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

Su Liu, Qiao Wu, Zheng-Ming Chen and Wen-Jin Su

The Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, The School of Life Sciences, Xiamen University, Xiamen 361005, Fujian Province, China

Supported by the National Outstanding Youth Science Foundation of China (B type), No.39825502 and the National Natural Science Foundation of China, No.39980015.

Corresponding to: Dr. Qiao Wu, the Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, The School of Life Sciences, Xiamen University, Xiamen 361005, Fujian Province, China. xgwu@xmu.edu.cn

Phone: +86-592-2182542, Fax: +86-592-2086630

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 transcriiptional 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 RARα 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-45/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

Original Research

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 superfamly, 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 transcrption 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[27]. 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 RARα 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 100×10⁻⁶ 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 32P-labeled probe as previously described[20]. 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, together 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 expression vector (pCH110, Pharmacia), and carrier DNA.

**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 RARα 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·L-1 agar in medium supplemented with 100 mL ·L-1 FCS was first prepared and hardened in 6-well plate. Cells 1×108 ·L-1, in culture medium containing 100 mL ·L-1 FCS, 5 g·L-1 agar, and 10-6 mol·L-1 ATRA (only for experimental groups), were seeded onto the underlayer. The plate was incubated for three weeks in CO2 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).

**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).

**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 | MKN/ RARα | BGC/ aRARα |
|-------------------|-----|-----|-----|-----|-----------|------------|
| Inhibitory rate (%) | 48.8b | 45.2b | 65.3b | 14.3b | 56.1b | 15.2b |

bP<0.01, vs control.

Regulation of ATRA on αRARE transcriptional activity

When transient transfection was performed with reporter gene, βRARE-κ-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 transformation of tumor cells, and can be induced by some agents for mitogen, such as TPA (12-O-tetradecanoylphorbol-13-acetate)⁴¹⁻⁻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.
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 plays 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 RARα 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 βRARE 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[4,42], 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.

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[1,13,30,34-36]. Effects of retinoic acid are mediated by its receptors RARs and RXRs[8-13]. In the present study, we demonstrated that the molecular mechanism by which RA inhibited the growth of gastric cancer cells was involved in RARα-mediated signal...
REFERENCES

1 Wu Q, Dawson MI, Zheng Y, Hobbs PD, Agadir A, Long L, Li Y, Liu R, Zhang XK. Inhibition of trans-retinoic-acid-resistant human breast cancer cell growth by retinoid X receptor-selective retinoids. Mol Cell Biol, 1997;17:6599-6608

2 Chen Y, Xu CF. All-trans-retinoic acid induced differentiation in human gastric carcinoma cell line SGC-7901. Xiamin Xuebao Xueyi Zazhi, 1997;5:91-92

3 Xia ZS, Zhu ZH, He SG. Effects of ATRA and 5-Fu on growth and telomerase activity of xenografts of gastric cancer in nude mice. Shijie Huaren Xiaohua Zazhi, 2000;6:674-677

4 Hao ZL, Li DG, Lu HM, Gu XH. The effect of retinoic acid on Ito cell proliferation and content of DNA and RNA. World J Gastroenterol, 1999;5:443-444

5 Chen YQ, Wu Q, Chen ZM, Su WJ, Chen F. Effects of retinoic acid on metastatic ability of gastric cancer cells MzGa38-3 in vivo and in vitro. Huaren Xiaohua Zazhi, 1998;6:869-872

6 Huang CC, Zhang JS, Zhang YE. Effects of retinoic acid on proliferation, phenotype and expression of cyclin-dependent kinase inhibitors in TGF-α1-stimulated rat hepatic stellate cells. World J Gastroenterol, 2000;6:819-823

7 Li LP, Zhang Z, Han SX. Retinoic acid in inhibition of cancer cell proliferation and induction of apoptosis. Shijie Huaren Xiaohua Zazhi, 2001;9:437-440

8 Zhang XK, Hoffmann B, Tran P, Graupner G, Pfahl M. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature, 1992;355:441-446

9 Zhang XK, Pfahl M. Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. Trends Endocrinol Metab, 1993;4:156-162

10 Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell, 1995;83:841-850

11 Benbrook D, Lernhardt E, Pfahl M. A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature, 1988;333:624-629

12 Brand N, Petkovich M, Krust A, Marchio A, Tiollais P, Dejean A. Identification of a second human retinoic acid receptor. EMBO J, 1992;11:1409-1418

13 Lied M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski R, Karin M, Pfahl M. Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation. New Biol, 1991;3:1206-1219

14 Bugge TH, Pohl J, Lonnon Y, Stunnenberg HJ. RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J, 1992;11:1409-1418

15 Wu Q, Li Y, Liu R, Agadir A, Lee MO, Liu Y, Zhang XK. Modulation of retinoic acid sensitivity in lung cancer cells by a dynamic balance of RAR and TR heterodimers to binding target sequences efficiently. Cell, 1992;68:377-395

16 Marks MS, Hallenberg PL, Nagata T, Segars JH, Apella E, Nikodem VM, Ozato K. H-2RIBBP (RXRβ) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J, 1992;11:1419-1435

17 Li Y, Hashimoto Y, Agadir A, Kagechika H, Zhang XK. Identification of a novel class of retinoic acid receptor β-selective retinoid a nagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. J Biol Chem, 1999;274:15360-15366

18 Gebert JF, Mognal N, Frangioni JV, Sugabaker DJ, Neel BG. High frequency of retinoic acid receptor β abnormalities in human lung cancer. Oncogene, 1991;6:1595-1606

19 de la Marchio A, Tiollais P, Dejean A. Differential expression and ligand regulation of the retinoic acid receptor α and β genes. EMBO J, 1989;8:429-433

20 Boylan JF, Lufkin T, Achkar CC, Tanqueja R, Chambon P, Gudas LJ. Targeted disruption of retinoic acid receptor α (RARα) and RARγ results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. Mol Cell Biol, 1995;15:843-851

21 Huang M, Ye YC, Chen SR, Chai JR, Lu JX, Zhao L, Gu LJ, Wang ZY. Use of all trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood, 1988;72:567-572

22 Castaigne S, Chomienne C, Daniel MT, Ballerini P, Berger R, Fenaur P, Degas L. All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. Blood, 1990;76:1704-1709

23 Warrell RP, Frankel SR, Miller WH, Scheinberg DA, Itti LM, Hittelman W N, Vyazs R, Mandel H, Tatifi A, Jakubowski A, Gabrilove J, Gordon M, Dimitrovsky E. Differentiation therapy of acute promyelocytic leukaemia with tretinoin (all-trans retinoic acid).

24 Nervi C, Volleberg TM, George MD, Zelent A, Chambon P, Jetten AM. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. Exp Cell Res, 1991;195:163-170

25 Gao ZL, Li DG, Lu HM, Gu XH. The effect of retinoic acid on Ito cell proliferation and content of DNA and RNA. World J Gastroenterol, 1999;5:443-444

26 Chen YQ, Wu Q, Chen ZM, Su WJ, Chen F. Effects of retinoic acid on metastatic ability of gastric cancer cells MzGa38-3 in vivo and in vitro. Huaren Xiaohua Zazhi, 1998;6:869-872

27 Xia ZS, Zhu ZH, He SG. Effects of ATRA and 5-Fu on growth and telomerase activity of xenografts of gastric cancer in nude mice. Shijie Huaren Xiaohua Zazhi, 2000;6:674-677

28 Wu Q, Dawson MI, Zheng Y, Hobbs PD, Agadir A, Jong L, Hobbs PD, Zhang XK. Inhibition of trans-retinoic-acid-resistant human breast cancer cell growth by retinoid X receptor-selective retinoids. Mol Cell Biol, 1997;17:6599-6608

29 Chen Y, Xu CF. All-trans-retinoic acid induced differentiation in human gastric carcinoma cell line SGC-7901. Xiamin Xuebao Xueyi Zazhi, 1997;5:91-92

30 Ka Y, Hashimoto Y, Agadir A, Kagechika H, Zhang XK. Identification of a novel class of retinoic acid receptor β-selective retinoid a nagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. J Biol Chem, 1999;274:15360-15366

31 Shen J, Yu M, Lindqvist H, Chen Z, Lin P, Hobbs PD, Zhang XK. Inhibition of trans-retinoic-acid-resistant human breast cancer cell lines: effect on growth inhibition and apoptosis induction. Int J Cancer, 1998;75:88-95

Edited by Hu LM