The Anti-estrogenic Effect of All-trans-retinoic Acid on the Breast Cancer Cell Line MCF-7 Is Dependent on HES-1 Expression*

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All-trans-retinoic acid has been shown to have an antiproliferative effect in the estrogen receptor α-positive breast cancer cell line MCF-7. The mechanism of this effect is not well understood. We have previously shown that 17β-estradiol down-regulates the basic helix-loop-helix factor Hairy and Enhancer of Split homologue-1 in MCF-7 and T47D cells (Ström, A., Arai, N., Leers, J., and Gustafsson, J. Å. (2000) Oncogene 19, 5851–5853) and that this down-regulation is essential for proliferation in response to 17β-estradiol. Treatment of the same cells with all-trans-retinoic acid prevented 17β-estradiol-mediated down-regulation of the factor. The antiproliferative effect of all-trans-retinoic acid correlated well with the prevention of Hairy and Enhancer of Split homologue-1 down-regulation. Increasing concentrations of all-trans-retinoic acid, in the range of 1–1000 nM, produced a dose-dependent inhibition of proliferation and prevented 17β-estradiol-mediated down-regulation of Hairy and Enhancer of Split homologue-1. By using a receptor-specific ligand we were able to show that the retinoic acid receptor α is important for regulation of the Hairy and Enhancer of Split homologue-1. Expression of a dominant negative form of Hairy and Enhancer of Split homologue-1 in MCF-7 cells abolished the growth-inhibitory effect of all-trans-retinoic acid in these cells. This finding indicates that Hairy and Enhancer of Split homologue-1 is a mediator of the antiproliferative effect of all-trans-retinoic acid in estrogen receptor α-positive breast cancer cell lines.

Retinoic acid and its derivatives are important during development, differentiation, and cell growth (1). These compounds have been used increasingly in cancer treatment where all-trans-retinoic acid (atRA)3 is used to treat various forms of cancer; one example is its use as a differentiating agent in the treatment of acute promyelocytic leukemia (2). Retinoids are also used as chemopreventive agents for treatment of skin, head, neck, and liver cancer. One study concerning colon cancer has shown that atRA can act as a chemopreventive agent on the formation of azoxymethane-induced aberrant crypt foci (3). Retinoids have also been demonstrated to have an antiproliferative effect on breast cancer cell lines (4, 5). Furthermore, it has been established that atRA functions as an anti-estrogen in terms of effect on 17β-estradiol-stimulated proliferation in estrogen receptor α (ERα)-expressing breast cancer cell lines (6).

The mechanism of the inhibition of proliferation by atRA is largely unknown, but one possibility is that atRA acts by inhibiting AP-1 activity (7). Another study shows that atRA increases the rate of D-type cyclin and Skp2 ubiquitinylation, where Skp2 is a protein that targets p27 for degradation. These events have been shown to occur in both T47D and MCF-7 cells (8). Resistance to atRA treatment has been reported in breast cancer cell lines like MDA-MB-231, which lacks the retinoic acid receptor α (RARα) (5). Another form of resistance to atRA can occur in cells lacking cellular retinoic acid-binding protein II (9). In an earlier report, we have shown that the Hairy and Enhancer of split homologue-1 (HES-1) is down-regulated by 17β-estradiol in breast cancer cell lines expressing ERα and that this down-regulation is essential for the proliferative effect of 17β-estradiol (10). In this study, we treated MCF-7 cells with 1 nM 17β-estradiol and increasing levels of atRA. We found that atRA prevented 17β-estradiol from down-regulating HES-1 expression and that this regulation correlated well with the effect of atRA on proliferation. Expression of a dominant negative form of HES-1 resulted in inhibition of this effect of atRA.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemicals—MCF-7 and COS-7 (ATCC, Manassas, VA) cells was grown in Dulbecco's modified Eagle's medium (atrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 0.1% kanamycin (Sigma). 17β-Estradiol and atRA (both from Sigma) were dissolved in ethanol and MeSO, respectively. The RARα-specific agonist RO 40-6055 (donated by Hoffman-La Roche) was dissolved in MeSO. Actinomycin D was purchased from Sigma and dissolved in MeSO. The construct used for stable transfection—containing dominant negative FLAG-tagged HES-1 (dn-HES-1), has mutations in the basic DNA binding domain where amino acids Glu-43, Lys-44, and Arg-47 were each mutated to Ala, and in addition the protein is truncated at amino acid 157 by using an internal SnaI site. Transfection of this construct into MCF-7 cells was done using FuGENE6 (Roche Molecular Biochemicals), and stable transfectants were selected using 800 μg/ml G418 (Invitrogen).

Proliferation Assay—Cells were plated at a density of 20,000 cells/cm². After 24 h, the medium was replaced with stripped medium (phenol red-free medium (Invitrogen) supplemented with 5% dextran-charcoal-treated FBS, 1% penicillin-streptomycin, 0.1% kanamycin) and different treatments (with/without 17β-estradiol and atRA). After 6 days of growth, the cellular ATP was extracted using 5% trichloroacetic acid followed by measurement of the amount of ATP, which is proportional to the cell number. The measurement of ATP was performed using Biothema's ATP kit and a microplate luminometer (Berthold).

Western Analysis—Cells were plated and grown in 150-mm plates at ~50% confluency for 24 h before medium replacement with stripped medium including different treatments. Extraction of nuclear proteins was done 72 h later according to standard protocols (12). 50 μg of proteins were loaded into each well. Separation, blotting, and visualization of the proteins were described previously (10). The relative expression was calculated using the NIH Image (version 1.62) program (rsb.info.nih.gov/nih-image/download.html).
Co-immunoprecipitation—Nuclei were isolated from MCF-7 cells stably expressing dn-HES-1 or from COS-7 cells transiently transfected with HES-1, inserted into pcDNA3 (Invitrogen), and dn-HES-1 using the DEAE-dextran method (13). Prior to nuclear extraction, COS-7 cells were grown for 48 h, whereas MCF-7 cells were grown for 24 h in the presence of 10 nM ICI 182,780 (Tocris). Nuclear proteins were diluted twice in dH20, incubated with M5 FLAG antibodies (Sigma) at 1:1000 dilution for 1 h, and added to protein G-Sepharose beads (Amersham Biosciences) for an additional hour. The beads were washed thoroughly with 1:1 dH20:High salt buffer (10 mM Hepes-KOH, pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol) and boiled with 1× SDS loading buffer. After centrifugation, the supernatant was separated, blotted, and visualized as described by Western analysis.

RNA Extraction and cDNA Synthesis—Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer’s instructions and digested with DNase I (Roche Molecular Biochemicals). 3 μg of total RNA were then reverse-transcribed using random hexamer priming and the SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions.

Real-time PCR—100 ng of cDNA was amplified in a real-time PCR using TaqMan® Universal Master Mix (PE Applied Biosystems), 200 nM primer for HES-1 and 300 nM fluorescent HES-1 probe. The real-time PCR reactions were performed in an ABI PRISM Model 7700 sequence detector (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The optimum concentration of primers and probe was determined in preliminary experiments. The sequences of HES-1 primers and probe are as follow: forward primer, 5′-AGG CGG ACA TTC TGG AAA TG-3′; reverse primer, 5′-CGG TAC TTC CCC AGC ACA CTT-3′; probe, 5′-FAM-TGT GCT CAG CGC AGC CAT CTG C-TAMRA-3′. Real-time PCR was done in triplicates. The 18 S rRNA (Pre-Developed TaqMan® Assay Reagents, Applied Biosystems) was used as reference gene.

Regulation of HES-1 Expression by atRA Correlates with Inhibition of Proliferation, and Expression of a Dominant Negative Form of HES-1 Inhibits the Antiproliferative Effect of atRA—Treatment of MCF-7 cells with 1 nM 17β-estradiol resulted in an increase in cellular proliferation, whereas addition of increasing amounts of atRA produced a dose-dependent inhibition of this proliferation (Fig. 1A). Analyses of HES-1 protein levels show an inverted correlation between HES-1 protein expression and atRA-mediated inhibition of proliferation (Fig. 1B). We show that 1 nM 17β-estradiol down-regulated HES-1 protein to ~5% of that in non-treated cells (Fig. 1B). As little as 10 nM atRA restored expression of HES-1 to more than 60% of non-treated levels, and following 100 nM atRA treatment, which significantly inhibits the estradiol-induced proliferation, 75% of HES-1 protein was present. By using 1 μM atRA, the expression of HES-1 was fully restored. Since this correlation could be coincidental we made a dominant negative form of HES-1 (dn-HES-1), which can form a heterodimer with endogenous HES-1 and inactivate its function. In contrast, non-transfected MCF-7 cells, cells that are stably transfected with the dominant negative form of HES-1 showed no responsiveness to atRA (Fig. 2A). The more than 100% increase in cellular proliferation induced by estradiol was not affected by as much as 1 μM atRA. MCF-7 cells transfected with empty plasmid behaved as normal cells in terms of response to atRA (Fig. 2B). This result suggests that HES-1 is an important factor participating in the inhibition of estrogen-stimulated proliferation of MCF-7 cells by atRA.

**FIG. 1.** The all-trans-retinoic acid-mediated inhibition of proliferation is correlated with HES-1 protein expression. Cells were cultured in phenol red-free medium supplemented with 5% hormone-depleted serum. A, proliferation was measured following 6 days of growth as described under “Experimental Procedures.” Values are means ± S.D. (n = 6). B, nuclear proteins were extracted 72 h after the different treatments and analyzed by Western blot. HES-1 protein expression levels following each treatment are expressed quantitatively as bars using NIH Image. E2, 17β-estradiol.

**FIG. 2.** No effect of all-trans-retinoic acid on proliferation in cells that express exogenous dominant negative HES-1. After 6 days of culturing in phenol red-free medium supplemented with 5% hormone-depleted serum, proliferation was measured as described under “Experimental Procedures.” Values are means ± S.D. (n = 6). A, cells stably expressing dn-HES-1. B, cells stably transfected with empty vector. E2, 17β-estradiol.
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**FIG. 3.** Co-immunoprecipitation of dominant negative HES-1 with HES-1. Cells were grown in medium supplemented with 10% FBS prior to extraction of nuclear proteins and co-immunoprecipitation as described under “Experimental Procedures.” A, co-immunoprecipitation of dn-HES-1 with HES-1 extracted from MCF-7 cells stably expressing dn-HES-1. 500 μg of nuclear proteins were used for co-immunoprecipitation and loaded in lanes 1 and 2. Lane 1 is a negative control since the FLAG antibody was excluded. 200 μg of nuclear proteins were used as a positive control and loaded in lane 3. B, co-immunoprecipitation of dn-HES-1 with HES-1 extracted from COS-7 cells transiently transfected with constructs expressing the named proteins. 300 μg of nuclear proteins were used in the assays and loaded in lane 1, the negative control, and in lane 2; whereas, in the positive control, lane 3, 200 μg of proteins were loaded. wt, wild type; wo, without; w, with; ab, antibody.

**FIG. 4.** atRA regulates HES-1 protein by activating RARα. Extraction of nuclear proteins was performed after a 72-h treatment of MCF-7 cells grown in phenol red-free medium supplemented with 5% hormone-depleted serum. Protein expression was analyzed by Western blot. The agonist RO 40-6055 is RARα-specific. E_2, 17β-estradiol.

**FIG. 5.** atRA regulates HES-1 transcriptionally. mRNA was extracted from cells grown in phenol red-free medium supplemented with 5% hormone-depleted serum, treated according to the protocols mentioned earlier, and analyzed using real-time PCR as described under “Experimental Procedures.” A, cells treated for 24 h. B, cells treated for 7 h. Gray bars are with 5 μg/ml actinomycin D, and black bars are without actinomycin D. E_2, 17β-estradiol.

**DISCUSSION**

Most breast cancer cell lines positive for ERα increase their proliferation in response to 17β-estradiol treatment. In this study, we investigated atRA-mediated inhibition of estradiol-stimulated proliferation and chose MCF-7 cells as a model for an estrogen-dependent cell line. Several reports have shown that atRA inhibits estrogen-dependent proliferation in this cell line (4, 15). In an earlier report, we showed that HES-1 is strongly down-regulated by 17β-estradiol treatment in the estrogen-dependent cell lines T47D and MCF-7. Furthermore, if this down-regulation is prevented by expression of HES-1 from a tetracycline-regulated expression vector, the proliferative effect of 17β-estradiol treatment is canceled (10). The mechanism by which HES-1 prevents 17β-estradiol-mediated proliferation of MCF-7 cells is currently not known. It is possible that the

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**dn-HES-1 Forms a Heterodimer with Endogenous HES-1**—To investigate whether the effect of dn-HES-1 is cell-specific, we used another cell line, T47D, that had been stably transfected with dn-HES-1 using a tetracycline-regulated system of expression. Also this cell line showed that the effect of atRA on proliferation was removed by inducing expression of dn-HES-1 (data not shown). These findings strongly suggest that HES-1 expression is important for the antiproliferative effect of atRA in these cells as well. To verify that the dn-HES-1 expressed in the MCF-7 cells is dimerizing with wild-type HES-1 we used nuclear extracts prepared from dn-HES-1-expressing MCF-7 cells and performed immunoprecipitation with a FLAG antibody. Fig. 3A shows that the FLAG epitope-fused dn-HES-1 indeed dimerized with endogenous HES-1. In addition, in COS-7 cells co-transfected with wild-type HES-1 and dn-HES-1, the same heterodimer was formed and could be immunoprecipitated with the FLAG antibody (Fig. 3B). Although no general statement about the mechanism of the antiproliferative effect of atRA in other tissues or cell lines can be made, the mechanism behind the antiproliferative effect of atRA in the breast cancer cell line MCF-7 is likely to depend on regulation of HES-1 expression.

**atRA Regulates HES-1 Expression through the Retinoic Acid Receptor α**—Since earlier reports have shown that RARα is the receptor responsible for the antiproliferative effect of atRA in MCF-7 cells (14) we wanted to investigate whether RARα could regulate HES-1 expression. To answer this question, we used the specific agonist RO 40-6055 for RARα (14). In MCF-7 cells,
inhibition takes place later in the G1 phase rather than early and may involve factors such as Cdc25A or other factors affecting Cdk2 rather than cyclin D1 activity. Foster et al. (16) have recently shown that 17β-estradiol regulates expression of Cdk inhibitor and the Cdk2-activating phosphatase Cdc25A independently of cyclin D1-Cdk4 function and cell cycle progression in MCF-7 cells.

HES-1 has been shown to inhibit proliferation in PC12 cells as well. In this cell type, an up-regulation of HES-1 by atRA has been observed (11), which is in agreement with previously published results that atRA treatment of this cell type leads to inhibition of proliferation (17). Furthermore, expression of HES-1 in NIH 3T3 cells also inhibits cellular proliferation; accordingly, HES-1 seems to affect proliferation in many cell types (11). Nevertheless, the effect of HES-1 may not be general since Sriruranpong et al. (18) have shown that HES-1 expression does not inhibit proliferation of small cell lung cancer cells.

In breast cancer cell lines, the retinoic acid receptor α has been shown to mediate the antiproliferative effect of atRA (14). This agrees with our finding that HES-1 is regulated by RARs since the α specific-agonist RO 40-6055 was able to up-regulate HES-1 expression. The regulation of HES-1 is transcriptional since real-time PCR clearly showed dose-dependent accumulation of mRNA after treatment with atRA, and furthermore actinomycin D prevented this accumulation of mRNA in response to atRA treatment. Analysis of the promoter region of human HES-1 shows that it does not contain any good consensus sequence for the retinoic acid receptor, but it does contain many AP-1 sites, which are potential retinoic acid response elements (19). Stimulation of transcription by atRA through an AP-1 element has been shown for the CSF-1 receptor gene (20). On the other hand, the antiproliferative effect of atRA has been suggested to be dependent on down-regulation of AP-1 activity (7). These conflicting findings might indicate that activation of an AP-1 element could lead to up- or down-regulation of downstream genes in response to atRA, depending on promoter context. At this point in the study, it is difficult to speculate how inhibition of HES-1 function might interfere with AP-1 activity, but it is possible that the effect of HES-1 might be indirect. That is, a component that is important for the inhibition of AP-1 may be dependent on HES-1 expression. Furthermore, retinoids have been shown to inhibit cyclin D1 expression in breast cancer cell lines (15). A recent publication by Dow et al. (8) describes that atRA treatment of breast cancer cell lines induces cyclin D1 ubiquitylation and subsequent degradation. Also, in the same study another protein, Skp2, is ubiquitylated, which leads to p27 accumulation since this protein targets p27 for degradation.

A scenario where atRA affects several components important for cell cycle progression is possible. This could mean that regulation of HES-1 expression is important for the effect of atRA on only one or a few of such components. More detailed studies are in progress to dissect the mechanism by which HES-1 affects proliferation of breast cancer cell lines.

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