Signal Relay by Retinoic Acid Receptors α and β in the Retinoic Acid-induced Expression of Insulin-like Growth Factor-binding Protein-3 in Breast Cancer Cells*

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Neither retinoic acid receptor-β (RARβ) nor insulin-like growth factor-binding protein-3 (IGFBP-3) is expressed in breast cancer cell line MCF-7. The expression of both proteins can be induced in response to all-trans-retinoic acid (atRA). By using an RARα-selective antagonist (Ro 41-5253), we demonstrated that RARβ expression was induced by atRA through an RARα-dependent signaling pathway and that RARβ induction was correlated with IGFBP-3 induction. However, MCF-7 cells transfected with sense RARβ cDNA expressed IGFBP-3 even in the presence of the RARα-selective antagonist Ro 41-5253. On the other hand, antisense RARβ cDNA transfection of MCF-7 cells blocked atRA-induced IGFBP-3 expression, indicating that RARβ is directly involved in the mediation of IGFBP-3 induction by atRA. Induction of IGFBP-3 expression by atRA occurs at the transcriptional level, as measured by nuclear run-on assays. Finally, we showed that atRA-induced IGFBP-3 is functionally active in modulating the growth-promoting effect of IGF-I. These experiments indicate that RARα and RARβ, both individually and together, are important in mammary gland homeostasis and breast cancer development. By linking IGFBP-3 to RARβ, our experiments define the signal intersection between the retinoid and IGF systems in cell growth regulation and explain why loss of RARβ might be critical in breast cancer carcinogenesis/progression.

Retinoids induce growth inhibition and apoptosis in a variety of tumor cells, including breast cancer cells (1). Recently, we proposed a mechanism by which all-trans-retinoic acid (atRA) synergizes with interferon to inhibit the growth of both estrogen receptor-positive and estrogen receptor-negative breast cancer cell lines (2). Here we studied mechanisms by which atRA counteracts the growth-promoting effects of insulin-like growth factors (IGFs) in breast cancer cells, focusing on the involvement by retinoic acid receptors (RARs).

It is known that the molecular actions of retinoids are primarily mediated by their nuclear receptors (RARα, β and γ, and the retinoid X receptors (RXRs) α, β, and γ), which function as liganded transcription factors (3). These receptors show both spatiotemporal patterns of expression during development and tissue-specific distribution in adults, suggesting that the various receptors play different roles in transducing retinoid signals. Among the RARs, RARα is expressed ubiquitously in adult tissues, RARγ is expressed mainly in skin, and RARβ is expressed primarily in epithelial cells, including those in mammary tissue (4). Expression of RARβ is lost in the majority of breast cancer cell lines; it can be induced by retinoic acid (RA) in estrogen receptor-positive breast cancer cell lines but not in estrogen receptor-negative cancer cell lines (4–7). The latter are believed to represent more advanced forms of breast carcinoma. Induction of RARβ expression correlates well with the growth-inhibitory and apoptotic effects of retinoic acid (8, 9), suggesting that loss of RARβ expression may be one of the critical events involved in breast carcinogenesis/progression and in responsiveness of breast cancer cells to retinoid chemotherapies. At the same time, there is strong evidence that RARα is the mediator of the growth inhibition of breast cancer cells by retinoids (10, 11). In general, RARα expression is lower in estrogen receptor-negative breast cancer cell lines than in estrogen receptor-positive lines; this corresponds to the responsiveness of these cell lines to RA. Taken together, these observations raise the possibility that both RARα and RARβ are involved in the physiological action of retinoic acid in breast cancer cells.

The insulin-like growth factor system includes IGF-I and IGF-II, their corresponding receptors, six IGF-binding proteins (IGFBPs), and four IGFBP-related proteins (12). IGF-I and IGF-II are thought to be important growth factors for breast cancer. IGF-I and -II receptors and IGFBP-2 and -4 proteins have been found in breast cancer cell lines and in tissue specimens (13). Although IGF-I and -II proteins are not expressed in breast cancer cell lines, they are expressed in breast cancer specimens, possibly by stromal cells (13), suggesting that IGFBPs, through a paracrine mechanism, promote breast cancer cell growth and underscoring the importance of IGFBPs for their ability to modulate IGF-I actions in the extracellular matrix.

In addition to the well established roles of IGFBPs in regulating IGF bioavailability and IGF-I receptor responsiveness to IGF-I, IGFBP-3 has also been recently proposed to function as a negative regulator of growth, independently of the IGF-I receptor (14, 15). Supporting its role as a growth inhibitory regulator, IGFBP-3 expression is up-regulated by growth-inhibitory (and apoptosis-inducing) agents, such as retinoic acid (16–19), vitamin D (20), transforming growth-factor-β (16, 21, 22), antiestrogens (23), tumor necrosis factor-α (24), and, most compellingly, the tumor suppressor gene p53 (25); IGFBP-3 expression is down-regulated by growth-promoting factors,
such as estrogen (26) and epidermal growth factor (27). All of these factors are involved in the regulation of IGFBP-3 expression, although the mechanism is not fully understood. In this study, we examined the role of retinoids in the regulation of IGFBP-3 expression.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Retinoids**—Cells of the breast carcinoma cell line MCF-7 (American Type Culture Collection, Manassas, VA) were grown in phenol red-free Eagle’s minimal essential medium (Sigma) supplemented with 5% charcoal-stripped calf serum (Sigma). Cells from <15 passages were used for experiments.

Retinoids were purchased from Sigma. The RAR-specific agonist Ro 13-7410, the RXR-specific agonist Ro 25-7386, and the RAR-selective antagonist Ro 41-5253 were generously provided by Hofmann-La Roche. Retinoids were dissolved in absolute ethanol under lights that were covered with a UV-blocking film (CLHC, Sydlin, Inc., Lancaster, PA). The integrity of atRA was routinely monitored by spectrophotometry.

**Preparation of Conditioned Medium**—MCF-7 cells were grown as described above for 24 h, washed with phosphate-buffered saline, and then transferred to phenol red-free Eagle’s minimal essential medium supplemented with 2 μg/ml fibronectin and 2 μg/ml transferrin (both from Sigma) for another 24 h before atRA treatment. The conditioned medium was then harvested with the addition of 0.2 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin (both from Sigma), dried under speed vacuum, and resuspended for analysis.

**Cell Growth Inhibition Assay**—MCF-7 cells (4 × 10^4 cells/well) were cultured in the conditioned medium described above in 96-well cell culture plates. Recombinant human IGF-I, recombinant human IGFBP-3 (both generous gifts of Celtrix, Palo Alto, CA), or medium from atRA-treated cell cultures was added alone or in different combinations to the cell cultures for 2 days. Cells were washed, fixed with 10% trichloroacetic acid for 1 h, and then stained with 1% sulfobetaine B for 1 h. Cells were washed again, and then 100 μl of 10 μM Tris-HCl, pH 10, was added to release the dye (28). The absorbance was measured at 562 nm.

**Immunodepletion**—Conditioned medium from atRA-treated or untreated cells was incubated with 2 μg/ml of anti-IGFBP-3 antibodies (goat polyclonal antibodies against human IGFBP-3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or normal goat serum (Santa Cruz Biotechnology) for 2 h. Protein A/Protein G PLUS-Agarose (Santa Cruz Biotechnology) was then added, and the media were rocked at 4°C overnight followed by filter sterilization of the supernatants. Immunoprecipitates were boiled for 3 min in SDS gel loading buffer and were used in a Western ligand blotting.

**Western Immunoblotting and Western Ligand Blotting**—Fifty μg of protein from cell lysates or conditioned medium was loaded onto 8–12% SDS-polyacrylamide gels under nonreducing conditions. After transfer, nitrocellulose blots were incubated with rabbit polyclonal antibodies against human RARβ (Santa Cruz Biotechnology). The blots were then incubated with secondary antibodies and developed using an ECL kit (Amersham Pharmacia Biotech). For Western ligand blotting, nitrocellulose blots were initially washed in 3% Nonidet P-40 (Fluka Chemical Corp., Ronkonkoma, NY) for 30 min, followed by blocking in 1% bovine serum albumin (Sigma) for 2 h and 0.1% Tween 20 (Sigma) for 15 min. Blots were then probed with 125I-labeled recombinant human IGF-II (Bachem California Inc., Torrance, CA) overnight followed by extensive washing with 1% Tween 20 before autoradiography.

**Transient Transfection**—A luciferase reporter gene construct under the control of a retinoic acid response element (DR5-tk-Luc, provided by Dr. R. M. Evans, Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA) was used to measure retinoid receptor-mediated gene activation. Ten μg of DR5-tk-Luc was co-transfected into MCF-7 cells with 2 μg of β-galactosidase expression vector (pCMVβ; CLONTECH, Palo Alto, CA) using Lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD) overnight followed by extensive washing with 1% Tween 20 before autoradiography.

**Stable Transfection**—Plasmid constructs for stable transfection experiments were pRCCMV-RARβ and pRCCMV-antisense RARβ (generous gifts from Dr. X.-K. Zhang, La Jolla Cancer Research Center, La Jolla, CA). MCF-7 cells grown to 50% confluence were washed with serum-free growth medium. Two μg of either empty vector or construct was mixed with Lipofectin reagent and added to cells for 5 h. Selection was initiated with 400 μg/ml of G418 (Life Technologies, Inc.) on the third day and continued for 17–21 days until drug-resistant colonies emerged. Single colonies were cloned and assayed for the expression of the inserted genes by Northern blotting, and the expression of RARβ receptor protein was measured by Western blotting.

**RESULTS**

**IGFBP-3 Expression Is Induced by atRA in a Dose- and Time-dependent Fashion in MCF-7 Cells**—To determine the effects of atRA on the expression of IGFBP-3 in our experimental system, MCF-7 cells were grown in the presence of 0, 10^-8, 10^-7, or 10^-6 M atRA for 72 h followed by Northern blotting analysis of IGFBP-3 mRNA. As shown in Fig. 1, A), MCF-7 cells did not express IGFBP-3 message in the absence of atRA, but as little as 10^-8 M atRA was effective in inducing the expression of IGFBP-3 mRNA. Higher levels of IGFBP-3 mRNA were detected with increasing concentrations of atRA (Fig. 1A). Fig. 1B shows the temporal effect of 10^-8 M atRA on the expression of IGFBP-3 message. IGFBP-3 mRNA was detected as early as 24 h after atRA treatment and was maximal at 48 h.

**atRA Activates IGFBP-3 Gene Transcription, and RAR, Rather Than RXR, Mediates This Process**—We next wished to determine whether the retinoic acid-induced expression of IGFBP-3 in MCF-7 cells was mediated by RAR or RXR and whether atRA directly activates the transcription of the IGFBP-3 gene. The second point was of interest because it is known that retinoids can regulate gene expression posttranscriptionally (29, 30). For these experiments, MCF-7 cells were incubated for 48 h with 10^-6 M of either atRA, the RAR-specific
...treated with 10⁻⁶ M of various retinoids (atRA, the RAR-specific agonist Ro 13-7410, or the RXR-specific agonist Ro 25-7386) for 48 h. The gene was not transcribed in cells treated with retinoids.

In a separate experiment, MCF-7 cells were grown in the presence of 10⁻⁶ M of various retinoids for 72 h. The results were normalized to β-actin mRNA as an indicator of equal loading. The results represent at least three independent experiments.

Agonist Ro 13-7410, or the RXR-specific agonist Ro 25-7386. Nuclei were isolated, and nuclear run-on assays were performed. As indicated in Fig. 2A, both atRA and the RAR-specific agonist Ro 13-7410 activated IGFBP-3 gene transcription, but the gene was not transcribed in cells treated with vehicle only or with the RXR-specific agonist Ro 25-7386. The β-actin gene was transcribed normally under all of these experimental conditions. These results indicate that 1) RAR but not RXR is involved in transducing the atRA signal to induce IGFBP-3 expression, and 2) atRA and Ro 13-7410 directly activate IGFBP-3 gene transcription. IGFBP-3 mRNA was measured in parallel experiments following treatment of MCF-7 cells with the various retinoids for 72 h (Fig. 2B). IGFBP-3 mRNA was only present in cells treated with atRA and Ro 13-7410, the RAR-specific agonist.

To verify the ability of the synthetic retinoids, Ro 13-7410 and Ro 25-7386, to activate retinoid receptors in our experimental system, a luciferase reporter gene under the control of a DR5 element, the canonical retinoic acid response element activated by RARs, was introduced into MCF-7 cells. Luciferase activity was measured 72 h later in the presence of 10⁻⁶ M of atRA, Ro 13-7410, or Ro 25-7386. As documented in Fig. 2C, Ro 13-7410, the RAR-specific agonist, activated the expression of luciferase gene at a level similar to that of atRA, but Ro 25-7386, the RXR-specific agonist, was not effective in activating the expression of luciferase gene. These results validate the use of the synthetic retinoids in our experimental system.

**RARβ Expression Is Induced by atRA in an RARα-dependent Pathway, and RARβ Relays the atRA Signal That Leads to the**

**Induction of IGFBP-3 Expression in MCF-7 Cells**—It has been shown that the transcription of the RARβ gene is induced rapidly after retinoid treatment, peaking by 6 h, and that it is independent of new protein synthesis (31, 32). Furthermore, the level of RARα expression in breast cancer cell lines appears to be correlated with the induced levels of RARβ expression (5, 8, 9). Thus, it is reasonable to postulate that RARβ is induced in MCF-7 cells by atRA through a signaling pathway mediated by RARα. To test this hypothesis, MCF-7 cells were grown in the presence or absence of 10⁻⁶ M atRA for 72 h. Total RNA was extracted, and 30 µg was used to measure mRNAs for RARα and RARβ by Northern blotting. As documented in Fig. 3, the levels of RARα expression in MCF-7 cells were similar in the presence or absence of atRA, whereas RARβ expression was detectable only after atRA treatment. These results indicate that RARα mediates the atRA-induced expression of RARβ.

These experiments led us to ask whether the signal leading to the induction of IGFBP-3 expression was mediated by RARα, or if the induced RARβ mediates IGFBP-3 induction. In order to answer this question, MCF-7 cells were cultured for 72 h in the presence of 10⁻⁷ M atRA plus 0, 10⁻⁶, 10⁻⁵, or 10⁻⁴ M of Ro 41-5253, an RARα-selective antagonist. A lower concentration of atRA was used because we wanted to minimize the cytotoxicity of retinoids that is observed at high concentrations. After incubation, 30 µg of total RNA was used to assay RARα mRNA. As documented in Fig. 4A, 1 molar excess of Ro 41-5253 blocked the induction of RARβ expression. With decreasing concentrations of Ro 41-5253, RARβ expression increased, indicating that the process is mediated by RARα. IGFBP-3 expression was measured in MCF-7 cells grown for 72 h in the presence of the same combinations of retinoids by Northern blotting (Fig. 4B). Paralleling the diminished expression of RARβ in the presence of 10⁻⁶ M of Ro 41-5253, IGFBP-3 expression was also abolished, indicating that retinoid-induced IGFBP-3 expression is correlated with RARβ expression.

In order to further document the direct involvement of RARβ in the atRA-induced expression of IGFBP-3, RARβ sense and antisense cDNA constructs were introduced into MCF-7 cells via expression vectors. Positive colonies were identified, cloned, and tested for RARβ expression by Western immunoblotting (Fig. 5). Three clones with average levels of expression of each sense (Fig. 5A, β1, β5, and β6) and antisense (Fig. 5B, As-β1, As-β5, and As-β6) RARβ were used for experiments similar to those described above. As exemplified by the results shown for β5 (Fig. 6A), the RARα-selective antagonist Ro 41-5253 was unable to block atRA-induced IGFBP-3 expression in the three clones of RARβ sense transfectants, indicating that RARα is not directly involved in this process. In contrast, in RARβ antisense transfectants, the induction of IGFBP-3 expression...
A, medium for 6 days in the presence or absence of 10^{-7} M atRA. For this purpose, MCF-7 cells were grown in conditioned medium for 4 days in the presence or absence of 10^{-6} M atRA, and the conditioned medium was collected. IGFBP-3 protein was immunodepleted in half of the conditioned medium from atRA-treated cells. The medium was then filter-sterilized and added to MCF-7 cells for 2 days in the presence of 1 nM IGF-I. Cell growth was measured by sulforhodamine staining and expressed as percentage of absorbance relative to control MCF-7 cell cultures treated with control medium supplemented with 1 nM IGF-I (100%) or 1 nM IGF-I plus 10 nM rhIGFBP-3 (0%) (Fig. 7C). Similar to the results described in Fig. 7B, the conditioned medium from atRA-treated MCF-7 cells was able to block the growth promotion of MCF-7 cells by IGF-I (Fig. 7C, CM/RA/BP3). When IGFBP-3 was depleted (Fig. 7C, CM/RA/BP3/S) the medium was no longer effective in blocking the growth promotion by IGF-I, whereas the normal goat serum-treated control (Fig. 7C, CM/RA/BP3/S) did not remove the growth inhibition effect, suggesting that atRA-induced inhibition of IGF-I-stimulated cell growth is mediated rather specifically by IGFBP-3, not IGFBP-2 or IGFBP-4, because IGFBP-3-depleted medium (CM/RA) was unable to counteract IGF-I even when IGFBP-2 and IGFBP-4 were still present (Fig. 7D, lane 2).

As shown in the Western blots in Fig. 7D, conditioned medium from the 4-day atRA-treated MCF-7 cells contained the IGFBPs 3, 2, and 4 (Fig. 7D, lane 1). After immunodepletion, only IGFBP-2 and IGFBP-4 were present (Fig. 7D, lane 2); when the immunoprecipitate was examined, only IGFBP-3 was found (Fig. 7D, lane 3). These experiments clearly demonstrate that atRA-induced IGFBP-3 is able to function as a downstream effector of RARβ to block the growth promotion by IGF-I in MCF-7 breast cancer cells. A semiquantitative Western blot analysis utilizing rhIGFBP-3 as a standard indicated that the concentration of IGFBP-3 in atRA-treated conditioned medium was ~3 nM (data not shown). This result is consistent with a partial block of IGF-I action by recombinant IGFBP-3, which resulted in ~50% inhibition at 2 nM concentration (Fig. 7B).
IGFBP-3 in MCF-7 cells. A, atRA-induced secretion of IGFBP-3 into the conditioned medium. MCF-7 cells were grown in conditioned medium for 4 days; cell growth was measured by the sulforhodamine B staining. The results represent the mean ± S.D. for quadruplicate experiments. B, growth effects of exogenous IGF-I and IGFBP-3 in MCF-7 cells. Recombinant human IGF-I or IGFBP-3 (BP3) was added alone or in different combinations to MCF-7 cells for 4 days; cell growth was measured by the sulforhodamine staining. The results represent the mean ± S.D. for quadruplicate experiments. C, growth effects of atRA-induced endogenous IGFBP-3 in MCF-7 cells. MCF-7 cells were grown in the conditioned medium containing 10⁻⁶ M atRA for 4 days. The medium (CM/RA/BP3) was collected, immunodepleted of IGFBP-3 (CM/RA) and applied to untreated MCF-7 cell cultures in the presence of 1 nM IGF-I. Medium from untreated cell cultures (CM/CTL) and from cultures that were treated but immunodepleted with nonspecific antibodies (CM/RA/BP3/S) were used as the control. The growth of cells treated with CM/CTL was taken as 100%, and that of cells treated with CM/CTL plus 10 nM rhIGFBP-3 (CM/CTL+1 nM IGF-I+10 nM BP3) was taken as 0%. The results represent the mean ± S.D. for quadruplicate experiments. D, Western ligand blot to confirm the occurrence of IGFBP-3 in atRA-treated conditioned medium (lane 1), IGFBP-3-immunodepleted medium (lane 2), and immunoprecipitates from IGFBP-3-immunodepleted medium (lane 3). The results represent at least three independent experiments.

FIG. 7. The secretion and biological activity of atRA-induced IGFBP-3 in MCF-7 cells. A, atRA-induced secretion of IGFBP-3 into the conditioned medium. MCF-7 cells were grown in conditioned medium in the presence or absence of 10⁻⁶ M atRA for 6 days. Conditioned medium was harvested at 2, 4, and 6 days, concentrated, and analyzed for IGFBP-3 secretion by Western ligand blotting. B, growth effects of exogenous IGF-I and IGFBP-3 in MCF-7 cells. Recombinant human IGF-I or IGFBP-3 (BP3) was added alone or in different combinations to MCF-7 cells for 4 days; cell growth was measured by the sulforhodamine staining. The results represent the mean ± S.D. for quadruplicate experiments. C, growth effects of atRA-induced endogenous IGFBP-3 in MCF-7 cells. MCF-7 cells were grown in the conditioned medium containing 10⁻⁶ M atRA for 4 days. The medium (CM/RA/BP3) was collected, immunodepleted of IGFBP-3 (CM/RA) and applied to untreated MCF-7 cell cultures in the presence of 1 nM IGF-I. Medium from untreated cell cultures (CM/CTL) and from cultures that were treated but immunodepleted with nonspecific antibodies (CM/RA/BP3/S) were used as the control. The growth of cells treated with CM/CTL was taken as 100%, and that of cells treated with CM/CTL plus 10 nM rhIGFBP-3 (CM/CTL+1 nM IGF-I+10 nM BP3) was taken as 0%. The results represent the mean ± S.D. for quadruplicate experiments. D, Western ligand blot to confirm the occurrence of IGFBP-3 in atRA-treated conditioned medium (lane 1), IGFBP-3-immunodepleted medium (lane 2), and immunoprecipitates from IGFBP-3-immunodepleted medium (lane 3). The results represent at least three independent experiments.

The tissue-specific distribution of retinoic acid receptors in adults and the spatiotemporal patterns of expression during development indicate that these receptors may play different roles. Yet the coexistence of two or three retinoic acid receptor subtypes in a specific tissue also suggests that some type of compensation/coordination may exist among retinoic acid receptors in transducing retinoid signals. Such a compensation/coordination of RARs in breast cancer cells was demonstrated in our experiments that showed that RARβ expression can be induced in MCF-7 cells by atRA via RARα mediation. The levels of RARα expression were similar in the presence or absence of atRA in MCF-7 cells, but RARβ expression, which was undetectable in the absence of atRA, was strongly induced by atRA. When the RARα-selective antagonist Ro 41-5253 was used, the induction of RARβ expression by atRA was blocked, indicating that RARβ induction is dependent on RARα.

Both RARα and RARβ have been implicated in tumor development. For example, it is well documented that acute promyelocytic leukemia is caused by a reciprocal chromosome 15:17 translocation in which the t(15:17) breakpoint occurs in the RARα gene (33). An involvement of RARβ in cancer development was originally suggested by the finding that RARβ is integrated by the hepatitis B virus in human hepatoma (34). Moreover, defective RARβ expression is believed to be an early event in epithelial carcinogenesis (35). Recently, it has been observed that RARβ expression is lost in many epithelial tumors and tumor cell lines, including breast cancer and breast cancer cells (5, 6, 35–39). Furthermore, transgenic mice carrying antisense RARβ2 develop carcinoma 14–18 months after birth (40), strongly supporting a role of RARβ in tumorigenesis. By demonstrating an RARα-dependent RARβ induction, our experiments further stress the importance of RARβ, which eventually becomes noninducible with progression of the tumor, as in estrogen receptor-negative breast cancer cells. The results of these experiments also allow us to clarify why both RARα and RARβ have been implicated in retinoid-induced growth inhibition of breast cancer cells.

RARβ has been suspected as a tumor suppressor for a long time, and loss of RARβ expression has been thought to be a critical event in the development of breast cancer. We suggest that RARβ functions as a tumor suppressor by regulating the expression of other critical cell growth regulatory factors. Our experiments show that the regulation (induction) of IGFBP-3 in MCF-7 cells is mediated by RARβ, because blocking RARβ expression by an RARα antagonist, Ro 41-5253, also blocked the expression of IGFBP-3. When MCF-7 cells were transfected with the sense cDNA of RARβ, IGFBP-3 was expressed, even in the presence of Ro 41-5253. At the same time, when the antisense cDNA of RARβ was transfected into MCF-7 cells, those cells were no longer able to respond to atRA by expressing IGFBP-3.

Using nuclear run-on assays, we showed that atRA directly activates IGFBP-3 gene transcription, supporting the recent finding that a major consensus sequence for retinoic acid is present in the promoter region of the IGFBP-3 gene (41). Whereas MCF-7 cells synthesize and secrete IGFBP-2 and IGFBP-4 into conditioned medium, the application of atRA not only induces the messenger for IGFBP-3, but also results in the appearance of secreted protein in the conditioned medium. IGFBP-3 secretion seems to occur while secretion of IGFBP-2 decreases and secretion of IGFBP-4 increases.

Among their diverse biological activities, IGFBPs are able to negatively modulate the actions of IGF by binding IGFs and preventing them from binding to the type 1 receptor (12, 42).
RARα and RARβ in IGFBP-3 Induction

Here, we demonstrated that the application of IGF-I stimulates cell growth in MCF-7 cells and that the application of exogenous rhIGFBP-3 can totally reverse this action. When we tested the biological activity of IGFBPContaining conditioned media in their ability to inhibit the IGF-I-stimulated growth of MCF-7 cells, we expected that changes in all of the IGF-3s (that is, IGFBP-3 induced by atRA along with an increase in IGFBP-4 and a decrease in IGFBP-2) would contribute to the growth inhibition effect. However, immunodepletion of IGFBP-3 from the conditioned medium removed all growth inhibitory activity, suggesting an IGFBP-3-specific growth inhibitory mechanism. The significance of atRA-induced changes in IGFBP-2 and IGFBP-4 and the reason why these changes do not help counteract the IG-F stimulation effect are not clear. Although IGFBP-3 induction by retinoids has been consistently observed, inconsistencies exist about retinoid-induced changes in IGFBPs 2 and 4 (17–19). In addition, as mentioned earlier, the biological activities of IGFBPs are not limited to negative effects on IGFs. Thus, changes in IGFBP-2 and IGFBP-4 may be germane to other, as yet unidentified mechanisms. In fact, the co-presence of IGFBPs 2, 3, and 4 in the cell culture medium may well indicate that these proteins possess different functions rather than simply representing functional redundancy. Another explanation is that IGFBP-3 may act through an IGF-independent pathway. IGF-independent actions of IGFBPs are being pursued vigorously. Of particular interest is the recent observation that IGFBP-3 can be translocated into the nucleus (44–46). Both exogenous (47) and endogenous IGFBP-3 have been shown to be translocated into the nucleus of breast cancer cells. Given the extremely selective nature of nuclear protein localization, it is reasonable to speculate that IGFBP-3 exerts profound biological activity in the nucleus.

In summary, our experiments show that both RARα and RARβ are involved in the growth inhibitory activity of retinoids by mediating the induction of IGFBP-3 expression. By linking IGFBP-3 to RARβ, our experiments have pinpointed an intersection between retinoid and IGF signals. This information also expands knowledge of the downstream effectors of RARβ and explains how RARβ might act as a tumor suppressor.

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