ABSTRACT

To investigate the role of AP-1 transcription factors in mediating retinoid-induced growth suppression of breast cells, we studied the sensitivity of MCF7 breast cancer cells with different levels of AP-1 activity to all-trans retinoic acid (aTRA). AP-1 activity was increased in MCF7 cells by stably transfecting c-jun DNA into these cells. Parental and vector-transfected MCF7 cells, which were sensitive to the growth-inhibitory effects of aTRA, exhibited aTRA-dependent retinoic acid receptor (RAR) transactivation and transrepression of 12-O-tetradecanoylphorbol-13-acetate-induced AP-1 activity. The c-jun-transfected MCF7 cells had increased basal AP-1 transactivation activity and increased expression of AP-1-regulated genes but were resistant to the antiproliferative effects of aTRA. However, MCF7 cells transfected with a deletion mutant of c-jun, TAM-67, which lacks most of the amino-terminal transactivation domain of cJun and is unable to activate AP-1-dependent gene expression, were sensitive to the growth-inhibitory effects of aTRA. These results suggest that the transactivation domain of cJun is required for induction of retinoid resistance in these breast cancer cells. aTRA did not activate RAR-dependent gene transcription or transrepress 12-O-tetradecanoylphorbol-13-acetate-induced AP-1 activity in these c-jun-overexpressing cells. Investigation of the RAR and retinoic acid X receptor expression level demonstrated that RARα and RARγ RNA expression was reduced in the c-jun-transfected MCF7 cells, whereas RARβ expression was upregulated. However, retinoic acid responsive element DNA binding activity was intact in c-jun-transfected cells. Therefore, the mechanism by which c-jun overexpression induces resistance to the growth-inhibitory effect of aTRA may be through interference with aTRA-dependent RAR transactivation or AP-1 transrepression, possibly through titration of essential coactivators. These results suggest that the antiproliferative effects of retinoids can be overcome by c-jun overexpression.

INTRODUCTION

The vitamin A-derived retinoids play an important role in regulating a broad range of biological processes including cell growth, differentiation, and development in variety of cell types and tissues (1). Retinoids also inhibit the growth and invasion of cancer cells (2, 3) and are clinically useful for the treatment and prevention of cancer (4). In breast cells, retinoids inhibit breast cancer cell growth (5–7) and induce apoptosis (8). One synthetic retinoid, N-(4-hydroxyphenyl)-retinamide, is being evaluated in a clinical trial for its ability to inhibit breast cancer cell development (9, 10).

Retinoids exert their effects by interacting with specific RAR3 and RXR receptors (RARα, β and γ; RXRα, β and γ) (11). Retinoid receptors are ligand-dependent DNA-binding proteins which regulate the expression of target genes by binding to the RAREs in the promoters of these target genes. The RAR and RXR isotypes are expressed differently during development and differentiation (12), and these various isotypes can heterodimerize to produce a variety of RAR:RXR complexes, which likely regulate different sets of retinoid-induced genes. This complexity is increased further by activation of the RAR and RXR receptors by different retinoid ligands. aTRA binds and activates RARs, whereas 9-cis-retinoic acid binds and activates both RARs and RXRs. RAR and RXR isotypes are expressed in breast cancer cells; however, their levels of expression vary widely. In general, RARα RNA expression is higher in ER+ cells than in ER− cells (13). RARβ RNA is frequently undetectable or expressed at a very low level in ER+ cells (14) but is detectable in some ER− cells (13). RARγ is expressed at similar levels in both ER+ and ER− cell lines (13). RXRα, RXRβ, and RXRγ RNAs have been detected in both ER+ and ER− breast cancer cells (15).

Many breast cancer cells are sensitive to the growth-suppressive effects of retinoids. The exact mechanism by which retinoids inhibit their growth is not well understood. Depending on the cell type and the specific retinoid ligand, retinoids have been shown to induce apoptosis, differentiation, or inhibit proliferation directly. Recent studies suggest that certain retinoid ligands, especially those which activate RXRs, are potent apoptosis-inducing agents (16), whereas other retinoids that activate RARs without activating RXR receptors regulate differentiation (16).

Studies in breast cancer cells (17) and human bronchial epithelial cells (18) suggest that retinoids may inhibit proliferation through inhibition of AP-1 transcription factor. The AP-1 transcription factor consists of heterodimers formed between Jun and Fos family members of protooncoproteins (19) or homodimers of Jun proteins. Activation of AP-1 transcription factors can regulate proliferation, differentiation, or apoptosis, depending on the cell type (19). In breast epithelial cells, AP-1 transcription factors likely regulate proliferation, because many hormones mitogenic for these cells, such as epidermal growth factor, transforming growth factor α, and insulin-like growth factors, activate this family of transcription factors (20). Hormones such as glucocorticoids, estrogens, and retinoids have been shown to inhibit the activity of the AP-1 transcription factor through transcription factor interaction (21–24). The antagonism between AP-1 and these nuclear hormone receptors may be mediated by competing or squelching essential coactivators such as CBP (25, 26). However, the exact mechanism of this transcription factor cross-talk is not fully understood, and more indirect mechanisms may also be involved. A recent study by Fanjul et al. (7) suggested that the ability of retinoids to inhibit AP-1 may be one mechanism by which retinoids inhibit breast cell growth. These workers demonstrated that specific synthetic retinoids, which fail to activate retinoid receptor-dependent gene expression but which can transrepress AP-1, inhibited the proliferation of several epithelial cell lines, including the T47D breast cancer cells.

To investigate the role of AP-1 in mediating the growth-inhibitory effects of retinoids, we have compared the sensitivity of breast cancer cells with different levels of AP-1 activity to aTRA. To generate comparable breast cancer cells with different levels of AP-1 activity, we overexpressed the c-jun gene in the MCF7 breast cancer cell line, a retinoid-sensitive cell line that has previously been found to have low basal AP-1 activity (20). The present studies demonstrate that overexpression of c-jun in breast cancer cells induces resistance to the
Fig. 1. A, atRA inhibits the growth of MCF7 breast cancer cells. MCF7 cells were exposed to 1 μM atRA or vehicle only (ETOH), which was added to the cells every other day. CellTiter 96 AQueous assay was used to measure cell proliferation at different days after retinoid treatment. Bars, SE. B, suppression of MCF7 cell growth is dose dependent. MCF7 cells were exposed to different doses of atRA (10^-9 to 10^-3 M and vehicle only), which was added every other day. Cell growth after 7 days of retinoid treatment was determined by CellTiter 96 AQueous assay. Bars, SE. C, activation of RAR-mediated transcription by atRA. MCF7 cells were transfected with a RARE luciferase reporter construct and the pCMV-β-gal plasmid and exposed to 1 μM atRA for 8 h. Cells were lysed, and luciferase and β-gal assays were performed as described in “Materials and Methods.” Bars, SE. D, activation of RAR is dose dependent. MCF7 cells were transfected with a RARE luciferase reporter construct and the pCMV-β-gal plasmid and exposed to different amounts of atRA (10^-9 to 10^-3 M) or vehicle (ETOH). Bars, SE. E, inhibition of AP-1 activity by atRA. MCF7 cells were transfected with the Col-Z-luciferase reporter construct containing an AP-1 binding site and the pCMV-β-gal plasmid and exposed to TPA and 1 μM atRA for 8 h. Cells were lysed, and luciferase and β-gal assays were performed as described in “Materials and Methods.” Bars, SE. F, inhibition of AP-1 activity by atRA is dose dependent. MCF7 cells were transfected with the Col-Z-luciferase reporter construct and exposed to different amounts of atRA (10^-9 to 10^-3 M) or vehicle only. AP-1 activity was measured as in E.
growth-inhibitory effects of atRA and interferes with retinoic acid-induced transactivation of retinoid receptors and transrepression of AP-1.

MATERIALS AND METHODS

Cell Lines and Reagents. The MCF7 human breast carcinoma cell line was obtained from Dr. Ken Cowan (National Cancer Institute, NIH). MCF7 cells were transfected with either pRC/CMV-c-jun or the pRC/CMV vector alone, and the stable clones were isolated after selection in G418 (1 mg/ml). Cells were grown in improved modified Eagle’s medium (Life Technologies, Inc., Gaithersburg, MD) with 10% FCS and 1% Pen/Strep antibiotics. The tumor promoter, TPA, and atRA were obtained from Sigma Chemical Co. All experiments with retinoids were performed in reduced light.

Plasmids. The eukaryotic expression vector pRC/CMV-c-jun was obtained by cloning the human c-jun cDNA into the pRC/CMV vector (Clonetech, Palo Alto, CA). The Col-Z-luciferase reporter construct, which contains a single AP-1 binding site in a portion of the human collagenase promoter (−1200 to +63), was kindly provided by Dr. Ion Kurie (M. D. Anderson Cancer Center, Houston, TX; Ref. 27) and was used to determine AP-1 transactivation activity. The RARE-luciferase reporter construct (28), which contains a portion of the RARβ promoter including a typical DR5 RARE, was used to determine RAR transactivation activity.

Cell Proliferation Assay. Breast cancer cell growth was measured using CellTiter 96 AQueous Non-Radioactive Cell Proliferation assay (Promega Corp., Madison, WI) according to the protocol provided by the manufacturer. Briefly, 1000–2000 cells in 100 μl of medium were seeded in a 96-well plate. One μM atRA was added the next day and replaced every other day. Twenty μl of 20:1 ratio of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate was added to the cells and incubated for 2 h at 37°C, and absorption at 490 nm was determined. Each data point was performed in quadruplicate, and the results were reported as mean absorption ± SE.

RNA Extraction and Northern Blotting. RNA was extracted by cell lysis in buffer containing 4 M guanidine isothiocyanate, 25 mM sodium acetate, and 0.12 M β-mercaptoethanol and pelleted through a CsCl cushion (29). Three to 15 μg of total RNA were separated by gel electrophoresis and blotted onto nitrocellulose membranes by Northern blotting procedures (30). The membranes were then hybridized with 32P-labeled probes prepared by the randompriming method (31). After washing with 2× SSC, 0.5% SDS for 10 min at 42°C; 0.5× SSC, 0.5% SDS for 15 min at 50°C; and 0.2× SSC, 0.5% SDS for 30 min at 50°C, the membranes were exposed to X-ray film. The intensity of hybridized bands was quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Analysis. Whole-cell extracts were prepared by suspending the cells in lysis buffer (250 mM Tris (pH 7.5), 60 mM KCl, 1 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 0.2 unit/ml aprotinin, and 10% SDS), and the protein concentration was determined by BCA assay (Pierce, Rockford, Illinois). Three μg of protein were fractionated on a 10% acrylamide denaturing gel and transferred onto a nitrocellulose membrane (Life Science, Athersham) by electroblotting. The membrane was blocked with 5% nonfat dry milk in TBST [50 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] overnight at 4°C and washed in TBST, and then the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Life Science, Amersham) by electroblotting. The membrane was washed in TBST and then developed using the enhanced chemiluminescence (ECL) procedure (Life Science, Amersham).

RT-PCR. cDNAs were synthesized from 1 μg of total RNA using Molloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) with a random hexamer primer (Life Technologies, Inc.) as described (32). The cDNAs were used as templates for PCR using primers (sense, 5′-AAGCT TOTCC ACAGC ACCAT GTTGG ACTGT ATGGATA TG-3′ and antisense, 5′-AGCCCT TTACA TCCCT CACAG-3′ for RARβ; and sense, 5′-GGCC GAGCTTT GTGAT CAAAG T-3′ and antisense 5′-GGCC GAGCTTT GTGAT CAAAG T-3′ for GAPDH as an internal control). The 5′ primers for PCR were first end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (DuPont NEN, Boston, MA) at 37°C for 30 min. PCR reactions were performed using 25 (GAPDH) or 30 (RARβ) cycles. The PCR products were fractionated on a 4% polyacrylamide gel and detected by autoradiography.

Transient Transfection and Reporter Assays. Cells were transfected with 2–5 μg of plasmid DNA by calcium phosphate precipitation for 16 h and were replaced with medium after washing with PBS twice to remove calcium phosphate precipitation. Ten nM TPA and 10−6 M atRA were added to the cells for 8 h, followed by lysis using a buffer containing 1 mM DTT, 100 mM potassium phosphate (pH 7.8), and 1% Triton X-100. Luciferase assays were performed by adding 10–100 μl of cell lysate to 100 μl of substrate A and 100 μl of substrate B using a standard protocol (Luciferase Assay kit; Tropix, Inc., Bedford, MA). β-gal assays were performed by adding 20 μl of either diluted or undiluted cell lysate to 80 μl of β-gal reagent buffer containing 88 mM phosphate buffer (pH 7.3), 11 mM KCl, 1 mM MgCl2, 54.7 μM β-mercaptoethanol, and 4.4 mM chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, Mannheim, Germany) at 37°C, and the absorption at 600 nm was determined. The luciferase results were then normalized using the β-gal assay results to control for transfection efficiency. All transient trans-
C

co

o

q

o

o

C

CD

CC

o.o

01 2345678

2.5

2.0

1.5-

1.0-

0.5-

0.0

2.5

2.0

1.5-

1.0-

0.5-

0.0

Days after Treatment

2.5

2.0

1.5-

1.0-

0.5-

0.0

2.5

2.0

1.5-

1.0-

0.5-

0.0

Relative Cell Growth (O.D. 490nm)

Fig. 3. Resistance of c-Jun-transfected cells to atRA. Vector-transfected cells (top) and c-Jun-transfected cells (bottom) were exposed to vehicle (ETOH) or 1 

μM atRA, which was added to the cells every other day. CellTiter 96 AQueous assay was used to measure cell proliferation at different days after retinoid treatment. Bars, SE.

fection studies were done in triplicates, and the data were plotted as mean ± SE from at least two independent experiments.

Mobility Shift Assay. Cells were treated with 10^-6 M atRA or vehicle (DMSO) as control for 24 h. Nuclear extracts were prepared as described (30).

Briefly, the cells were lysed in lysis buffer (10 mM HEPES, 1 mM EDTA, 60 mM KCl, 0.5 mM DTT, 0.5% NP40 and protease inhibitors), and the extracts were centrifuged at 5000 rpm to isolate nuclei. The isolated nuclei were resuspended in nuclear suspension buffer [250 mM Tris (pH 7.8), 400 mM KCl, 0.5 mM DTT, 20% glycerol, and protease inhibitors] and lysed by freezing and thawing. The protein concentration was determined by Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA).

The DR5 RARE oligonucleotides (Life Technologies, Inc.) contains RARE DR5 (response elements are underlined). Their sense and antisense sequences and the sequences of mutated DR5 RARE are as follows: DR5 RARE sense, TCGAGGGTAGGGTTCACCGAAAGTTCACTCG; DR5 RARE antisense, AGCTCCCATCCCAAGTGGCTTTCAAGTGAGC; mutated DR5 RARE sense, TCGAGGGTAGGCTTACCCGAAAGTTCACTCG; and mutated DR5 RARE antisense, AGCTCCCATCCGAATGGGCTTTCAAGTGAGC.

The double-stranded oligomer probes were prepared by annealing sense and antisense oligonucleotides at 90°C for 10 min and at room temperature for 1 h, followed by 32P-end labeling with T4 polynucleotide kinase (Life Technologies, Inc.). One hundred pg of probe were incubated with 10 μg of nuclear protein extract and 2 μg of poly(dI-dC) in binding buffer (20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM MgCl2, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM DTT, and 1% glycerol) at 4°C for 30 min. The reaction mixture was subjected to electrophoresis on 5% nondenaturing polyacrylamide gel at 4°C. The gel was exposed to X-ray film for autoradiography, and the retarded bands were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

atRA Activates RAR-dependent Gene Expression and Inhibits AP-1-dependent Gene Expression in atRA-sensitive Breast Cancer Cells. To investigate the mechanism by which retinoids inhibit breast cell growth, we studied the ability of atRA to affect RAR-dependent transactivation and AP-1-dependent transactivation in the MCF7 breast cancer cell line, previously shown to be sensitive to the growth-inhibitory effects of atRA (33, 34). As seen in Fig 1A, 1 μM atRA inhibited the growth of MCF7 by 50% (P < 0.05) at day 7. Fig. 1B demonstrates that this inhibition is dose dependent, with an IC50 of 3 x 10^-8 M. To determine whether the ability of atRA to inhibit growth correlates with activation of the RARs (transactivation) or suppression of AP-1 activity (transrepression), we transfected MCF7 cells with either a retinoid-responsive luciferase reporter construct (RARE-luciferase) containing an RARE linked to the luciferase gene or an AP-1-dependent reporter gene (Col-Z-luciferase) containing a portion of the collagenase promoter (-1200 to +73), which contains an AP-1-dependent TPA-response element. Previous studies have demonstrated that this AP-1 binding site is critical for TPA- and growth factor-induced activation of this portion of the collagenase promoter (27). As shown in Fig. 1, C and D, atRA activates RARE-dependent gene expression in a dose-dependent manner in MCF7 cells. One nm atRA induced a 4-fold increase in luciferase activity (P < 0.05), whereas maximum induction (a 9-fold increase) was seen at 1 μM atRA (P < 0.05; Fig. 1C). The EC50 of atRA-induced transactivation was 1 x 10^-8 M (Fig 1D). Using the AP-1-dependent
Fig. 4. atRA dose response in MCF7 transfectants. Vector-transfected cells and c-Jun-transfected cells were exposed to different doses of atRA (added to the cells every other day). Cell proliferation was measured at day 7 after retinoid treatment. Bars, SE.

reporter construct, we demonstrated that atRA could also inhibit basal and TPA-induced AP-1 activity in MCF7 cells (Fig. 1, E and F). One μM atRA inhibited basal AP-1 activity by 25% (P < 0.05) and TPA-induced AP-1 activity by 50% (P < 0.05), with IC50 of 2 × 10⁻⁸ M (Fig. 1, E and F). atRA also inhibited the growth of T47D cells, activated RAR-dependent transactivation, and inhibited AP-1 activity in T47D cells (data not shown). These results demonstrate that atRA activates RAR-dependent gene transcription and inhibits AP-1-dependent gene transcription in breast cancer cells that are growth inhibited by atRA.

Construction of c-Jun-overexpressing MCF7 Cells. To address whether inhibition of AP-1 activity is necessary for atRA-induced growth suppression, we overexpressed c-Jun in MCF7 cells and measured their proliferation in the presence of atRA. To isolate c-Jun-overexpressing MCF7 cells, we transfected MCF7 cells with the human c-jun gene under the control of a strong constitutive promoter (pRC/CMV-c-jun). We obtained three independent c-Jun-overexpressing cell lines, designated as cJun 1, cJun 2, and cJun 3, and three vector-transfected control cell lines (vector 1, vector 2, and vector 3). cJun Overexpression Blocks atRA-induced Growth Suppression of MCF7 Cells. We next measured the proliferation of these c-Jun-overexpressing MCF7 cells in the presence of atRA. The growth rate of these c-Jun-overexpressing cells in the absence of retinoids is slightly slower than that of vector-transfected cells (Fig. 3). In the presence of 1 μM atRA, the growth of the vector-transfected cells was inhibited (Fig. 3). However, 1 μM atRA failed to inhibit the growth of each of the c-Jun-transfected MCF7 clones (Fig. 3). Vector-transfected clones have an IC50 of approximately 1–2 × 10⁻⁸ M atRA (Fig. 4) and are equally sensitive to atRA as parental MCF7 cells (compare with Fig. 1A). However, the growth of the c-Jun-transfected cells was not inhibited (cJun clone 1) or was minimally inhibited (cJun clones 2 and 3) by atRA, even at the doses up to 10⁻⁵ M (Fig. 4).

to investigate whether expression of the full-length c-Jun gene is required to induce resistance to atRA, we transfected MCF7 cells with a deletion mutant of c-Jun, TAM-67, which lacks most of the NH₂-terminal transactivation domain of c-Jun (amino acids 3–122 have been deleted). This c-Jun mutant has been shown previously to be unable to activate AP-1-dependent gene expression (35) but able to bind DNA and dimerize with cJun and cFos (36, 37). MCF7 cell lines were stably transfected with pCMV-TAM-67, and three independent clones that have high expression of the TAM-67 protein (data not shown) were tested for their sensitivity to atRA. The growth of these cells in the presence of atRA is shown in Fig. 5. Each of the three TAM-67-transfected MCF7 clones is sensitive to the growth-inhibi-
Fig. 5. TAM67-transfected cells are sensitive to atRA. Vector-transfected cells and mutant c-jun (TAM67)-transfected cells were exposed to vehicle (ETOH) or 1 μM atRA (added to the cells every other day). Cell proliferation was measured as described in Fig. 3. Bars, SE.

Fig. 6. atRA does not induce RAR transactivating activity in c-jun-transfected cells. c-jun-transfected cells and vector-transfected cells were transfected with the RARE luciferase reporter construct and the pCMV-β-gal plasmid and exposed to 1 μM atRA for 8 h. Cells were lysed, and luciferase and β-gal assays were performed as described in "Materials and Methods." Bars, SE.

atory effects of atRA. These results suggest that the transactivation domain of cJun is required for induction of retinoid resistance in these breast cancer cells.

atRA-induced RAR-dependent Transactivation and AP-1 Transrepression Are Blocked in cJun-overexpressing Cells. To investigate the mechanism by which cJun overexpression induces resistance to atRA, we examined the ability of atRA to activate RAR-dependent gene expression in the cJun-overexpressing cells. The results shown in Fig. 6 demonstrate that atRA-induced RAR-dependent transactivation is blocked in cJun-overexpressing cells as compared to either parental MCF7 cells (Fig. 1C) or vector-transfected MCF7 cells (Fig. 6). However, the basal level of RAR-dependent transactivation is not down-regulated in cJun-overexpressing cells when compared to the basal level in vector-transfected cells (data not shown).

atRA-induced AP-1 transrepression is also blocked in the c-jun-transfected MCF7 cells, as shown in Fig. 7. In all three c-jun-transfected clones, atRA failed to inhibit TPA-induced AP-1 activity, whereas it did inhibit TPA-induced AP-1 activity in each of the vector-transfected cells by 50–60% (Fig. 7), just as was seen with the parental MCF7 cells (Fig. 1D).

RARα and RARγ Expression Is Down-Regulated, and RARβ Expression Is Up-Regulated in cJun-overexpressing Cells. To further investigate the mechanism by which cJun overexpression induces resistance to atRA, we next measured the expression of RARs in the cJun-overexpressing cells. Fig. 8 shows Northern blot analysis of RNA from vector- and c-jun-transfected cells treated with vehicle (DMSO) or atRA (1 × 10⁻⁶ M) for 24 h. The RNA was then analyzed for expression of the RARs (RARα, RARβ, and RARγ) or RXRs (RXRα, RXRβ, and RXRγ). As shown in Fig. 8, the expression of RARα mRNA is down-regulated in cJun-overexpressing cells (a 3–4-fold decrease as quantitated by Phosphoimager) as compared to vector-transfected cells, either in the absence or presence of atRA. The expression of RARγ and RXRα mRNA is also decreased 2-fold in the c-jun-transfected cells as compared to vector-transfected cells. RXRβ expression is increased in two of three c-jun-transfected clones, and RXRγ expression is increased in one of three c-jun-transfected clones.
cJUN OVEREXPRESSION INDUCES RETINOID RESISTANCE

Fig. 7. atRA failed to inhibit AP-1 transactivating activity in c-jun-transfected cells. cJun-transfected cells and vector-transfected cells were transfected with the Col-Z-luciferase reporter construct containing an AP-1 binding site and the pCMV-β-gal plasmid and exposed to TPA and 1 μM atRA for 8 h. Cells were lysed, and luciferase and β-gal assays were performed as described in “Materials and Methods.”

Therefore, there is no consistent change in the expression of the RXRβ or RXRγ in the c-jun-transfected cells as compared to the vector-transfected cells. In addition, we saw no induction of RARα, RARγ, or RXRα, RXRβ, RXRγ in response to atRA in either the vector-transfected or the c-jun-transfected cells.

Because RARβ RNA was not detected by Northern blot analysis, we used RT-PCR to measure the expression of RARβ in these cells. As shown in Fig. 9, RARβ RNA is undetectable, even using RT-PCR in vector-transfected cells, but is detectable in c-jun-transfected cells (cJun 1, cJun 2, and cJun 3). Thus, RARβ expression is up-regulated in these cJun-overexpressing MCF7 cells.

RARE Binding Activity Is Intact in cJun-overexpressing Cells.

To investigate the mechanism by which cJun overexpression blocks atRA-induced RAR transactivation activity in c-jun-overexpressing cells, we used a mobility shift assay to determine whether the RARs present in the vector- and c-jun-transfected cells can bind DNA containing a RARE DR5 consensus sequence. Fig. 10A shows the RARE DNA binding activity present in one vector-transfected MCF7 clone (vector 2). As shown in this figure, band 1, band 2, and band 3 are RARE-specific binding complexes because the binding of these complexes was competed by a nonradiolabeled wild-type RARE oligonucleotide but was only minimally competed by a nonradiolabeled mutant RARE oligonucleotide. Fig. 10B shows the RARE DNA binding activity of all vector- and c-jun-transfected MCF7 clones in the absence (−) or presence (+) of atRA. RARE-binding complexes are present in all three cJun-overexpressing cells as well as in vector-transfected cells. The vector- and c-jun-transfected cells had similar RARE-DNA binding activity. Specifically, the amount of RARE DNA binding activity in band 1 and band 3 was similar between the vector-transfected and c-jun-transfected cells, whereas there was an approximately 40% decrease in the amount of RARE-DNA binding activity in band 2 in the c-jun-transfected cells (relative amount in the absence of atRA for: vector 1, 1.0; vector 2, 1.1; vector 3, 0.9; cJun1, 0.6; cJun2, 0.6; cJun3, 0.6). atRA treatment did not increase RARE binding activity in either vector- or c-jun-transfected cells. This decrease expression in band 2 may be due to the decreased expression of RARα or RARγ shown above in Fig. 8. However, the total RARE DNA binding activity was only slightly decreased in c-jun-transfected
cJun overexpression induces retinoid resistance

Vector cJun

RARβ

GAPDH

Fig. 9. RARβ RNA expression in cJun-overexpressing cells detected by RT-PCR. cDNAs were synthesized from 1 μg of total RNA by Moloney murine leukemia virus reverse transcriptase with a random hexamer primer and were used as templates for PCR using primers for RARβ and for GAPDH as an internal control. The 5′ primers for PCR were first end-labeled with [γ-32P]ATP by T4 polynucleotide kinase. PCR reactions were performed as described in “Materials and Methods.”

These results demonstrate that the RARE binding activity does not correlate with RAR transactivation activity in these cJun-overexpressing MCF7 cells because despite considerable levels of RARE-DNA binding activity, atRA failed to activate RAR-dependent transcription.

DISCUSSION

The above results show that overexpression of cJun, which increases AP-1 activity, in breast cancer cells induces resistance to the growth-inhibitory effects of atRA and prevents retinoid-induced activation of RAR-dependent transactivation and transrepression of AP-1-dependent transactivation. MCF7 cells transfected with a c-Jun mutant lacking the transactivation domain are sensitive to atRA, demonstrating that the transactivation domain of cJun is required for this resistance. The results also suggest that inhibition of atRA-induced RAR-dependent transactivation in cJun-overexpressing cells is not due to the loss of RARs/RXRα or the lack of RARE DNA binding (although the expression of RARα and RARγ receptors is down-regulated). Therefore, a defect in atRA-induced RAR transactivation or AP-1 transrepression may contribute to retinoid resistance in these cells.

Previous studies have demonstrated that transient overexpression of cJun can affect retinoid signaling in F9 mouse embryonal carcinoma cells (23). Yang-Yen et al. (23) showed that transient transfection of c-jun into F9 cells caused inhibition of retinoid-induced transactivation of a retinoid-dependent reporter gene. Conversely, hormones such as glucocorticoids and retinoids have been shown to inhibit the activity of the AP-1 transcription factor through transcription factor “cross-talk” (21–24). This transcription factor cross-talk is reciprocal because AP-1 transcription factors have also been shown to inhibit the activity of glucocorticoid receptor and retinoid receptors (22). Recent studies suggest that the antagonism between AP-1 and RAR may be mediated through competitive binding or “squelching” of essential coactivators such as CBP, which is required for both AP-1-dependent and RAR-dependent transcription (25, 26).

Other investigations using anti-AP-1 retinoid analogues have also shown that retinoid-induced inhibition of AP-1 can suppress the growth of breast cancer cells. Fanjul et al. (7) showed that such anti-AP-1 retinoid analogues, which inhibited AP-1 without activating retinoid-dependent gene transcription, could inhibit the growth of several different cancer cell lines, including the breast cancer cell line, T47D.

Fig. 10. RARE binding activity in cJun-overexpressing cells. Vector- and c-Jun-transfected cells were treated with 1 μM atRA or vehicle (DMSO) for 24 h. Nuclear extracts were prepared, and gel shift assays using the oligonucleotides containing a DR5 RARE were performed as described in “Materials and Methods.” A, a gel shift assay was performed using nuclear extracts from the vector-transfected cell line (vector 2) incubated with a radiolabeled RARE oligonucleotide probe and different amounts of nonradiolabeled RARE and mutant RARE oligonucleotides. The three thin arrows show specific RAR/RXR-DNA binding complexes, because they are competed preferentially by the wild-type DR5 RARE. The unbound probe is indicated by the broad arrow. B, a gel shift assay was performed using nuclear extracts from vector- and c-Jun-transfected cells treated with vehicle (DMSO; marked “−”) or 1 μM atRA (marked “+”) for 24 h. The thin arrows show specific RAR/RXR-DNA binding complexes.
Many of the previous studies investigating the mechanism of AP-1/RAR interaction have shown AP-1/RAR interaction at the level of DNA binding using in vitro gel shift experiments or in cells transfected with reporter genes. Such studies do not address the physiological importance of interaction between AP-1- and RAR-dependent signal transduction pathways in controlling cellular processes such as proliferation. The results presented here demonstrate that c-Jun overexpression in breast cancer cells may allow the development of specific antiproliferative retinoids that may be effective chemotherapeutic or chemopreventive agents. The observation that increased c-Jun expression induces retinoid resistance may also allow prospective identification of tumors that will or will not respond to retinoid therapy based on their expression of AP-1 family members. Just as expression of the estrogen receptor in breast tumors guides present therapy, expression of retinoid receptors and/or AP-1 family members may guide future therapy with retinoids.

ACKNOWLEDGMENTS

We thank Dr. Jon Kurie (M.D. Anderson, University of Texas Health Science Center) for providing us with the Col-Z-luciferase and RARE-luc plasmids, GAPDH, and RARβ PCR primers. We also especially thank Drs. Jon Kurie, Douglas Yee, and John Ludes-Meyers for helpful critiques of the manuscript.

REFERENCES


Induction of Retinoid Resistance in Breast Cancer Cells by Overexpression of cJun

LiMin Yang, HeeTae Kim, Deborah Munoz-Medellin, et al.