Role of AP-1 Antagonism in Growth Inhibition of Cervical Cancer Cell Lines by Retinoids

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Abstract: Retinoid compounds induce multiple molecular effects that need to be taken into consideration in the development of these agents as pharmaceuticals for cancer chemoprevention. Inhibition of cancer cell growth by retinoids is thought to occur through at least two mechanisms of transcriptional regulation resulting from direct binding of retinoids to nuclear retinoid receptors (RARs and RXRs). First, the nuclear receptors induce or repress expression of specific genes by direct binding to retinoic acid response elements (RAREs) in gene promoters. Second, the nuclear receptors antagonize transactivation of activator protein-1 (AP-1) promoter elements by the AP-1 transcription factors. The objective of this study was to determine the contributions of RARE transactivation and AP-1 antagonism to the mechanism of growth inhibition in cervical cancer cell lines by: the 9-cis isomer of retinoic acid (9-cis-RA), which binds both RARs and RXRs; and a series of synthetic retinoids, called heteroarotinoids, that possess various receptor specificities and reduced toxicity. The effects of these compounds on reporter gene expression and proliferation were measured in CC-1 and SiHa cell lines stably transfected with RARE or AP-1 reporter plasmids or a retrovirus harboring an inducible AP-1 protein that is a dominant negative mutant of c-Jun (TAM67). In the CC-1 cell line, which exhibited high AP-1 activity, retinoid growth inhibition significantly correlated with both RARE transactivation and AP-1 repression, was antagonized by superinduction of AP-1 with TPA (12-O-tetradecanoylphorbol-13-acetate) and was enhanced by TAM67. In the SiHa cell line, which exhibited low AP-1 activity, RARE transactivation also significantly correlated with growth inhibition, but AP-1 induction, in contrast to AP-1 repression, correlated with growth inhibition. Furthermore, TPA superinduction of AP-1 did not attenuate retinoid growth inhibition activity in SiHa. In conclusion, repression of AP-1 with retinoids may only be effective against a subset of tumors with high AP-1 activity.

Key words: AP-1, retinoids, cervical cancer, growth inhibition

INTRODUCTION

Retinoic acid (RA) and synthetic analogs, called retinoids, are promising anti-cancer agents based on their abilities to regulate growth, differentiation, apoptosis, angiogenesis and the immune system[1]. These activities are mediated by two classes of nuclear retinoid receptors (RARs and RXRs), which act as transcription factors by binding to retinoic acid response elements (RAREs) as dimers[2,3]. Natural isomers of RA and synthetic retinoids can selectively bind to and regulate RARE transactivation by retinoid receptors. The all-trans and the 13-cis isomers of RA (trans-RA and 13-cis-RA, respectively) bind only to the RAR’s while the 9-cis isomer (9-cis-RA) regulates both the RARs and RXRs[4]. Since the first isomer studied, trans-RA, was too toxic for most clinical applications, efforts to reduce the toxicity by designing synthetic retinoids selective for receptors and activities were pursued by restricting the flexibility of the tetraene side chain of trans-RA through incorporation into aromatic rings[5,6]. Thousands of different aromatic retinoids, called arotinoids, have been synthesized and many were found to be selective for retinoid receptors and retinoid activities[7,8]. The first arotinoid thoroughly evaluated, TTPNB, was found to be 1000-fold more toxic than trans-RA[9,10]. Incorporation of a heteroatom into the cyclic ring of arotinoids resulting in compounds called heteroarotinoids reduced the toxicity 1000-fold in the diaryl TTNPB structure and 3000 fold in the monocaryl heteroarotinoid structure[11]. Therefore, this class of compounds has the potential for clinical application as anti-cancer drugs. In support of this, the Chemoprevention Working Group to the American Association for Cancer Research concluded that retinoids are considered one of the most promising classes of cancer chemoprevention agents[12].

Although the toxicities have been reduced, the complexities of retinoid activities still need to be deciphered in order to understand and optimize the activities involved in the anti-cancer mechanisms. In addition to RARE transactivation, retinoids inhibit transactivation by the Activator Protein 1 (AP-1) transcription factors which include the Jun, Fos and CREB families of transcription factors, as well as other leucine zipper proteins[13]. AP-1 transcription factors also bind to DNA response elements (AP-1 sites) as dimers and regulate growth, differentiation and apoptosis[14]. Induction of AP-1 is an immediate early response in cells stimulated to grow or stimulated with a stress-induced signal, such as DNA damage[14]. The AP-1 transcription factor called cJun is considered an oncogene and is highly regulated through post-translational modifications including phosphorylation and oxidation of the transcriptional activation and DNA binding

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RARE and AP-1 transactivation and repression: The reporter cell lines were plated in 6-well plates at 5×10^5 cells well⁻¹ and incubated overnight to allow cells to become attached. The next day, the media were replenished and drugs were added. After 48 hrs of treatment, cell lysates were prepared according to published procedures[23] and assayed for the CAT expression using the CAT ELISA Kit (Roche Molecular Biochemicals, Indianapolis, IN). The protein concentration of each lysate was determined with the Protein Assay reagent (BioRad, Richmond, CA). All results were normalized for protein concentration.

Growth assays: Cultures were plated in 96-well microtiter plate at 400 cells well⁻¹ and incubated over night. The next day, the media in each well were carefully removed with a multi-channel pipetman and replaced with media containing appropriate concentration of drugs. For the 3-day growth assay, the media and drug were not replenished for the duration of the treatment period. For the 7-day growth assay, the media and drugs were replenished every two days. A the end of the treatment period, growth was assayed by the MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium inner salt) assay (Promega, Madison, WI). All treatments were performed in triplicate. The growth index was determined by dividing the average OD of the treated wells by the average OD of the control untreated wells. The results were presented as the average and standard deviation of 3 to 10 experiments.

Retroviral expressions of dominant negative cJun: Eight micrograms of the TAM67 expression vector (gift from Dr. Michael Birrer, National Institutes of Health) was transfected into Phoenix A cells using Fugene 6. After 48 hrs, the media was replenished and cells were incubated at 32°C for 48 hrs to accumulate virus particles. The media was then collected, spun at 10,000 rpm for 10 min to eliminate any phoenix A cells and then either directly used to infect other cells or frozen at −70°C for future use. Before infection, CC-1 and SiHa cells were plated at 5X10^5 cells well⁻¹ on 6-well plates, 24 hrs before infection. The media in each well were then removed and a cocktail consisting of 0.5 mL virus-containing media and 6 μg mL⁻¹ polybrene (hexadimethrine bromide) (Sigma, St. Louis, MO) in MEM media. After 6 hrs of incubation at 37°C, the cocktail was removed and replaced with fresh MEM media. The cultures were then incubated at 37°C for another 48 hrs to allow the expression of blasticidin S deaminase. The cells in each well were then trypsinized and plated at low density and

MATERIALS AND METHODS

Cell lines and drug treatment: CC-1 and SiHa cell cultures were maintained in Eagle’s minimal essential media (MEM) containing Earle’s salts and L-glutamine (Cellgro, Mediatech, Herndon, VA) supplemented with nonessential amino acids, sodium pyruvate, anti-biotic-anti-mycotic and 10% fetal bovine serum. Phoenix A cell cultures were maintained in Dubeccal's modified eagle media (DMEM) containing pyridoxine hydrochloride and high glucose supplemented with L-glutamine, anti-biotic-anti-mycotic and 10% fetal bovine serum. Media were replenished every 2 days. The synthetic retinoids (heteroarotinoids - gifts of K. Darrell Berlin, Oklahoma State University) were synthesized as previously described[11,21,22]. The 9-cis-RA (BIOMOL, Plymouth Meeting, PA), heteroarotinoids and TPA (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) at 1000× stock solutions, so that the final concentration of DMSO in all cultures was less than 0.1%, which is not cytotoxic and does not induce differentiation. Drugs were replenished every 2 days, with the media. The reporter cell lines were established by co-transfection of the pCoICAT reporter plasmid, which contains the collagenase AP-1 DNA binding site and the thymidine kinase (tk) promoter driving the chloramphenical acetyl transferase (CAT) gene, or the RARE-tk-CAT reporter plasmid, which contains the RARE from the RARβ gene in place of the AP-1 site in the same plasmid. CC-1 and SiHa cultures were plated in 6-well plates at a concentration of 5×10^5 well⁻¹ before the day of transfection. The next day, 2 μg of each reporter plasmid was mixed with 0.2 μg pXT1neo' plasmid (in order to convey the Genetecin resistance) and diluted in 100 μl serum-free MEM media containing 3 μl of Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN). Genetecin-resistant colonies were isolated and tested for CAT expression.
blasticidin S was added to isolate resistant colonies. To induce TAM67 expression, 1 µg mL⁻¹ of doxycycline was added to the culture media simultaneously with retinoid or TPA treatment.

**Electrophoretic mobility shift assay (EMSA):** A double stranded oligonucleotide probe encoding the collagenase gene promoter AP-1 response element (GATCCTAAGGCATGACTAGCACCG) was radioactively labeled using MuMLV Reverse Transcriptase and α-P³²-dCTP (New England Nuclear, City, State) and used in the binding assay. A probe mixture was prepared by mixing 2 µl of the labeled oligonucleotide, 88 µl of the Gel-Retardation Buffer (25 mM HEPES pH 7.9, 150 mM KCl, 1 mM EDTA, 5 mM DTT and 10X Denhardts reagent) and 10 µl 2.5 mg mL⁻¹ poly-dIdC. For each binding reaction, exactly 1 µg of nuclear proteins was mixed with 15 µl of GRB, 5 µl probe mixture and appropriate amount of nuclear extraction buffer so that the final volume was 25 µl. The mixture was incubated at 4°C for 20 minutes. Reactions containing 100 fold excess specific competitor (unlabeled same oligonucleotide) and non-specific (unlabeled and mutated AP-1 binding site) and antibody against cJun were also prepared to test the specificity of binding. All reactions were then directly loaded onto an 8 % non-denaturing polyacrylamide gel and electrophoresed for 2 hrs at 200 volt in 0.5XTBE (Tris, Borate and EDTA) buffer. The gel was dried and the bands were detected and quantitated by either using a phosphoimage system (Dynamic Molecules) or exposing a Kodak X-ray film and densitometric scanning of the bands. Each experiment was repeated between 1 to 3 times to confirm the results.

**Western analysis:** Cultures in 6-well plates were trypsinized and collected into 1.5 mL eppendorf tubes and washed twice with PBS. The pellets were re-suspended in suspension buffer (0.1 mol L⁻¹ NaCl, 10 mmol L⁻¹ Tris-HCl (pH7.6), 1 mmol L⁻¹ EDTA (pH8.0), 1 µg mL⁻¹ Aprotinin, 100 µg mL⁻¹ PMSF) and lysed by adding SDS loading buffer (0.1 mol L⁻¹ Tris-HCl (pH6.8), 0.2 mol L⁻¹ DTT, 4% SDS, 20% Glycerol, 0.2% Bromophenol Blue). Samples were then heated at 100°C for 10min, vortexed vigorously and frozen at -20°C. Samples were quickly thawed and centrifuged at 12,000 rpm for 15 min before loading on 10% denaturing gel (0.375 mol L⁻¹ Tris-HCl (pH 8.8), 0.1% SDS in Tris-Glycine buffer (3.03 g L⁻¹ Tris, 14.4 g L⁻¹ Glycine, 0.1% SDS, pH 6.6) and electrophoresed for 1 hour. Separated proteins were transferred from gel to PVDF membrane (Millipore IMMOBIOLONTM-P) using a BioRad TRANSBLOT semi-dry transfer cell at 200mA for 30 min. The membrane was then blocked in 10% blocking solution (10% dry nonfat milk, 20 mmol L⁻¹ Tris-HCl (pH 8.0), 100 mmol L⁻¹ NaCl, 0.05% Tween-20) for 1 hour at room temperature, washed with washing buffer (20 mmol L⁻¹ Tris-HCl (pH8.0), 100 mmol L⁻¹ NaCl, 0.05% Tween-20, 2 mmol L⁻¹ CaCl₂) for 15min, incubated in primary antibody (1 mg mL⁻¹ in washing buffer) for 1 hour, washed in washing buffer for 15 min, incubated in secondary antibody (Anti-Rabbit-POD from Roche Molecular Biochemicals, Indianapolis, IN, 0.02 U mL⁻¹ in washing buffer) for 1 hour and finally washed 3 times in washing buffer. The membrane was soaked in substrate solution (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 minute before film exposure. Each Western was repeated once to confirm the results.

**Statistical analysis:** Statistical analysis was performed using GraphPad Software (Prism).

**RESULTS**

**Correlation of RARE and AP-1 transactivation with growth inhibition:** Transactivation of RARE and AP-1 DNA elements by transcription factors endogenous to cancer cells was measured in CC-1 and SiHa cell lines that were stably transfected with reporter plasmids. These plasmids encode the chloramphenicol acetyl transferase (CAT) gene under the control of an RARE or an AP-1 DNA binding sites, so that the levels of CAT produced in the cells represent a measure of transcriptional regulation from the RARE or AP-1 DNA binding sites. The CC-1 and SiHa RARE reporter cell lines exhibited similar dose-response curves of RARE transactivation by 9-cis-RA that were saturated at the 6-7 fold induction level in the µM concentration range (Fig. 1A). In contrast, AP-1 induction by TPA was 10 fold higher in the CC-1 reporter cell line than in the SiHa reporter cell line, but both reached saturation between 0.4 and 0.8 ng mL⁻¹ TPA (Fig. 1B). This 10-fold difference was consistently observed with several CC-1 and SiHa AP-1 reporter cell lines tested. These reporter cell lines were used to screen a series of 9 synthetic retinoids and the natural 9-cis-RA for their ability to regulate RARE and AP-1 transactivation and to inhibit growth in the same cell lines. Linear regression analysis demonstrated a statistically significant direct correlation between reductions in the growth indexes and RARE transactivation caused by the retinoids in both CC-1 (P = 0.0003) and SiHa (P = 0.0026 ) (Fig. 1C and 1D, respectively). Although retinoid repression of AP-1 induction directly correlated with growth inhibition in CC-1 (P = 0.0003), it inversely correlated with growth inhibition in SiHa (P = 0.145)[Fig. 1E and 1F, respectively].

**Mutual antagonism of retinoids and TPA on RARE and AP-1 transactivation:** The natural 9-cis-RA exhibited a dose responsive repression of AP-1 activity in the CC-1 and SiHa AP-1 reporter cell lines (Fig. 2A and 2B, respectively). TPA concentrations that induced maximal AP-1 activity reduced, but did not prevent dose-responsive AP-1 antagonism caused by 9-cis-RA in both cell lines. Conversely, 9-cis-RA concentrations that maximized RARE transactivation reduced, did not prevent TPA-induction of AP-1 activity in the CC-1 reporter cell line (Fig. 1B).

The DNA binding activities of the AP-1 transcription factors in CC-1 and SiHa, were evaluated with EMSA's performed on nuclear protein extracts isolated from cultures...
Fig. 1: Correlation of retinoid regulation of RARE transactivation, AP-1 transactivation and growth. A, RARE transactivation by 9-cis-RA in CC-1 and SiHa RARE reporter cell lines. B, AP-1 transactivation by TPA in CC-1 and SiHa AP-1 reporter cell lines. The effects of 10 µM 9-cis-RA on TPA transactivation of AP-1 is shown for CC-1. C-F, Correlation analysis. The reporter cell lines CC-1 RARE (C), the SiHa RARE (D), CC-1 AP-1 (E) and SiHa AP-1 (F) were treated with 10 different retinoids. The growth index after 3 days of treatment and the RARE transactivation after 2 days of treatment were plotted and correlated using GraphPad Software.

Fig. 2: Mutual antagonism of 9-cis-RA and TPA on AP-1 Transactivation and DNA binding. A-B, Repression of AP-1 induction by 9-cis-RA in the absence and presence of 0.4 ng mL⁻¹ TPA in the CC-1 AP-1 (A) and SiHa AP-1 (B) reporter cell lines. C-D, EMSAs of the collagenase AP-1 DNA binding site oligonucleotide incubated alone (lane 0), or with nuclear extracts from cultures that were untreated (lanes 1, 5, 9), treated with 10 µM 9-cis-RA (lanes 2, 6, 10), with 0.4 ng mL⁻¹ TPA (lanes 3, 7 and 11), or both agents (lanes 4, 8, 12) for 2 days. Bar graphs below the EMSAs represent densitometric quantitation of the specific bands in lanes 1 (DMSO), lane 2 (9-cis-RA), lane 3 TPA) and lane 4 (TPA + RA).
Retinoid Growth inhibition in the context of TPA-antagonized AP-1 repression: The mutual antagonism of TPA and retinoids on AP-1 activity was utilized to test if retinoids can inhibit growth in the absence of AP-1 repression. Cultures were treated with a range of 9-cis-RA concentrations in the presence and absence of the concentration of TPA that saturated AP-1 induction (0.4 ng mL⁻¹). TPA alone inhibited the growth of CC-1 by 8 % and SiHa by 15 %. After normalizing for the effects of TPA alone, it was still apparent that TPA antagonized retinoid growth inhibition in CC-1 (Fig. 3A), but had only slight effect in SiHa (Fig. 3B). Since TPA activation of PKC has many other effects besides the activation of cJun and AP-1 transactivation, the TPA interactive effects with retinoids may be due to AP-1 independent effects of TPA. To determine if TPA and 9-cis-RA effects were additive, indicating that they work through the same mechanism of action, or if the were synergistic, indicating that they work through complementary mechanisms of action, the fractional product method was used. In this method if the effect of combined treatment (E₉-cis-RA X E₉-cis-RA) is greater than the product of the effects of the individual treatments (E₉-cis-RA X E₉-cis-RA), the two drugs are considered to work synergistically. In CC-1, the products of growth index of TPA alone and 9-cis-RA alone were not significantly different from the growth indexes of the combined treatment at all 9-cis-RA doses tested. Therefore the antagonism of 9-cis-RA growth inhibition in CC-1 is additive and not synergistic, suggesting that both agents regulate CC-1 growth through the same molecular mechanism of actions, i.e. regulation of AP-1 activity.

The ability of saturating levels of TPA to counteract growth repression by the 9 synthetic retinoids was evaluated in the CC-1 and SiHa reporter cell lines and the effects of the combined treatments were normalized for the effects of TPA alone. When used in combination, TPA alleviated growth repression by most compounds in CC-1 (Fig. 3D), but not in SiHa (Fig. 3E).

Retinoid growth inhibition in the context of quenched AP-1: To further test the hypothesis that retinoids can regulate growth independent of their AP-1 repression, a dominant negative mutant of one of the AP-1 transcription factors, called cJun, was utilized to quench AP-1 activity. This mutant, called TAM67, is deleted in the protein domain responsible for

![Fig. 3: TPA antagonizes retinoid growth inhibition in CC-1, but not in SiH.](image-url)
transactivation and therefore will dimerize with the full-length cJun and all of the other AP-1 transcription factors, resulting in nonproductive complexes that can bind DNA but cannot transactivate AP-1 sites[16]. The TAM67 mutant was introduced into CC-1 and SiHa cell lines using a retrovirus vector that contains the Blasticidin S resistance gene and Blasticidine S resistant colonies were isolated. The retrovirus was induced to express TAM67 using a drug called doxycycline (dox). In CC-1 cultures containing the TAM67 retrovirus, Western Analysis demonstrated leaky expression of TAM67 in the absence of dox treatment and induction of TAM67 in the presence of dox (Fig. 4A). Consistent with the known positive autoregulation of cJun expression, dox decreased the expression of cJun demonstrating loss of cJun transactivation activity in the presence of TAM67 (Fig. 4A). Despite leaky expression of TAM67 in SiHa cultures containing the retrovirus (SiHaT67), the TAM67 protein was not inducible by dox treatment in 8 separate clones isolated and evaluated (4 of the clones are shown in Fig. 4A). The SiHa clones exhibited a very low to undetectable levels of wild type cJun expression in comparison to CC-1.

The effects of TAM67 expression on 9-cis-RA growth inhibition was tested by inducing TAM67 expression with dox treatment. Although dox had no effect on growth of the parental CC-1 cell line (Fig. 4B), it significantly enhanced the growth inhibition caused 9-cis-RA in the CC1 TAM67 clone (Fig. 4C). This effect was due to more than an additive effect of dox and 9-cis-RA on CC1 TAM67 growth because the growth indexes in the combined treatment curve were normalized for the effect of dox alone. To further demonstrate that the effects of dox-induced TAM67 expression and 9-cis-RA treatment were synergistic and not additive, the fractional product method was used[24]. The product of the growth indexes of dox and 9-cis-RA single agents was plotted on the same graph as the growth index curves of 9-cis-RA alone and in combination with dox (but not normalized for dox alone) (Fig. 4E). The combined treatment curve is clearly below the calculated fractional product curve (dashed line), demonstrating synergistic effects of TAM67 and 9-cis-RA and indicating that these two agents act through separate but complementary mechanisms of action. As expected, TAM67 expression and dox treatment had no effects in SiHa (data not shown), most likely due to its low level of endogenous AP-1 activity.

**DISCUSSION**

The results of this study demonstrate that the contribution of AP-1 antagonism to the mechanism of retinoid growth inhibition is dependent upon the cellular environment. While RARE transactivation correlated with growth inhibition in both cell lines, AP-1 antagonism only appeared to play a major role in the context of high AP-1 activity. In the CC-1 cell line, which exhibited high levels of AP-1 activity, the contribution of AP-1 antagonism to retinoid growth inhibition was demonstrated by the findings that: 1) AP-1 antagonism significantly correlated with growth inhibition activities of 10 retinoids, 2) TPA induction of AP-1 additively antagonized retinoid growth inhibition, and 3) TAM67 inhibition of AP-1 enhanced retinoid growth inhibition. The SiHa cell line, which exhibited high levels of AP-1 activity, the contribution of AP-1 antagonism to retinoid growth inhibition was demonstrated by the findings that: 1) AP-1 antagonism significantly correlated with growth inhibition activities of 10 retinoids, 2) TPA induction of AP-1 additively antagonized retinoid growth inhibition, and 3) TAM67 inhibition of AP-1 enhanced retinoid growth inhibition. The SiHa cell line, which exhibited a low level of AP-1 however, did not exhibit significant contribution of AP-1 antagonism to the mechanism of retinoid growth inhibition. In SiHa, AP-1 antagonism inversely correlated with growth inhibition and TPA induction of AP-1 did not significantly attenuate retinoid growth inhibition. Since RARE transactivation correlated with growth inhibition in both CC-1 and
SiHa, it is likely that this activity is primarily responsible for growth inhibition in SiHa and is overshadowed by AP-1 antagonism in CC-1.

The SiHa cell line exhibited the greatest sensitivity to retinoid growth inhibition, but also exhibited the lowest level of basal and induced AP-1 activity. TPA-induction in SiHa was 10-fold lower than in CC-1 and Western Analysis confirmed a very low level of c-Jun expression in SiHa in comparison to CC-1. This trend of lower AP-1 associated with increased retinoid sensitivity was also observed in the retinoid-sensitive MCF7 breast carcinoma cell line, which has low basal AP-1 activity, but that can be made retinoid-resistant by overexpression of c-Jun.9-10 cis-RA dose-responsively repressed the low level of AP-1 activity in SiHa, this activity inversely correlated with growth inhibition. This might be explained by the ability of AP-1 to induce apoptosis in specific cellular environments.

**CONCLUSION**

The transcriptional mechanism of retinoid growth inhibition is dependent upon the cellular context. In a cellular environment with high AP-1 activity, AP-1 antagonism can play a major role in retinoid growth inhibition, but in a cellular environment with low AP-1 activity, AP-1 antagonism can counteract retinoid growth inhibition. Therefore, development of retinoids to specifically target AP-1 may only be effective against a subset of tumors with high AP-1 activity.

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