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World J Gastroenterol 2001;7(5):662-666
World Journal of Gastroenterology
 ISSN 1007-9327

• ORIGINAL RESEARCH •

The effect pathway of retinoic acid through regulation of retinoic acid receptor α in gastric cancer cells

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Received 2001-02-20 **Accepted** 2001-06-30

Abstract

AIM **To evaluate the role of RARα gene in mediating the growth inhibitory effect of all-trans retinoic acid (ATRA) on gastric cancer cells.**

METHODS **The expression levels of retinoic acid receptors (RARs) in gastric cancer cells were detected by Northern blot. Transient transfection and chlorophenicol acetyl transferase (CAT) assay were used to show the transcr iptional activity of β retinoic acid response element (βRARE) and AP-1 activity. Cell growth inhibition was determined by MTT assay and anchorage-independent growth assay, respectively. Stable transfection was performed by the method of Lipofectamine, and the cells were screened by G418.**

RESULTS **ATRA could induce expression level of RARα in MGC80-3, BGC-823 and SGC-7901 cells obviously, resulting in growth inhibition of these cell lines. After sense RARα gene was transfected into MKN-45 cells that expressed rather low level of RARα and could not be induced by ATRA, the cell growth was in hibited by ATRA markedly. In contrast, when antisense RARá gene was transfected into BGC-823 cells, a little inhibitory effect by ATRA was seen, compared with the parallel BGC-823 cells. In transient transfection assay, ATRA effectively induced transcriptional activity of βRARE in MGC80-3, BGC-823, SGC-7902 and MKN/RARα cell lines, but not in MKN-45 and BGC/aRARα cell lines. Similar results were observed in measuring anti-AP-1 activity by ATRA in these cancer cell lines.**

CONCLUSION **ATRA inhibits the growth of gastric cancer cells by up-regulating the level of RARα; RARα is the major mediator of ATRA action in gastric cancer cells; and adequate level of RARα is required for ATRA effect on gastric cancer cells.**

Subject headings receptor; retinoic acid/pharmacology; stomach neoplasm/drug therapy; stomach neoplasm/pathology

Liu S, Wu Q, Chen ZM, Su WJ. The effect pathway of retinoic acid through regulation of retinoic acid receptor α in gastric cancer cells. *World J Gastroenterol*, 2001;7(5):662-666

INTRODUCTION

Retinoic acid (RA) exerts profound effects on the growth, differentiation and apoptosis of normal, premalignant and malignant epithelial cells *in vivo* and *in vitro*[1-7]. The effects of retinoic acid are mainly mediated by two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs)[8-13], which belong to steroid/thyroid receptor superfamily, and are encoded by three distinct genes, α, β and γ. RXRs form homodimers (RXR/RXR) and heterodimers (RAR/RXR) with RARs receptively, then bind to specific RA response elements (RARE), and regulate positively and negatively their transcriptional activities of target genes[9,13-17]. These receptors, thus, display distinct patterns and exert specific functions on anti-cancer effects in various cancer cell lines.

There have been sufficient evidences showing a link between the alteration of RARs activity and some diseases[18-22]. t (15;17) chromosomal translocation leads to the forming of PML-RARα fusion and abnormal RARα transcription in acute promyelocytic leukemia[23-26] . High frequency of the deletion next to RARα gene in chromosome 3P is observed in human lung cancer. Lack of RARα expression is responsible for the resistance of RA in breast cancer cells[1,20,21,27- 29]. Investigation the functions of retinoic acid receptors, therefore, is essential to elucidate their anticancer effects of RA. In the present study, we evaluate the role of RARα gene in mediating the effect of all-trans retinoic acid (ATRA) in gastric cancer cells. The results indicated that RARá is required for ATRA to exert its growth inhibition on gastric cancer cells.

MATERIALS AND METHODS

Cell lines and culture conditions

The human gastric cancer cell lines, BGC-823, SGC-7901 and MKN-45, were purchased from Institute of Cell Biology, Shanghai, China. MGC80-3 cell line was established by Cancer Research Center in Xiamen University. All of four cell lines were maintained in RPMI1640 medium, supplemented with 100 mL·L^{-1} FCS, 1 mmol·L^{-1} glutamine, and 100×10^3 U·L^{-1} penicillin.

RNA preparation and Northern blot

Total RNA was prepared by guanidine hydrochloride/ultracentrifugation method. About 30 μg total RNA was fractionated on 10 g·L^{-1} agarose, then transferred to nylon, and probed with ^{32}P-labeled probe as previously described[30]. The probes of RARα, RARβ, RARγ and RXRα were provided

by Dr. Zhang (The Burnham Institute, CA, USA). 28S and 18S were shown in quantitation of RNA.

Transient transfection and CAT assay

Cells were seeded in six-well plates with approximately 70% confluent at the time of transfection. Cells were transient transfected by Lipofectamine™ (Gibco/BRL). Transient transfection was performed utilizing βRARE-tk-CAT reporter gene plasmid, containing the βRARE linked with tk-CAT promoter[29], or -73col-tk-CAT receptor gene plasmid, containing an AP-1 binding site located between residues -73 and -63 in collagenase promoter[31,32]. Transfection condition was as follows: 6 μL Lipofectamine™ in 1.0 mL standard medium was added to each well, togelher with 1.0 mL of standard medium containing 400 ng reporter gene plasmid, 400 ng β-galactosidase expression vector (pCH110, Pharmacia), and carrier DNA (pBluescript) added up to 1000 ng total DNA. CAT activity was normalized for transfection efficiency to the corresponding β-galactosidase activity as described elsewhere[1,30-32].

Stable transfection

Sense RARα- and antisense RARα expression vectors (provided by Dr. Zhang) were stably transfected into gastric cancer cells, MKN-45 and BGC-823, respectively, by Lipofectamine™ (Gibco/BRL) as described above, and then screened with 600 μg of G418. Expression of endogenous RARá was determined by Northern blot.

MTT assay

Cells were seeded at 1000 cells per well in 96-well plates, and treated with ATRA (Sigma) at various concentrations. Medium was changed and ATRA was added every other day. After treatment for one week, cells were stained with 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma) for 3 h-4 h. Cell viability was determined by the MTT assay[1,30,32]. An underlayer of 5 $g \cdot L^{-1}$ agar in medium supplemented with 100 mL $\cdot L^{-1}$ FCS was first prepared and hardened in 6-well plate. Cells $1\times10^{8} \cdot L^{-1}$, in culture medium containing 100 $mL \cdot L^{-1}$ FCS, 5 $g \cdot L^{-1}$ agar, and 10^{-6} $mol \cdot L^{-1}$ ATRA (only for experimental groups), were seeded onto the underlayer. The plate was incubated for three weeks in CO_2 incubator. Number of colonies with diameter >80 μm was counted under microscope[5].

RESULTS

Expressions of RARα, RARβ, RARγ and RXRα in gastric cancer cells

Northern blot analysis showed that the level of RARα expression was high in MGC80-3, BGC-823 and SGC-7901 cells, while rather low level in MKN-45 cells. After treated with ATRA, MGC80-3, BGC-823, and SGC-7901, cells exhibited a marked increase in RARα expression, whereas MKN-45 cells had no change in RARα expression. RARβ expressed in MGC80-3, BGC-823, and SGC7901 cells, but not in MKN-45 cells. As for RARγ, none of the four cell lines expressed RARγ (data not shown). All cell lines showed a relatively low-level expression of RXRα. However, the expressions of RARβ, RARγ and RXRα could not be induced by ATRA in these four cell lines (Figure 1).

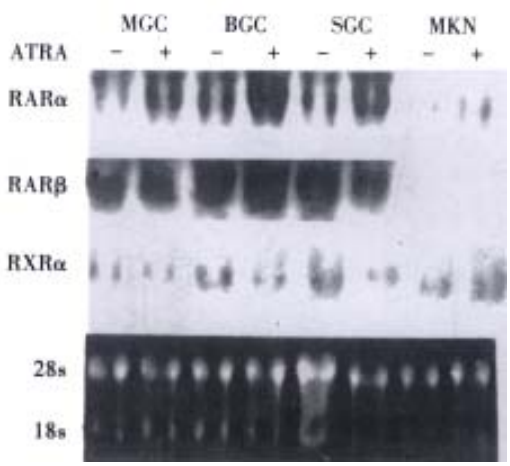


Figure 1 Expressions of RARα, RARβ and RXRα in gastric cancer cell lines detected by Northern blot. Cells were treated with 10^{-6} $mol \cdot L^{-1}$ ATRA.

Transfection and expression of RARα gene in gastric cancer cells

Based on these results mentioned above, we transfected antisense RARα gene and sense RARα gene into BGC-823 and MKN-45 cells, respectively. It was demonstrated by Northern blot that when antisense RARα gene was transfected into BGC-823 cells, RARα expression was repressed, and could not be induced by ATRA, compared with parallel cells BGC-823 (Figure 2A). On the contrary, MKN/RARα cells that transfected with sense RARα gene had a higher expression of RARα than parallel MKN-45 cells, and the expression of RARα could be induced by ATRA (Figure 2B).

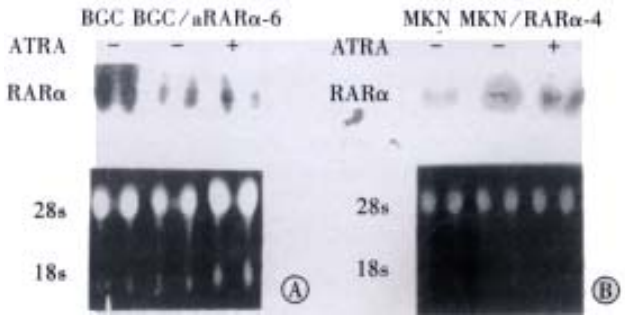


Figure 2 A. Expression of RARα mRNA in BGC-823 cells transfected with antisense RARα gene. B. Expression of RARα mRNA in MKN-45 cells transfected with sense RARα gene.

Effect of ATRA on the growth inhibition of gastric cancer cells

ATRA could effectively inhibit the growth of MGC80-3, BGC-823 and SGC-7901 cells, but had a rather weak effect on MKN-45 cells (Figure 3A). As for the transfected cells, BGC/aRARα, the inhibition rate by ATRA dropped obviously from 61.0% to 18.4%. The opposite result was seen in another transfected cell, MKN/RARα, in which ATRA could effectively suppress the growth of MKN/RARα cells, with an enhanced inhibition rate from 3.9% to 31.7% (Figure 3B).

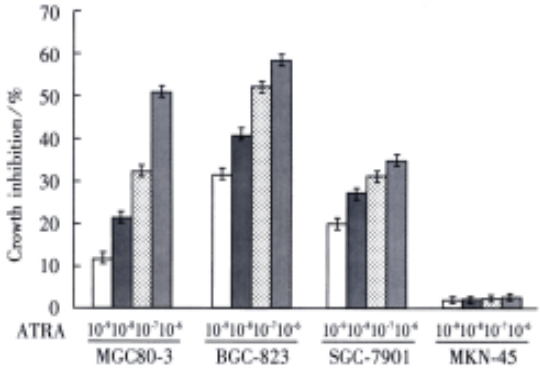


Figure 3A Growth inhibitory effect of ATRA on gastric cancer cell lines measured by the method of MTT. Cells were treated with various concentrations of ATRA indicated.

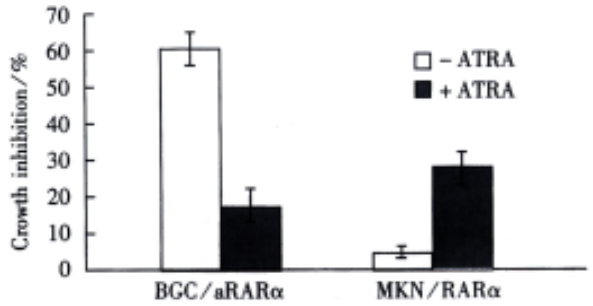


Figure 3B Growth inhibitory effect of ATRA on BGC-823 cells transfected with antisense RARα gene and on MKN-45 cells transfected with sense RARα gene, respectively.

Effect of ATRA on cell clone formation in soft agar

ATRA could inhibit the ability of clone formation in four cell lines and the inhibition for MKN-45 cells was lowest among four cell lines (Table 1). In contrast, in the transfected cells, the highest inhibition on MKN/RARα cells transfected with sense RARα gene was observed, compared with BGC/aRARα cells transfected with antisense RARα gene (Table 1).

Table 1 Inhibitory rate of clone formation of cells treated with 10^{-6} mol·L^{-1} ATRA in soft agar

Cell lines	MGC	BGC	SGC	MKN RARα	MKN / aRARα	BGC / aRARα
Inhibitory rate (%)	48.8[b]	45.2[b]	65.3[b]	14.3[b]	56.1[b]	15.2[b]

[b]$P<0.01$, *vs* control.

Regulation of ATRA on αRARE transcriptional activity

When transient transfection was performed with reporter gene, βRARE-tk-CAT, MGC80-3, BGC-823 and SGC-7901 cells exhibited a stronger induction of CAT activity by ATRA than MKN-45 cells, with an increased induction (CAT activity induced by ATRA deletes CAT activity in control) by 3.67, 3.44 and 2.25 fold, respectively, compared with that of MKN-45 cells by 1.04 (Figure 4A). However, ATRA could not significantly induce CAT activity in BGC/aRARα cells, and the induction was 1.76 fold, compared with 3.40 fold in MKN/RARα cells whose CAT activity was induced by ATRA obviously (Figure 4B).

Inhibitory effect of ATRA on AP-1 activity

AP-1 (activator protein-1) activity is associated with proliferation and trans fomation of tumor cells, and can be induced by some agents for mitogen, such as TPA (12-O-tetradecanoylphorbol-13-acetate)[31-33]. Detection of AP-1 activity by transient transfection and CAT assay was carried out in gastric cancer cells. As shown in Figure 5, the AP-1 activity (CAT activity) induced by TPA was suppressed by ATRA in MGC80-3, BGC-823 and SGC-7901 cells, with an ATRA-dose dependent manner. However, the suppressive effect of ATRA could not be observed in MKN-45 cells (Figure 5A). In the transfected cells, ATRA treatment resulted in a decrease of AP-1 activity induced by TPA in MKN/RARα cells transfected with sense RARα gene, but with a little effect in BGC/aRARα cells transfected with antisense RARα gene (Figure 5B).

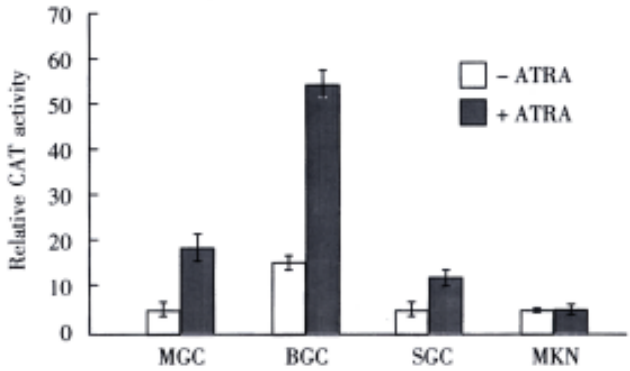


Figure 4A Regulation of ATRA on βRARE transcriptional activity in gastric cancer cell lines detected by CAT assay.

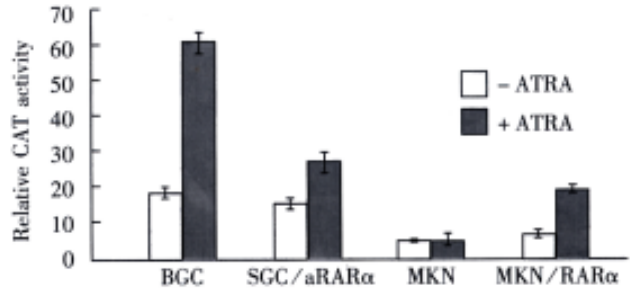
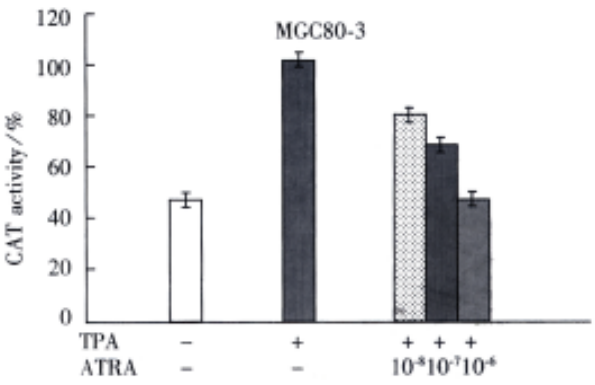


Figure 4B Regulation of ATRA on βRARE transcriptional activity in BGC-823 cells transfected with antisense RARα gene and in MKN-45 cells transfected with sense RARα gene, respectively.



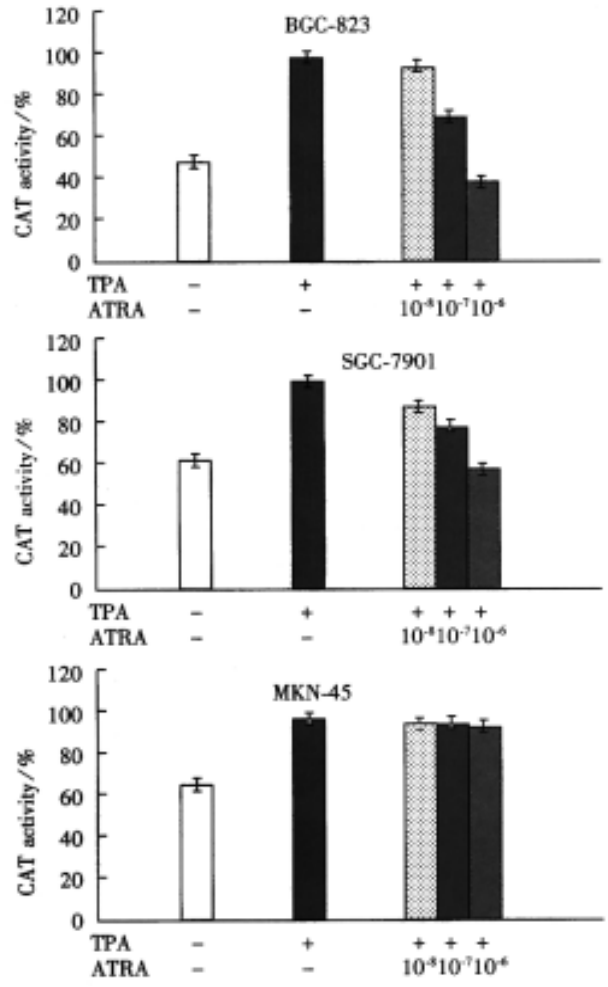


Figure 5A Inhibitory effect of ATRA on AP-1 activity in gastric cancer cell lines at various concentrations of ATRA shown by CAT assay.

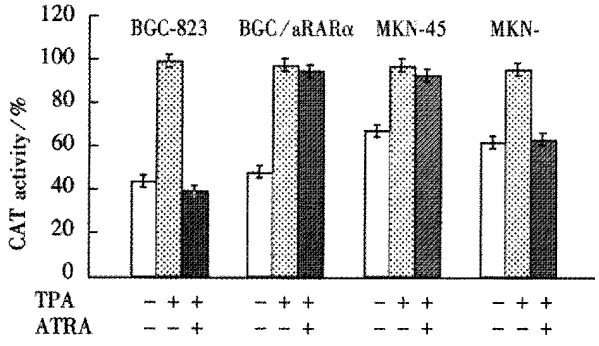


Figure 5B Effect of ATRA on AP-1 activity in BGC-823 cells transfected with antisense RARα gene and in MKN-45 cells transfected with sense RARα gene, respectively.

DISCUSSION

Retinoicacid (RA) is known to inhibit the growth of cancer cells *in vitro*, including cells of breast cancer, lung cancer, gastric cancer and liver cancer[1,15,30,34-36]. Effects of retinoic acid are mediate d by its receptors RARs and RXRs[8-13]. In the present study, we demons trated that the molecular mechanism by which RA inhibited the growth of gastric cancer cells was involved in RARα-mediated signal transduction pathway. Although ATRA did not show any inhibitory effects on MKN-45 cells (Figure 3A, Table 1), the expression of exogenously transfected sense RARα gene at elevated level in MKN-45 cells resulted in acquisition of sensitivity to growth inhibition by ATRA (Figures 2B, 3B, Table 1). In contrast, exogenous transfection of antisense RARα gene into BGC-823 cells, which expressed RARα, and RARα could be induced by ATRA (Figure 1, 2A), failed in growth inhibition by ATRA (Figure 3B, Table 1). These data suggested that the growth inhibitory effect of ATRA is due to the presence of RARα. In addition, we noted that although RARα mRNA was detected in MKN-45 cells, its mRNA level was rather low, compared with that in MGC80-3, BGC-823 and SGC-7901 cells (Figure 1). This may be the reason why ATRA could not exert its anti-proliferation effect on MKN-45 cells. RARα, thus, plays a major role in mediating growth inhibition of ATRA on gastric cancer cells, and adequate level of RARα is required for such action.

AP-1 is a transcriptional factor mainly composed of the products of cJun and cFos[31,37,38], which relate with proliferation and transformation of tumor cells. Our observation that ATRA could effectively inhibit AP-1 activity induced by TPA in MGC80-3, BGC-823 and SGC-7901 cells, but not in MKN-45 cells (Figure 5A) indicated that the suppression of AP-1 activity might contribute to cell growth inhibition by ATRA in gastric cancer cells. The anti-AP-1 effect of ATRA was mediated by the activation of RARα. When transfecting sense RARα gene into MKN-45 cells, a clear inhibition of AP-1 activity was seen (Figure 5B), thus leading to growth inhibition of MKN-45 cells (Figure 3B, Table 1). However, a little effect by ATRA in BGC/ aRARα cells observed in this study (Figure 5B) was associated with a weakened inhibition in BGC/aRARα cell proliferation (Figure 3B, Table 1). Thus, anti-AP-1 activity is one of the mechanisms for ATRA to inhibit growth of gastric cancer cells, and RARα does play a critical role.

RARα, once activated by RA, forms a heterodimer with RXR, then bind to retinoic acid response element (such as βRARE), and regulates transcription and expression of target genes[13-17]. In acute promyelocytic leukemia cells and RA-resistant breast cancer cells, RA could up-regulate the expression of RARα via modulation of RARE motif located in RARα promoter[39-41]. The fact that when the reporter gene βRARE-tk-CAT was transfected into MGC80-3, BGC-823 and SGC-7901 cells, a marked increase in βRARE transcriptional activity induced by ATRA was observed (Figure 4A) suggested that RARs are functional in these cell lines, i.e., to activate βRARE transcriptional activity in the presence of ATRA, and then to stimulate cell growth inhibitory signals to repress the growth of cancer cells. However, when the same reporter gene was transfected into MKN-54 cells, the âRARE transcriptional activity induced by ATRA was relatively low (Figure 4A), indicating the abnormality of βRARE transcriptional regulation or functional loss of RARα in MKN-45 cells, which caused the failure of growth inhibition of MKN-45 cells by ATRA. The similar results were further confirmed by transient transfection assay in transfected gene cell lines, BGC/aRARα and MKN/ RARα, respectively (Figure 4B). All these data are consistent with those observed in breast cancer cells and lung cancer cells[1,42], and imply that low-level expression of retinoic acid receptors in cancer cells is closely associated with the development of malignant tumor. RARα might serve as a candidate marker to determine which gastric cancer patient would respond to and benefit from the retinoid therapy, and this is also useful for the synthesis of RARα-selective retinoids. Of course, some further experiments to verify this issue are needed.

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Edited by Lu HM