Effect of All-trans-Retinoic Acid on c-fms Proto-oncogene [Colony-stimulating Factor 1 (CSF-1) Receptor] Expression and CSF-1-induced Invasion and Anchorage-independent Growth of Human Breast Carcinoma Cells

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ABSTRACT

Abnormal expression of c-fms proto-oncogene, which encodes for the macrophage colony-stimulating factor-1 (CSF-1) receptor, has been observed in a variety of carcinomas of epithelial origin, including those of the breast. Here, we have investigated the effect of retinoic acid (RA), an important regulator of normal differentiation of mammary epithelial tissues, on the expression of the c-fms gene and CSF-1/CSF-1 receptor-induced invasion and anchorage-independent growth in breast carcinoma cells. We have demonstrated that all-trans-RA (atRA) significantly increases levels of c-fms transcripts in the estrogen receptor-negative but RA receptor α-positive breast carcinoma cell lines BT20 and SKBR3. The atRA-induced increase in fms transcript levels was completely abolished by RO41-5253, a synthetic RA receptor antagonist. Our results indicate that atRA could enhance fms expression by up-regulating the activity of the first promoter of the fms gene. DNase I protection, mobility shift, and mutational analysis revealed that a potential activator protein 1 (AP-1) site in the first fms promoter sequence could mediate the observed atRA effect on fms transcription. Our results also showed that atRA, by itself and in the presence of CSF-1, can increase the ability of breast carcinoma cells to invade in vitro. Furthermore, we demonstrated that atRA is able to abolish the CSF-1-induced increase in anchorage-independent growth of breast carcinoma cells without affecting the anchorage-dependent growth. In summary, our findings suggest that retinoids may play conflicting roles throughout breast cancer progression, depending on the stage of cancer development. Although retinoids might suppress growth at the early stages of tumor formation, they might promote malignant transformation at later stages by stimulating the invasive capacity of certain cell variants in the breast tumor population.

INTRODUCTION

Retinoids, the natural and synthetic analogues of vitamin A, are essential for regulating a broad range of normal cellular phenotypes, including cell growth, differentiation, and development in a variety of cell types and tissues (1). Retinoids also inhibit the growth and invasion of cancer cells and are clinically useful for treating and preventing cancer (2). In breast cancer, preclinical studies have shown that retinoids inhibit chemically induced mammary carcinogenesis in rat models and suppress the in vitro growth of certain human breast carcinoma cell lines (3–5). In particular, most estrogen-dependent, ER1-positive breast cancer cells, which express significant levels of the functional RAR, are sensitive to the growth-inhibitory effects of retinoids, whereas most ER-negative cells, which usually express low levels of RAR, tend to be retinoid resistant (6–9).

atRA and 9-cis-RA, the active derivatives of vitamin A, interact with two classes of nuclear receptors belonging to the steroid/thyroid/retinoid hormone receptor superfamily, RARs (RARα, β, and γ), and RXRs (RXRα, β, and γ; Ref. 1). The activation of RAR by its ligand atRA results in the formation of heterodimers with RXRs that bind to RARE (10). Whereas RAR transactivation requires DNA binding to a RARE, a distinct regulatory mechanism that involves protein-protein interaction between RAR and a family of AP-1 transcription factors allows retinoids to modulate AP-1-responsive genes in various biological processes (11). Both regulatory mechanisms may be involved in tumorigenesis, but experiments suggest that the antitumor effects of retinoids may be mediated by interaction with AP-1 (12).

Retinoids play important roles in regulating the expression of genes involved in the progression of breast cancer. These target genes include tumor suppressor genes, such as p53 and RB, as well as proto-oncogenes, such as c-myc and c-erbB-2 (13–16). In the present study, we investigated the possibility of regulation of c-fms proto-oncogene expression by RA in hormone-independent breast carcinoma cell lines, which express functional RARs (17, 18).

The proto-oncogene c-fms encodes a growth factor receptor with an intracellular tyrosine kinase domain whose activating ligand is the macrophage CSF-1 (19–21). CSF-1 and its receptor are required for macrophage proliferation and differentiation and for the regulation of normal uterine implantation and placental development (22–24). They also play important roles in the normal invasive processes of both macrophages and trophoblasts (22–24). Furthermore, several recent findings have implicated a role for CSF-1 and its receptor in the mammary gland during pregnancy (25–27). In lactating breast epithelium, very high levels of CSF-1 and CSF-1R antigens are expressed (26), as opposed to the undetectable levels of CSF-1R and low levels of CSF-1 found in resting ductal epithelium (26). These results are very intriguing given the recently demonstrated clinical significance of CSF-1/CSF-1R in human breast carcinoma, i.e., the abnormal expression of CSF-1R by malignant epithelial cells of breast and other carcinomas has correlated with high histological grade, advanced stage, and poor prognosis (28–32). Also, previous studies have investigated a potential autocrine mechanism of transformation caused by the overexpression of the normal c-fms gene in nonmalignant cells synthesizing endogenous CSF-1. These studies revealed that the overexpression of the normal c-fms gene (cloned into a retroviral vector and transfected into CSF-1-expressing normal cells) can be sufficient to induce a fully transformed phenotype in epithelial cells (33). Given the known importance of the effect of steroids and other hormones in the biology of normal and neoplastic breast development, we and others have extensively studied the hormonal regulation of the c-fms gene (34, 35). The first evidence for such regulation derives from our own studies, where we have previously demonstrated that glucocorticoids, which are potent physiological modulators of normal mammary epithelial cell differentiation (28, 35), increase levels of c-fms transcripts and protein in two different breast carcinoma cell lines.
lapping cosmid clones (cos2-e and cos1-22) containing the human c-fms gene expression in breast carcinoma cells (28). In contrast, however, estrogen, prolactin, and insulin are capable of up-regulating CSF-1 gene expression in breast carcinoma cells, a result that further suggests the important role of CSF-1/Csf1r in the regulation of normal lactogenic differentiation.

In this communication, our goal is to study the potential effect of retinoids, which are another class of potent physiological modulators of mammary epithelial cells, on the expression of CSF-1R and on the CSF-1/Csf1r-induced physiological end points in breast carcinoma cell lines.

MATERIALS AND METHODS

Cell Lines and Cell Culture Methods. The human breast carcinoma-derived cell lines BT20 and SKBR3 were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were propagated at 37°C and 5% CO2 to RPMI 1640 supplemented with 10% charcoal-treated fetal bovine serum (BT20) or McCoy’s 5A medium with 15% charcoal-treated fetal bovine serum (SKBR3) and grown to 90% confluence. Before harvesting the cells for RNA extraction, the culture medium was decanted and replaced with serum-free DMEM/Ham’s F-12 medium for 24 h. Proliferation assay was performed as described previously (33). Soft agar colony assays were carried out in 12-well plates. Cells (104) were plated in 0.3 ml of 0.3% agarose in DMEM/Ham’s F-12 medium supplemented with 10% charcoal-treated fetal bovine serum. An underlayer of 1% agarose was used to prevent the attachment and spread of cells. The number of colonies was scored after 21 days.

For hormone induction experiments, cells were incubated in the serum-free DMEM/Ham’s F-12 medium supplemented with either 10-7 m atRA (Sigma) or 10-6 M dexamethasone (synthetic glucocorticoid; Sigma) for the times indicated in the figure legends.

RNA Extraction and Northern Blot Analysis. Total cellular RNA was isolated by the guanidinium/cesium chloride method as described previously (33). For Northern blots, RNA samples were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Genescreen; DuPont). Filters were hybridized overnight at 42°C in the presence of 10% dextran sulfate, 50% formamide, 1.5 M NaCl, 1% SDS, 100 µg/ml salmon sperm DNA, and 32P-labeled c-fms probe (a 1.2-kb EcoRI fragment purified from the pA3CSF1R1 plasmid; Ref. 37). After autoradiography, filters were stripped and rehybridized with the GAPDH probe (a 0.8-kb PstI/XhoI fragment of HHCH32 obtained from the American Type Culture Collection; catalogue number 78105). Specific hybridization was visualized by Kodak XAR-5 film autoradiography, whereas quantitation of the relative intensity of hybridized bands was carried out with the Phosphorimage detection and quantitation system (Molecular Dynamics) according to the protocol provided by the manufacturer.

Construction of Plasmids Containing Genomic Sequences Linked to Reporter Genes. All constructs were subcloned previously (35) from overlapping cosmid clones (cos-e and cos-1-22) containing the human c-fms locus originally prepared by Visvader and Verma (38). A 1.4-kb genomic BamHI fragment containing the first exon of the c-fms gene was subcloned into pBlueScript SK+ (Stratagene), and the resulting clone was used for the amplification of the first promoter fragments. To prepare the E1-P construct, a 380-bp fragment was amplified using the vector-specific antisense primer and a gene-specific sense primer (5’-CCCCAGTGCTGAGAAGCAGAG-3’). The resulting PCR products were blunt-end ligated into pGL2 basic luciferase vector (Promega). To generate the mutated consensus AP-1 site in the E1-P site in the E1-P construct, the QuikChange Site-directed Mutagenesis Kit (Stratagene) was used. Complementary primers (ccaggtccaggTGACGCGatagcgcceccaggg), which incorporated the three nucleotide alterations (italics) in the AP-1 site (uppercase letters), were designed according to the criteria in the QuikChange protocol.

To prepare the second promoter construct (E2-P), a 1.4-kb genomic fragment of the most distal portion of the first intron and the second exon of the c-fms gene was amplified from the cos-1-22 cosmid clone with two c-fms gene-specific primers (5’-GAATGGACCGGAGCGTGGACG-3’ as the anti-sense primer and 5’-CCTACTGCACCTCCAGGCTGG-3’ as the sense primer) and blunt-end ligated into the pGL2 basic luciferase vectors. The correct sequences and orientations of all the above constructs were verified by restriction mapping and direct sequence analysis.

Preparation of Nuclear Extracts and DNase I Footprinting. SKBR3 cells were grown to 90% confluence in growth media. Before harvesting the cells, the culture medium was removed and replaced with serum-free media for 24 h, and then 10-7 M atRA was added to the media at various time intervals. A modification of the method described by Schreiber et al. (39) was used to prepare CNEs from small numbers of cells. Briefly, cells washed with cold PBS were resuspended in 4 volumes of cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT with freshly added 0.4 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin, 1 mM sodium orthovanadate, and 4 µg/ml pepstatin]. After incubation on ice for 15 min, 6.25 µl of 10% NP40 were added per 100 µl of buffer A, and the samples were vortexed for 10 s. Nuclei were pelleted by a 30-s centrifugation and resuspended in a volume of cold buffer C [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and freshly added protease inhibitors in concentrations identical to those in buffer A] approximately equal to the original packed cell volume. The suspension was incubated at 4°C on a rocking platform for 20–30 min. Debris were pelleted by centrifugation for 5 min at 4°C in a microcentrifuge. Supernatants were taken as the CNE. Protein concentrations were measured by the Bio-Rad Protein Assay and typically ranged from 5–10 mg/ml. Aliquots of the CNE were frozen at -70°C.

DNase I footprinting assays were adapted from the method described by Tabor et al. (40). Briefly, a 380-bp cloned portion of the first c-fms promoter (E1-P fragment), including the 179-bp exon 1 and the 201-bp of pre-exon 1, was used to prepare a double-stranded DNA probe. The plasmid was linearized at the 5’ end of the insert with HindIII, end-labeled with the Klenow enzyme in the presence of [32P]dATP, and then digested with the BamHI restriction enzyme at the 3’ end of the insert, resulting in a double-stranded fragment with only the 3’ end of the antisense strand labeled. The labeled insert was separated from the plasmid vector by agarose gel electrophoresis and gel purified. The 32P-labeled DNA probe and 15 µg of CNE were incubated on ice for 45 min in the presence of 30 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 2% polyvinyl alcohol, 0.1 mM EDTA, 5 mM MgCl2, 0.25 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride plus 0.5 µg of poly(dI-dC) in the final volume of 50 µl. DNA-protein mixtures were equilibrated to 10°C, and an equal volume of 10 mM MgCl2 and 5 mM CaCl2, plus 20 µg of DNase I (bovine pancreas DNase I; ECN) were added and incubated at 10°C for 1 min. Reactions were stopped with 100 µl of 0.2 M NaCl, 20 mM EDTA, 1% SDS, and 0.25 mg/ml yeast tRNA, and extracted with an equal volume of phenol:chloroform (1:1 mixture). DNA was alcohol-precipitated, resuspended in 60% formamide buffer, and electrophoresed in an 8% denaturing acrylamide gel. DNase I-protected areas were visualized by Kodak XAR-5 film autoradiography, Maxam-Gilbert G and C+G DNA sequencing reactions were performed according to Pichiroy (41) on the same labeled DNA and run in adjacent lanes on the same gels to determine the sequence of DNase I-protected areas.

EMSA and Preparation of 32P-labeled DNA Probes. A shorter c-fms fragment (gcagttagctgagctcgctgatgctgacgcceccaggg; 48 bp) was prepared by annealing two 45-mer complementary oligonucleotides (overlap of 42 nucleotides with 3 nucleotide 5’ overhangs at each end) and labeling both ends with [32P]dATP using the Klenow DNA polymerase to fill in with a complementary sequence. The labeled probe was purified by using the Sephadex G25 spin column (Boehringer Mannheim).

In a 10-µl volume, CNE (2–10 µg) was mixed with 0.1–0.3 pmol of the 48-bp 32P-labeled DNA probe in the presence of 10 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 3 µg of BSA, 6.25 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 mM EGTA, and 1 µg of poly(dI-dC). After a 15-min incubation at 30°C, DNA-protein complexes were separated from unbound DNA in nondenaturing 4% acrylamide (80:1 acrylamide:bis-acrylamide; National Diagnostics), 2.5% glycerol
REGULATION OF *fms* EXPRESSION BY RETINOIDS

atRA Increases *c-fms* Transcript Levels in Two Breast Carcinoma Cell Lines. The effect of atRA on the expression of the *fms* gene was analyzed in two *fms* transcript-positive human breast carcinoma cell lines (SKBR3 and BT20; Refs. 17 and 18) by the Northern blot technique. In agreement with our previous data (35), both BT20 and SKBR3 expressed readily detectable levels of a 4-kb *fms* transcript (Fig. 1). After \(10^{-7}\) M atRA treatment (a dose within physiological range), the level of this transcript was increased as early as 2 h and further increased in a time-dependent manner for up to 6 h in both breast cell lines (Fig. 1, A and B). In both cell lines, steady-state *c-fms* transcript levels reached a maximum by 6 h, at which time they were 25-fold higher than the levels in untreated cells. After 6 h, *fms* transcript levels in SKBR3 cells decreased gradually, and at 24 h of atRA treatment, they were only 5-fold higher than they were in untreated cells. In contrast, levels of *fms* transcripts in BT20 cells declined more rapidly, and at 24 h after the addition of atRA, they were similar to the levels we had observed in untreated cells. Furthermore, these RA-induced increases of *c-fms* transcript levels in both cell lines were completely abolished by prior treatment of the cells with RO41-5253 (Fig. 2, A and B), a synthetic retinoid that acts as a selective RAR\(\alpha\) antagonist (43, 44).

We have also studied the atRA effect on *c-fms* gene expression in two ER-positive cell lines (MCF7 and T-D47) and found no evidence that atRA can stimulate *c-fms* gene expression in these cell lines (data not shown).

**RESULTS**

**A.** SKBR3+atRA

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**B.** BT20+atRA

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Fig. 1. Northern blot analysis of *c-fms* mRNA induction by RA in (A) SKBR3 and (B) BT20 cells. SKBR3 and BT20 breast carcinoma cells were exposed to \(10^{-7}\) M atRA for 0, 2, 4, 6, 24 (A and B, Lanes 1–5), and 48 h (A, Lane 6). Total cellular RNA was isolated, and 10 \(\mu\)g of RNA were electrophoresed and thereafter probed with \(^{32}\)P-labeled *fms* and GAPDH cDNA. Total RNA (10 \(\mu\)g) from BT20 cells treated with \(10^{-8}\) M dexamethasone for 24 h (A and B, Lanes C), was included as a positive control for the expression of the *fms* transcript (35).

**RA Stimulates the Transcription Rate of *fms* Transcript.** Our next question was whether the increased level of *fms* transcript in atRA-treated cells was due to the increased activity of the *fms* promoters (transcriptional control) or to prolongation of the *fms* transcript half-life (posttranscriptional control). We have previously demonstrated that the *fms* transcript basal half-life is \(>8\) h in BT20 and SKBR3 cells (35), a result which suggests that prolongation of transcript half-life could not possibly account for the changes in *c-fms* transcript levels we have observed in the first 4 h of atRA treatment. For this reason, we have focused primarily on the possibility that atRA can induce the transcriptional activity of the *fms* gene.

To better characterize possible effects of atRA on the transcription rate of the *c-fms* gene, we have used previously created reporter constructs (35) in which the two known *c-fms* promoters (36) were cloned upstream of the firefly luciferase reporter gene. The promoter/reporter gene constructs were transiently transfected into SKBR3 cells, and the luciferase activity was measured before and after atRA treatment. The activity of the first promoter of the *c-fms* gene was assayed with a reporter gene construct containing the pre-exon 1 and exon 1 sequences linked upstream from the luciferase gene (E1-P). The activity of the second promoter was studied with a construct containing pre-exon 2 and exon 2 sequences (E2-P). The activities of the various *c-fms* promoters were determined from measurements of luciferase activity normalized to the level of \(\beta\)-galactosidase expression from a cotransfected \(\beta\)-galactosidase expression vector (pSV\(\beta\)-galactosidase; Promega).

As a negative control, the luciferase activity of a promoterless luciferase plasmid (pGL2 basic) was measured. For a positive control, the pGL2 promoter control vector (with the SV40 promoter) was included in each transient transfection experiment (pGL2 promoter). No effects of atRA treatment were observed on the level of expression of the luciferase gene for any of the positive (Fig. 3) or negative control plasmids (data not shown).

In the absence of atRA, a relatively high level of luciferase gene expression was observed with the first promoter construct (E1-P) in SKBR3 cells (Fig. 3). However, luciferase experiments with the second promoter construct revealed a significantly lower promoter activity. These results are in good agreement with our previous observations, which showed that the proximal promoter adjacent to exon 1 is responsible for most of the transcriptional activity of the *fms* gene in mammary epithelial cells (35).

The *fms* first promoter activity increased 2–3-fold within 2 h of the addition of atRA and remained elevated 4 h after atRA treatment in SKBR3 cells, whereas the second promoter activity did not change significantly after atRA treatment (Fig. 3). We have also repeated the above experiments in BT20 cells and have observed a very similar
effect of atRA on the activity of the first and second fms promoters (data not shown).

**atRA May Modulate AP-1 Activity in the First fms Promoter.**

To investigate the molecular basis for the atRA-induced increase in the activity of the fms first promoter, we analyzed the 5’ flanking sequence of the c-fms gene for potential RARE sequences. A computer search did not reveal any likely RARE sequences, but we did find one consensus AP-1 sequence (from bp 41–48). As mentioned earlier, the RA-induced effect could be mediated through an AP-1 sequence (11). To prove this hypothesis, DNase I footprinting was performed. For this experiment, we used a DNA fragment consisting of a cloned portion of the fms first promoter plus exon 1. Equal amounts of CNEs prepared from SKBR3 cells treated with atRA for various times were incubated with labeled DNA probe followed by limited digestion with DNase I enzyme. Digestion products were separated on denaturing acrylamide gels and compared with digests of DNA that had not been mixed with nuclear extracts. As shown in Fig. 4, only one region of the c-fms DNA fragment interacted with nuclear proteins from untreated and atRA-treated SKBR3 cells protecting a single site (bp 35–52) in the first fms promoter. The protected site contains the AP-1 consensus sequence. Analyses of the protected pattern in the untreated and atRA-treated samples showed very minor differences (if any) for the indicated time periods. Also, further analyses of the nuclear proteins from untreated and atRA-treated SKBR3 cells showed no protection in any other regions within the 380-bp DNA fragment.

Because >50% of the target site-bearing labeled DNA molecules need to be occupied by the specific DNA-binding protein to yield a protected site in DNase I footprinting, this assay is not considered to be sensitive enough to detect small changes in the DNA binding pattern (45). For that reason, we have also performed EMSAs concentrating on the AP-1 region of the fms first promoter. A 48-bp 32P-labeled DNA probe based on the sequence identified by the DNase I footprinting experiment was generated, and CNEs from SKBR3 cells treated with atRA for various times (0, 10, 30, 60, and 90 min) were incubated with labeled DNA. Fig. 5 (Lanes 1–5) shows that two DNA-protein complexes are formed. The intensity of both bands is clearly dependent on the time of treatment with atRA, showing a dramatic increase in binding at 60 min followed by a decrease at 90 min. The addition of an unlabeled consensus AP-1 double-stranded DNA (Fig. 5, Lane 6) cgctgtaTGAGTCAgccggaa (uppercase letters indicate the consensus binding site) resulted in a significant decrease in both complexes, suggesting that the AP-1 site is the target for protein binding and therefore implicating the involvement of Jun family proteins in transcriptional regulation. The AP-1 site in the fms first promoter is studied in more detail elsewhere.4

**Mutation at the AP-1 Site Abolished the atRA-induced Increase in the First fms Promoter Activity.** To further prove that the AP-1 site can mediate the observed atRA effect on the activity of the fms promoter, we mutated this AP-1 site by changing 3 bp of the core promoter, manuscript in preparation.

Fig. 2. Northern blot analysis of c-fms mRNA in (A) SKBR3 and (B) BT20 cells. The cells were treated with 10−7 M atRA for 0 and 6 h (Lanes 1 and 2) or pretreated with RO 41-5253 (5 × 10−5 M) for 30 min before the addition of atRA (Lane 3). Total cellular RNA was isolated, and 10 μg of total RNA were electrophoresed and sequentially probed with 32P-labeled fms and GAPDH cDNA. Total RNA (10 μg) from BT20 cells treated with 10−6 M dexamethasone for 24 h (A, Lane DEX) was included as a positive control for the expression of the fms transcript (35).
sequence (see “Materials and Methods”). We then used this mutated construct (E1-MAP) in our luciferase reporter assay. Transient transfection of this altered construct into SKBR3 cells revealed a basal promoter activity that was significantly lower than that of the original, undeleted construct (E1-P; Fig. 6). Furthermore, atRA treatment had no effect on the activity of the E1-MAP construct (Fig. 6), a result that suggests that the AP-1 sequence indeed plays a crucial role in mediating the effects of atRA on the activity of the first fms promoter.

RA Enhances CSF-1-induced Invasion but Inhibits CSF-1-induced Stimulation of Growth in Soft Agar. We have previously shown that CSF-1 can affect the invasive capacity and anchorage-independent growth of BT20 breast carcinoma cells through its receptor (33). These CSF-1-induced effects can be further enhanced by treatment with dexamethasone, an agent that can up-regulate the expression of the CSF-1R in this cell line (46). In our following studies, we have asked whether the atRA-induced increase in the CSF-1R level could affect the CSF-1-induced anchorage-independent growth and invasive capacity of breast carcinoma cells. As a model for these experiments, we used BT20 cells, the only known cell line that expresses measurable amounts of the CSF-1R without expressing its ligand, CSF-1 (28). Our other model cell line, SKBR3, expresses significant amounts of endogenous CSF-1 (28).

The invasive potential of BT20 cells in the presence of CSF-1, dexamethasone, or atRA was determined by measuring invasion through a barrier of the reconstituted basement membrane, Matrigel, over a 72-h period (Fig. 7). BT20 cells were able to invade through Matrigel without the addition of CSF-1 to the culture medium. However, in the presence of CSF-1, the invasive activity of BT20 cells was increased ~6-fold ($P < 0.001$), whereas in the presence of dexamethasone, invasion increased by ~2-fold ($P = 0.01$). CSF-1 plus dexamethasone increased the invasive capacity of BT20 cells by ~10-fold ($P = 0.001$). These data are in good agreement with the
previously reported stimulatory effect of CSF-1 and dexamethasone on the invasion of this cell line (46).

In our next experiments, we studied the effect of atRA on the invasive capacity of the CSF-1-treated and untreated BT20 cells. Invasion by BT20 cells was increased <2-fold by atRA alone (P = 0.1). However, when we added atRA and CSF-1 together, the invasion was enhanced by ~8-fold (P = 0.0002).

Anchorage-independent growth in vitro is a cellular phenotype closely linked to in vivo tumorigenicity. BT20 cells were assayed for anchorage-independent growth in soft agar in the presence and absence of exogenous CSF-1 and atRA. Table 1 presents the quantitative data on the colony numbers. BT20 cells grew readily in soft agar and formed discrete colonies (Table 1; Fig. 8A). The addition of exogenous CSF-1 caused a <2-fold increase in the number of the colonies (P = 0.09); however, the size of the colonies was four times larger than the size of untreated colonies (P = 0.009; Table 1; Fig. 8B). The treatment of BT20 cells with 10^{-7} M atRA did not produce any significant effect on the colony formation (P = 0.5), but interestingly, it inhibited the CSF-1-induced increase in the size but not the number of the soft agar colonies (Table 1; Fig. 8, C and D).

RA Has No Effect on the Proliferation Rate of BT20 Cells. To interpret data from invasion and soft agar experiments, it is important to know whether CSF-1 and atRA can affect the proliferation rate of this cell line. The effect of atRA on proliferation rates of BT20 cells was determined by treating the cells for 5 days with 10^{-7} M atRA alone or in combination for 72 h. Invasion studies were performed by adding 1 × 10^4 cells to the upper part of invasion chambers coated with 1 μg/mm² Matrigel. Cells that invaded through the Matrigel were stained and counted after 72 h. Bars, mean ± SE from at least six independent experiments; in each experiment, six filters were used for each condition.

DISCUSSION

The results described in this communication demonstrate that atRA, an inhibitor of proliferation and an inducer of differentiation in a wide variety of normal and neoplastic cell types (1), increases levels of c-fms transcripts in the two ER-negative, RARα-positive breast carcinoma cell lines, BT20 and SKBR3. The atRA-induced increase in fms transcript levels was completely abolished by RO41-5253, which is a synthetic retinoid that acts as a selective RARα receptor antag-
onist (43–44), suggesting that atRA induction of fms transcription can be mediated by RARα. Our results also indicate that RA could enhance fms expression by up-regulating the first promoter transcriptional activity in these breast carcinoma cells, which is consistent with recent observations that fms gene transcription is primarily controlled from the first promoter in these cells (35). Although our computer analysis was not able to identify any RARE in the fms promoter, our gel shift assay revealed that a potential AP-1 site might mediate the observed atRA effect on fms transcription. This hypothesis was further analyzed by mutating this AP-1 element in the fms promoter, which completely eliminated atRA stimulation of the promoter activity, suggesting that the effect of atRA on fms expression can be mediated through an AP-1 consensus sequence.

Furthermore, our results also showed that atRA by itself can increase the ability of BT20 cells to invade through basement membrane matrices, but it does not have a significant effect on the proliferation rate or the anchorage-independent growth of breast carcinoma cells. In the presence of CSF-1, however, atRA treatment further increased the invasiveness of BT20 cells, but interestingly, the CSF-1-induced increase in the size of BT20 soft agar colonies was abolished by atRA.

The increase in invasive capacity of BT20 cells by atRA treatment was surprising at first because retinoids have been shown to inhibit invasion and metastasis in various tumors, including mammary epithelial tumors, both in vivo and in vitro (47–49). In contrast, however, in certain neuroblastoma cells, which are resistant to the terminal differentiation effect of retinoids, atRA was shown to be able to increase invasion significantly through the tissue-type plasminogen activator pathway (50). Furthermore, it was also demonstrated that in mammary epithelial cells, retinoids are capable of both stimulating and inhibiting invasion capacity. Bracke et al. (51) reported that the opposing retinoid effects on the invasive phenotype of MCF-7 sublines (derived from the same tumor) could depend on which subtype was being used in the experiments. Our result of atRA-induced invasive capacity in BT20 cells further suggests that at least in certain breast carcinoma cells, retinoids could indeed promote rather than inhibit malignant behavior.

RARs can interact with a family of AP-1 transcription factors formed by dimers of Jun proteins and heterodimers of Jun and Fos proteins, thereby allowing two distinct regulatory systems involved in programs of cell differentiation and proliferation to mediate fine control of gene expression (52). AP-1-responsive genes include the proto-oncogenes c-erbB-2 (15) and c-myc (16) and various matrix-degrading proteinases (such as vimentin, tissue plasminogen activator, and urokinase plasminogen activator; Refs. 53–55); the interaction between RARs and AP-1 may play an important role in regulating certain protease cascades and tumorigenesis. The AP-1 site found in the fms promoter could be a potential target site for RARs for that purpose because previous data demonstrated that the overexpression of the fms proto-oncogene can dramatically increase the invasive capacity of mammary epithelial cells in vivo and in vitro through a urokinase plasminogen activator-dependent pathway (33).

ER-positive human breast cancer cells are hormonally regulated, and their growth is inhibited by retinoids, whereas most ER-negative breast cancer cells are not (6). For example, the growth of our model cell line BT20, which is ER negative, is resistant to growth inhibition by retinoids in our experiment, a finding that agrees with an observation previously reported for this cell line (16). Furthermore, our results suggest that the role of atRA in anchorage independence in BT20 breast carcinoma cells is complex and dependent on the presence of CSF-1 and probably other growth factor(s). Anchorage-independent growth in soft agar has been used extensively in clinical and experimental oncology as an in vitro indicator of malignancy (56). Darro et al. (57) studied the effect of different retinoid derivatives on the anchorage-independent and -dependent growth of an ER-positive breast carcinoma cell line (T-47D) and found that both cellular processes can be inhibited by these retinoids. Our data showing that atRA inhibited only the CSF-1-induced anchorage-independent growth provide strong evidence for the importance of the CSF-1/CSF-1R pathway in mediating atRA inhibition of anchorage-independent growth.

Retinoids, which have shown significant antitumor activity in acute promyelocytic leukemia (58) and squamous cell carcinoma of the head and neck (59), are considered promising chemopreventive and chemotherapeutic agents for the treatment of solid tumors, including lung and breast cancer. In an ongoing trial, a synthetic retinoid (4-hydroxyphenyl retinamide) is being evaluated for its ability to prevent contralateral breast cancer in patients with early-stage disease (60). The role of retinoids as a chemotherapeutic agent in advanced breast cancer has yet to be established. Phase II trials of retinoids in patients with metastatic disease have been disappointing (61). Our results provide an interesting addition to these clinical observations because they indicate that RA can induce up-regulation of fms proto-oncogene expression along with in vitro invasiveness in a subset of breast carcinoma cells. The results suggest that retinoids may play a progressive role in advanced-stage carcinoma. Similar to retinoids, glucocorticoids have been shown to induce the invasive characteristic of breast carcinoma cells in vitro (46). Pharmacological doses of glucocorticoids are often being used to palliate the symptoms of breast cancer patients with very advanced or near-terminal disease. Although we have no data as to whether glucocorticoids have a stimulatory effect on the invasiveness of breast cancer cells in these patients, it was reported that certain potent antagonists of glucocorticoid and progestin action (such as mifepristone, RU486) can exert significant antineoplastic activity for breast carcinomas (62, 63).

Although there is convincing evidence that RA can inhibit the growth of some but not all RAR-positive breast carcinoma cells, the question remains as to whether RA plays different roles depending on the stage of cancer development. RA might suppress growth at early stages of tumor formation but might be promoting malignant transformation at later stages by stimulating the invasiveness of certain cell variants in the breast tumor population. Our results may provide practical information that would prove useful in the design and interpretation of clinical trials with RA as a chemotherapeutic drug in the future and may begin to provide an explanation for the lack of success of retinoid treatment thus far.

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REFERENCES

REGULATION OF fms EXPRESSION BY RETINOIDS


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