RAR-βand p14 gene expression in comparison to the control group. The flow cytometry results showed that ATRA can significantly induce cell apoptosis at 48 hours. The cell viability in both 24 and 48 h treated groups were significantly lower than the control group. In order to these results we concluded that ATRAcan up regulate theRAR-βand p14 gene expression in OVCAR-3 ovarian cancer cell line.

Keywords:RAR-β, p14, ATRA, OVCAR-3

INTRODUCTION

Ovarian cancer is the seven most common cancer among women(1) and each year caused the 152,000 deaths around the world(2, 3). Because this cancer is completely asymptomatic in the early stages, diagnosed in advanced stages (grade III, IV) and the mortality rate in these grades is relatively high. Common treatments today, include surgery and chemotherapy using platinum and taxane compounds. Studies show that in 70% of patients after treatment, the disease returns again(4). Therefore, it is necessary to find new methods to treat this deadly malignancy. One of the most important causes of cancers, including ovarian cancer is down regulation of tumor suppressor genes. Retinoic Acid Receptor-β (RAR-β) isa tumor suppressor gene thatLocated on chromosome 3p24.2(5) and many researchers investigated its role on various malignancies. Several studies show the down regulation of this gene in different cancers such as prostate, colon,leukemia and breast(6).Gregory *et al* showed the mRNA level of RAR-β gene is reduced in breast cancer and reactivation of this gene increases the cancer cells(7, 8). Down regulation of the RAR-β has also reported in ovarian cancer by several studies(9, 10).The reasons that led to down regulation of RAR- β gene is still poorly understood, but it seems that one of the most important, is the epigenetic alterations in promoter of this tumor suppressor

gene(11, 12).Khodyrev*et al* showed the down regulation of RAR-βin ovarian cancer cells due to epigenetic changes in promoter of this gene(12). Another study showed that benzo[a]pyrenediol epoxide(BPDE), a carcinogen present in tobacco smoke and environmental pollution can induce the methylation of the RAR-β gene promoter(13, 14). In addition,the expression of RAR-β depends on the levels of retinoids in the cells, becausethesereceptors are ligand inducible genes , thereforethe expression of RAR- β is reduced in organs that have vitamin A-deficiency(15). Vitamin A is achieved from plant and animal sources and has an important role in many aspects of cell biology and physiology(16) Stephen *et al* showed **vitamin A metabolism is impaired in human ovarian cancer(17).** All Trans Retinoic Acid (ATRA) is one of the derivatives of vitamin A thatused in prevention and differentiation therapy for many types of cancers. This agent has a critical role in growth and differentiation of normal and malignant cells.

ATRA can cause up regulation of the RAR-β gene in cancer cells by various mechanisms. Studies showed that ATRA can modify the epigenetic changes in some malignancies(18).Heo showed that ATRA can modify the hypomethylation of tumor suppressors and induce cell apoptosis in human hepatocytes(19).Binding of ATRA to related receptor also can reactivate RAR-β tumor suppressor gene and prevents mitosis and induces cell differentiation in different cancers (20-23).Wu *et al* showed treatment of ovarian cancer cells by ATRA lead to up regulation of the RAR-β and subsequent cell apoptosis in CA-OV3 ovarian cancer cells(24).

Although some numbers of apoptotic molecules that ATRA activate them and induce cell apoptosis are determined, but the detailed mechanism by which ATRA induces apoptosis in cancer cells is not fully understood. Recent studies showed that down regulation of theRAR- βcan reducethe activation of p14 gene.p14/ARF (Alternative Reading Frame) or p19 ARF in the mouse, is located predominantly in the nucleolus and on chromosome 9p21 in humans.. Normal cells have low levels of p14, but when they are stimulated by various stresses, serum starvation and oncogenes, the concentration of p14 significantly increases(25). P14 is inactivated as a result of epigenetic alterations in many cancers (26). P14 plays a major role in cell cycle, so that blocks the cell cycle in G_1 and G_2 phase and arrests the growth of abnormal cells and induces apoptosis in p53 dependent and independent pathways(25, 27, 28).Indeed,some studies show that, there is a mutual and unrecognized cross talk between RAR- βand the p14 gene in cancer cells(29).

In this study, we treated the OVCAR-3 ovarian carcinoma cells with ATRA, as a ligand of RAR-β and investigated the RAR- β and p14 tumor suppressor gene expression to evaluate the relations between these tumor suppressor genes.OVCAR-3 is a highly metastatic human ovarian cancer cell line that its p14 gene expression is very low, and thus it is an ideal cell line for this study.

MATERIALS AND METHODS

Cell culture and drug treatment

The OVCAR-3 cell line was purchased from the National Cell Bank of Iran‑Pasteur Institute. The cells were cultured in RPMI1640 (Sigma) with 10% fetal bovine serum(Gibco,Germany), 100U/ml penicillin(Bioidea,Iran), 100 µg/ml streptomycin(Bioidea,Iran) and incubated at 37°C in a humidified atmosphere with 5% CO2. For experiments after trypsinization (trypsin-ethylene diamine tetra acetic acid(EDTA,Bioidea,Iran)), the cells were seeded in T_{75} flasks(SPL,South Korea). ATRAwas purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO, sigma,USA) to the appropriate concentrations according to half maximal inhibitory concentration (IC50) assay. After the cells were >80% confluent, OVCAR-3 cells were treated with ATRA, based on IC50 concentration and in the certain times(24,48h). The cells that did not treat with the drug, considered as the control group.

IC50 assay

The IC50 values for the ATRA in OVCAR-3 cells were achieved after 24 h of treatment. After

trypsinization, 10^4 cells were placed into each well of a 24-well plate and afterwards,then treated with various ATRA concentrations (5,10, 15, 25, 50 and 75 μM/L) for 24 h, and then 3(4,5-dimethylthiazol-2-yl)2,5- diphenyl-tetrazoliumbromide(MTT, sigma, USA) survival assay was done for investigating the cell viability with various drug concentrations in each group. All experiments were repeated 3 times, with at least three measurements.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was done to quantitatively evaluate the mRNA expression of RAR-β and p14 in OVCAR-3 cells after treatment with ATRA at 24 and 48 h, total RNA from cells were extracted, using the RNeasy mini kit and following the instructions from the manufacturer (Qiagen, Hilden, Germany). After treatment with DNase I, total RNA (100 ng) was reverse‑transcribed to cDNA by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. The Maxima SYBR Green/RoxqPCRMaster Mix kit (Fermentas) was used for real‑time PCR. Primer sequences are shown in Table1. Real‑time PCR reactions were performed with StepOnePlus (Applied Biosystem, USA). The program of real-time PCR lasted 10 min at 95°C followed by 40 cycles of denaturation step at 95°C for 15 s following by annealing and extension for 1 min at 60°C. The data were analyzed by Comparative CT (ΔΔCT) method. The RAR-β and p14 gene expression levels calculated by determining a ratio between the amount of every gene expression and that of endogenous control. Melting curve analysis (60°C to95°C increment of 0.3°C) was used to determine the melting temperature of specific amplification products and primer dimmers. These experiments were carried out in triplicate and were independently repeated at least 3 times.

Flowcytometryassay

The percentage of cell apoptosis was determined by flow cytometry following AnnexinV (FL1-H) and PI (FL2-H) labeling. A minimum of 5 × 105 cells/ ml were selected for each sample. Cells were treated with ATRA (50μmol/L) for 24and 48 hours and then washed in PBS and re-suspended in binding buffer (1×; 5 μl). AnnexinV-FITC was added to 195 μl cell suspension and then analysis was carried out according to the manufacturer's protocol (BMS500F1/100CE AnnexinV-FITC, eBioscience, USA). Finally the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). These experiments were carried out in triplicate and were, independently, repeated at least 3 times.

MTT assay

1×10^4 cells were plated in 24-well plates. After 24 h the medium was changed and the ATRA were added to the medium at a concentration of 50μl. At the indicating time (24 and 48 h) the cell viability was measured using MTT in RPMI-1640 medium for 3 h. After lysis with DMSO, a 100μl aliquot of the soluble fraction was transferred in 96-well plates, and the optical density (OD) was measured by plate reader. The percent of cells stained by MTT are determined by comparing the OD of each sample with the OD of the control group.

STATISTICAL ANALYSIS

All the quantitative data were presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) with the Tukey post hoc test was performed to determine statistical significance among different groups by using SPSS software package 20.0. Significance was accepted at a level of $P < 0.05$.

RESULTS

IC50 assay

After the treatment of OVCAR-3 cells with MTT solution, the darker blue formazan crystals were seen in viable cells, which indicated their metabolic activity.

The reduction in the number of cells was directly dependent on the drug doses as shown by the IC50 in figure 1. The essential drug concentration to obtain the IC50 in OVCAR-3 cells at 24 hours was 50 μM/L [Figure 1].

This experiment was repeated three times for each group.

Real time PCR

To examine the effect of ATRA (based on IC50) at 24 and 48 hours on expression of RAR-β and p14 genes in OVCAR-3 cells, we used real-time quantitative PCR. The expression of RAR-β and p14 gene in 48h treated groups was dramatically up-regulated by ATRA. So that there was significant differences between 48h treated groups and both the 24h treated and untreated (control) group. Although the RAR-β and p14 mRNA levels increase in 24h treated groups in comparison with the control group, but these differences was not significant (Fig.4).

Flowcytometry

The flowcytometry assay was used to determine the percentage of apoptosis in the cells which treated with ATRA. 4×10^5 cells/ml were analyzed for each group. The results showed that the 50 µM concentration of ATRA at 48 hours could significantly induce apoptosis in OVCAR-3 cells progressively (P<0.05) (Fig.3). Whereas there is no significant difference in 24 h treated group in comparison with the control group.

MTT assay

The effects of ATRA on the viability of OVCAR-3 cells were determined by MTT assay. 50 µM of ATRA, was used according to the IC50. Untreated cells have been considered as a control group. To measure the changes in the number of cells in the wells during the experiment, cell proliferation had to be determined 24 and 48 h after the treatment period. The cell viability in both 24 and 48 h treated groups were significantly lower than the control group. The cell vitality in the 24h treated group was significantly more than 48h treated group. The percentage of living cells at 24 h and 48 h treated groups were 48.33 and 34.33 respectively (fig2).

DISCUSSION

Abdominal mass, abdominal distension and gastrointestinal problems are the main symptoms of ovarian cancer in the later period patient, but unfortunately in the early period, this malignancy is asymptomatic and the diagnosis is very poor. Now day surgery, radiotherapy and chemotherapy are the common methods for treatment of ovarian cancer, but the 5-year survival rate in the patients with ovarian cancer is still remain about 30%(30, 31) , so in recent decades investigators have focused on finding the new approaches and drugs to cure this deadly malignancy.

In this research, we investigated the effects of ATRA on the expression of RAR-β and p14 tumor suppressor genes in ovarian cancer cells. Among the different types of ovarian cancer cell lines, we used OVCAR-3, because it is a highly invasive cell line and previous studies showed that OVCAR-3 is more sensitive to ATRA in comparison to other ovarian cancer cell lines (24).

The principal finding in our study was that, the increase of the RAR-β gene expression inOVCAR-3 ovarian cancer cells is associated with the increase of the p14 tumor suppressor gene.

Many studies in recent years showed that ATRA can up regulate the RAR-β tumor suppressor gene in various cancer cells(17, 32-34). ATRA binds to the RAR-β as a ligand of this cell receptor and reactivates this tumor suppressor gene. Our real time findingsalso showed that 48 hour treatment of OVCAR-3 cells with ATRA causes an increase of mRNA level of the RAR-β gene in comparison to the control group.

P14 is another tumor suppressor gene that has been extensively studied in the progression of different types of cancers. It can keep mdm_2 localized in nocleulus and as a result mdm_2 can not degrade p53(26) so cell apoptosis induce in the p53 pathway. P14 can also inhibit the growth of different cancer cells lacking p53 in (in vivo) and (in vitro) models (19). The p53 independent tumor suppression mechanisms of p14 are a contribution to the DNA damage response and promotion of autophagy. Any alteration of these pathways, can induce various malignancies, therefore If we increase the level of p14 gene expression in cancer cells, we can induce apoptosis.

ATRA is one of the derivatives of vitamin A that seems can directly and indirectly increase the p14 gene expression. Dahl *et al* showed that ATRA can increase the mRNA levels of p14 on the melanoma cells through increasing the RAR-β

gene and induce cell apoptosis. Indeed Dhal proved that there is a RAR-β – p14 signaling axis in melanocytes and claimed that retinoic acid (including ATRA) is the first naturally inducer of p14(17). Our real time results confirm these findings, so that there was no significant difference in RAR-βgene expression between 24h treated group and the control, as the p14 gene expression in the 24h treated group did not significant difference with the control group. But the mRNA level of the RAR-βand p14 in 48h treated groups was significantly more than both the control and 24h treated groups. These results show that there are high levels of p14 gene expression in groups that have high levels of RAR-β. So it seems that a special and unrecognized relationship between RAR-β and the p14 that Dahl *et al* have shown in melanoma, exist in OVCAR-3 cells. Meanwhile, our real time results in 24h treated groups showed that, long-term exposure is needed to ATRA can activate the RAR-β and p14 related molecular pathways.

It should be noted that ATRA can also directly increase the p14 gene expression. Previous studies have demonstrated the ability of ATRA on modification of epigenetic alterations(18). Heo*et al* showed that ATRA can modify promoter hypermethylation of p14 and increase this gene in hepatocytes and induce cell apoptosis in p53 dependent pathway (19). Our real time results confirm these findings, in our investigation, treatment of OVCAR-3 ovarian cancer cell with ATRA causes the significant up regulation of the P14 gene in 48 h treated group in comparison to control the group. Therefore, it seems that ATRA can also up regulate the p14 gene in a direct manner and more studies are needed to find exact molecular mechanisms that how ATRA can up regulate the p14 mRNA level in the cells.

Our MTT assay results show that the cell viability of ovarian cancer cells in treated groups progressively decreased, so that in both 24 and 48h treated groups, the cell viability is significantly lower than the control group. These results are similar to Heo study on hepatocytes and Dahl on melanocytes.

Our flow cytometry results showed that 24 hours exposure with ATRA did not significantly increase the cell apoptosis in OVCAR-3 cells, whereas in 48h treated groups, the percent of cell apoptotic cells was significantly more than the control group. These findings similar to real time results show that long term exposure is needed to induce cell apoptosis in OVCAR-3 cells. In order to our results we concluded that ATRA can up regulate the RAR-βand p14 gene expression in OVCAR-3 ovarian cancer cell line.

CONCLUSION

In order to our results we believe that ATRA can up regulate the RAR-βand p14 gene expression in OVCAR-3 ovarian cancer cell line.

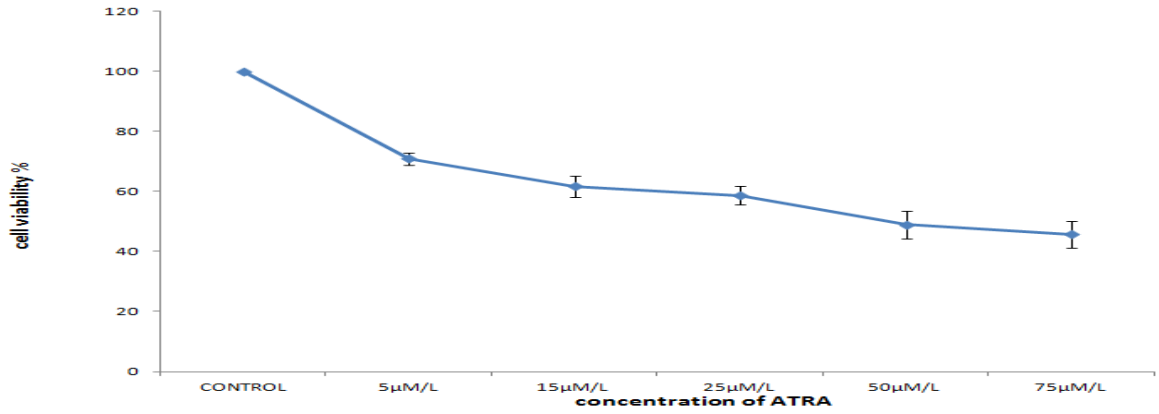


Fig. 1.IC50 assay of a; ATRA in OVCAR-3 cancer cell lines. Cells incubated with/without the drug in various concentrations and the relative amount of viable cells determined by measuring the absorbance of MTT solution. Graph of viability versus drug concentration used to calculate IC50 values.

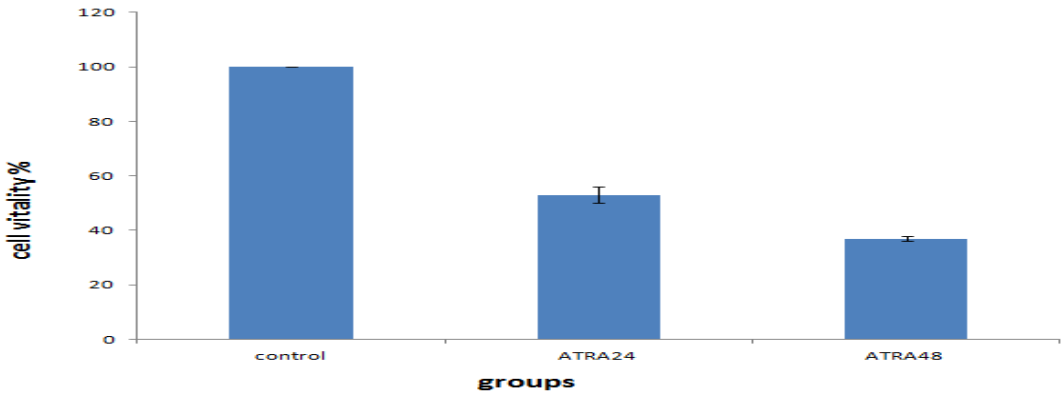


Fig. 2.MTT assay in IC50 concentrations of ATRA at 24and 48 h after treatment

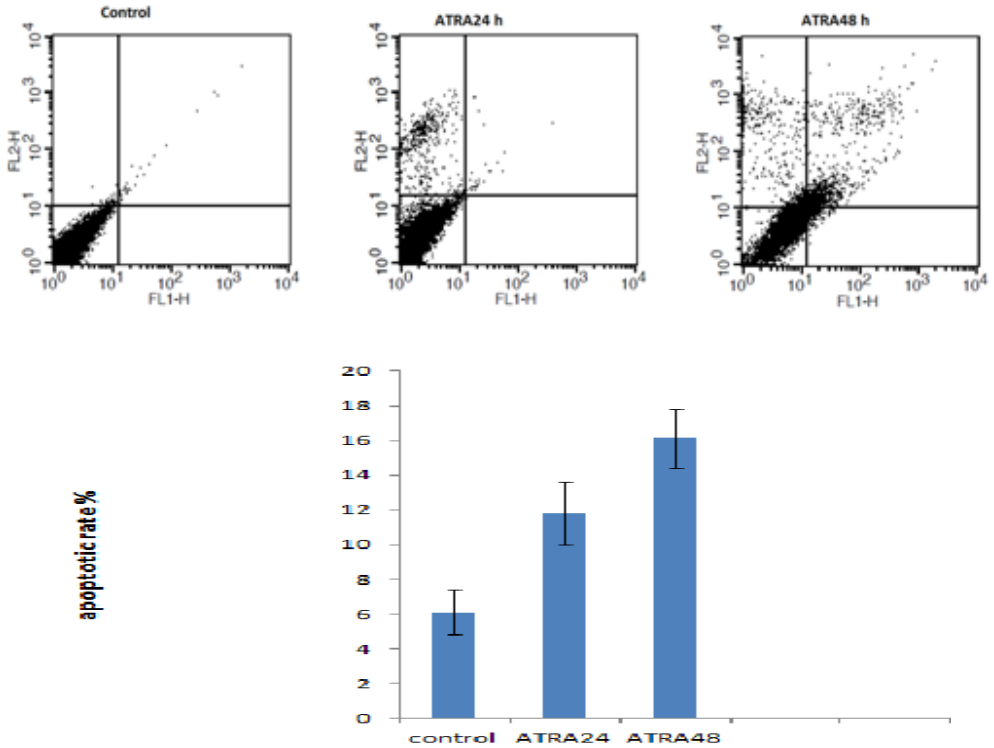
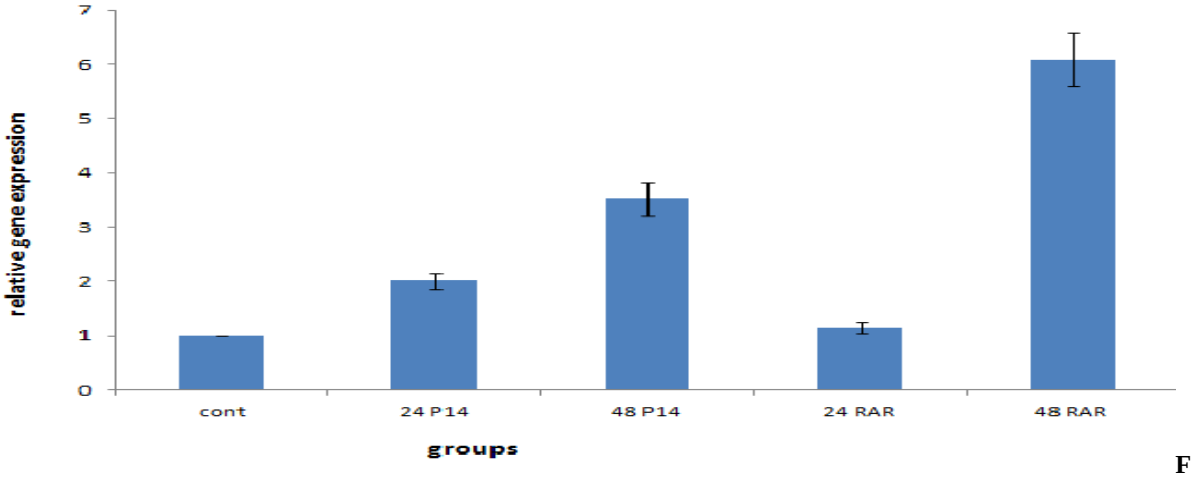


Fig 3. Effects of ATRA on apoptosis of OVCAR-3 cells in 24 and 48 h.



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ig. 4. Effects of ATRA on the levels of RAR-β_2 and p14 expression in OVCAR-3 cells in 24 and 48h after treatment.

Table 1. Primers used in real-time PCR

Primers ID	Primers Sequences
P14 forward	GAAGGTCCCTCAGACATCCC
P14 reverse	GAAAGCGGGGTGGGTTGT
RAR-βforward	CTGCCTGGACATCCTGATTCTTAG
RAR-βreverse	GGCGGTCTCCACAGATTAAGC
GAPDH forward	AAGCTCATTTCCTGGTATG

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