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Insulin-like growth factor binding protein-3 prevents retinoid receptor heterodimerization: implications for retinoic acid-sensitivity in human breast cancer cells☆

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Abstract

Insulin-like growth factor binding protein-3 (IGFBP-3) has both IGF-dependent and -independent effects on cell growth, which are frequently growth-inhibitory. Interestingly, the development of a more aggressive phenotype in breast cancer cells (BCCs) correlates positively with elevated expression of IGFBP-3 and is often associated with all-*trans*-retinoic acid (atRA)-resistance. IGFBP-3 was previously demonstrated to interact directly with retinoid X receptor (RXR). In this study we have shown that IGFBP-5 also interacts with RXR and that both IGFBPs interact with retinoic acid receptor (RAR). To investigate whether the presence of IGFBP-3 regulates breast cancer cell responsiveness to atRA, we immuno-neutralized the IGFBP-3 expressed by the atRA-resistant Hs578T and MDA-MB-231 BCCs (which express IGFBP-3 constitutively) and showed that they become more sensitive to the growth-inhibitory effects of atRA. Similarly, in Hs578T cells expressing a reporter gene under the control of an RAR response element (RARE), depletion of IGFBP-3 resulted in the induction of reporter gene expression in response to atRA. In investigating possible mechanisms for IGFBP-3 regulation of atRA-sensitivity, we found that IGFBP-3 blocked the formation of RAR:RXR heterodimers and disrupted the ligand-inducible receptor complex. Thus, IGFBP-3 has the potential to reduce the RARE-mediated transactivation of target genes and modulate the atRA-response in BCCs.

Keywords: IGFBP; Retinoid receptors; Breast cancer; Retinoic acid resistance

The insulin-like growth factors (IGF-I and IGF-II) are potent mitogens for many cell types, including normal and malignant mammary epithelial cells [1]. They exert their mitogenic effects principally through the type I IGF receptor. The IGFs also have high affinity for a family of six structurally related IGF binding proteins, IGFBP-1 to IGFBP-6, which are responsible for regulating the bioavailability of the IGFs in the circulation and the extracellular environment [2]. In addition, a number of IGFBPs have effects on cell function that are IGF-independent. In the case of IGFBP-3, these effects are frequently growth-inhibitory [3,4]. Although the mechanisms for the IGF-independent effects of IGFBP-3 are not fully understood, some effects may be related to the nuclear actions of IGFBP-3. Nuclear translocation of IGFBP-3 (and the highly homologous IGFBP-5) have now been reported in numerous cell lines [5–7]; however, the roles of these IGFBPs in the intracellular environment remain unclear.

Retinoic acid (RA) is a non-steroidal hormone essential for the maintenance of normal growth and differentiation of epithelial cells [8]. In addition, the retinoids are potent growth-inhibitory and pro-apoptotic factors for many normal and malignant cell types. The actions of RA are mediated by a family of nuclear proteins, retinoic acid receptor (RAR)-α, -β, and -γ,

☆ *Abbreviations:* IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; VDR, vitamin D receptor; TR, thyroid hormone receptor; PPAR, peroxisome proliferator activated receptor; ER+ve, estrogen receptor-positive; BCC, breast cancer cells; atRA, all-*trans*-retinoic acid; ER−ve, ER-independence; RARE, RAR response element; GST, glutathione-*S*-transferase; His_6, polyhistidine tag.

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which heterodimerize with one of the three isoforms of the nuclear retinoid X receptor (RXR)-α, -β or -γ, to form ligand-activated transcription factor complexes [9]. RXR also forms homodimers or can heterodimerize with other nuclear receptors, such as the vitamin D receptor (VDR), the thyroid hormone receptor (TR), and the peroxisome proliferator activated receptors (PPAR), thereby constituting the functional form of these receptors [10].

The majority of estrogen receptor-positive (ER+ve) breast cancer cells (BCCs) are sensitive to the growth-inhibitory effect of all-*trans*-retinoic acid (atRA) [11]. However, most breast cancers become resistant to atRA upon progression to ER-independence (ER−ve), a phenomenon that may be partly explained by the observed decrease in their levels of RAR-α [12]. Interestingly, a number of studies have observed that the development of estrogen-insensitivity and aggressive phenotype in BCCs and tumors correlates positively with elevated expression of IGFBP-3 [13–15]. If IGFBP-3 is capable of blocking RAR response element (RARE)-mediated signaling, it may be contributing to the RA-resistant phenotype in these cells.

IGFBP-3 has been shown in vitro to interact directly with RXR-α (co-localizing in the cytoplasm and nucleus) and to enhance the transactivation caused by an RXR-specific ligand [16]. In contrast, IGFBP-3 inhibited the transactivation of the RARE by atRA. The present study examines a possible role for IGFBP-3 in modulating the atRA-responsiveness in BCCs and demonstrates that IGFBP-3 may attenuate atRA-signaling by interfering with the formation of RAR:RXR heterodimers. This may be important in the atRA-resistant phenotype observed in certain cancers.

Materials and methods

Materials. Recombinant human IGFBP-3 and IGFBP-5 were produced in 911 human retinoblastoma cells [17,18]. Recombinant human IGF-I was provided by Genentech (South San Francisco, CA). Rabbit antiserum against IGFBP-3 and non-immune serum were prepared in this laboratory and the IgG fraction was purified on protein A–Sepharose (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Plasmids expressing hRXR-α fused to glutathione *S*-transferase (GST) and hRAR-α under the control of the CMV promoter (pCMX-hRAR-α) were provided by Thorsten Heinzel (Georg Speyer Institute, Germany) and Ron Evans (Howard Hughes Medical Institute, USA), respectively. hRAR-α was amplified by PCR from pCMX-hRAR-α and inserted into pGEX-2T (Amersham Biosciences) or pRSET (Invitrogen, Carlsbad, CA) to generate GST-RAR-α and His_6-RAR-α, respectively. atRA was purchased from Sigma (St. Louis, MO) and [^{3}H]atRA was from NEN Life Science Products (Boston, MA).

GST pull-down assay. GST, GST-RXR-α, and GST-RAR-α were expressed in *Escherichia coli* and captured from cell lysates using glutathione–Sepharose beads (Amersham Biosciences). The immobilized proteins were incubated with IGFBP-3 or IGFBP-5 (1 μg) in binding buffer (50 mM sodium phosphate, 0.1% BSA, and 2 mM (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate: CHAPS), pH 6.5) for 2 h at 22 °C with gentle rotation. The bound IGFBPs were separated on 10% SDS–PAGE prior to membrane transfer and detected by Western ligand blotting using [^{125}I]IGF-I.

In another series of experiments, GST-RXR-α (1.5 μg) was immobilized on glutathione beads and pre-incubated in binding buffer without or with IGFBP-3 (7.5 μg) for 1 h at 22 °C with gentle rotation. His_6-RAR-α was expressed in *E. coli* and purified from cell lysates on a Ni–NTA agarose column (Qiagen, Valencia, CA). Equimolar amounts of His_6-RAR-α (8.7 μg) were added to each reaction and incubated as above for a further 30 min. This concentration of His_6-RAR-α was required for its detection by immunoblotting. Therefore, to compete with equimolar amounts of IGFBP-3, a relatively high level of IGFBP-3 was required. Bound His_6-RAR-α was analyzed on reducing SDS–PAGE and Western immunoblotting using a monoclonal anti-polyhistidine antibody (Sigma). Imaging was carried out using a Fuji-Film FLA3000 gel imaging system, with quantitation performed using the Image Gauge 3.11 software.

Cell culture. The human breast cancer cell lines MDA-MB-231 and Hs578T were maintained in RPMI medium supplemented with 10 μg/ml bovine insulin (Sigma) and 10% fetal calf serum.

Retinoid response following immuno-depletion of IGFBP-3. MDA-MB-231 and Hs578T human BCCs were grown to subconfluence in 6-well plates before the medium was changed to serum-free medium supplemented with 0.1% BSA. The following day one set of samples was counted to establish baseline cell numbers (day 0). The remaining cells were changed to fresh serum-free medium containing BSA, protease inhibitors with or without affinity-purified IGFBP-3 antiserum or non-immune serum (50 μg IgG/ml) and treated with the RAR-specific agonist, atRA (10^{-6} M) or vehicle. Viable cell numbers were determined at days 3 and 6. Samples counted at day 6 were retreated with anti-IGFBP-3 IgG or non-immune IgG and atRA or vehicle at day 3. Each experimental condition was performed in triplicate and repeated twice. Values are expressed as means ± SD.

Transcriptional reporter assays. Hs578T cells were seeded in 24-well plates and transiently co-transfected with 0.675 μg reporter plasmid (DR5-thymidine kinase (TK)-luciferase) kindly provided by Michael Hayman (State University of New York, USA) [19] and 0.075 μg of internal control reporter plasmid (pRL-TK) (Promega, Madison, WI). DR5-TK-luciferase expresses the firefly luciferase gene under the control of a RARE-DR5 (AGGTCAtgtctAGGTCA) element. The pRL-TK vector expresses the *Renilla* luciferase gene and was used to control for transfection efficiency. Hs578T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were treated with affinity-purified IGFBP-3 antiserum or non-immune serum (50 μg IgG/ml) in serum-free media for 24 h prior to stimulation with atRA (10^{-6} M) or vehicle. After a further 24 h, cell lysates were prepared and assayed for firefly and *Renilla* luciferase activity using the Dual-Glo Luciferase Assay System (Promega). Luminescence was quantified with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Each experimental condition was performed in triplicate and applied in two independent experiments. Values are expressed as means ± SD.

Ligand-binding assay. The ligand-binding activity of RAR:RXR heterodimers was assessed using a GST pull-down assay [20]. Preformed GST-RXR-α:GST-RAR-α or GST-RXR-α:His_6-RAR-α heterodimers were immobilized on glutathione beads. The bound heterodimers were incubated in binding buffer with or without IGFBP-3 (1 μg) in the presence of [^{3}H]atRA (1 nM) and increasing amounts of unlabeled atRA ranging from 0 to 100 nM. The reaction mix was incubated for 1 h at 22 C with gentle rotation and the samples were rapidly washed at 4 °C. The receptor/ligand complex was released and collected following centrifugation and bound [^{3}H]atRA was determined by scintillation counting.

Statistical analysis. Data were analyzed by an unpaired *t* test or by analysis of variance followed by Fisher's protected least significant difference test using Statview 4.02 (Abacus Concepts, Berkeley, CA).

Results

IGFBP-3 and IGFBP-5 interact with RXR-α and RAR-α

We investigated the ability of IGFBP-3 and IGFBP-5 to interact with the RXR-α and RAR-α using a GST pull-down assay (Fig. 1). Unfused GST, GST-RXR-α, and GST-RAR-α proteins were immobilized on beads and incubated with IGFBP-3 (lanes 2–4, respectively) or IGFBP-5 (lanes 6–8, respectively). In comparison to GST alone, both GST-RXR-α and GST-RAR-α bound IGFBP-3 and IGFBP-5 strongly.

IGFBP-3 modulates the atRA-sensitivity in BCCs

Numerous studies have shown that the ER−ve Hs578T and MDA-MB-231 BCCs are resistant to the growth-inhibitory effects of atRA [21,22]. As expected, exposure to atRA did not decrease Hs578T (Fig. 2A) or MDA-MB-231 (Fig. 2B) cell growth. In fact, in the case of MDA-MD-231 cells, cell growth was significantly increased by treatment with atRA ($p < 0.0005$ and $p < 0.02$, after 3 and 6 days of treatment, respectively). We have shown that both these cell types express IGFBP-3 constitutively and IGFBP-3 levels are unchanged following treatment with atRA. Furthermore, immuno-depletion of IGFBP-3 from Hs578T cell cultures significantly decreased nuclear IGFBP-3 content (0.43 ± 0.18 ng IGFBP-3/μg total protein) compared to cells treated with non-immune serum (1.6 ± 0.43 ng IGFBP-3/μg total protein) as determined by IGFBP-3 RIA. Because IGFBP-3 is internalized into the cytosol and nucleus from the extracellular environment (6, 7), these results suggest that immuno-depletion of extracellular IGFBP-3 has reduced the intracellular levels of IGFBP-3 by blocking its cellular uptake.

To determine whether the constitutive expression of IGFBP-3 contributed to the RA-resistant phenotype of Hs578T and MDA-MB-231 cells, we immuno-depleted IGFBP-3 from the cell culture medium and then investigated changes in cell growth following treatment with atRA. After exposure to atRA for 6 days, Hs578T (Fig. 2A) and MDA-MB-231 (Fig. 2B) cell numbers were reduced by 38% and 93%, respectively, in cells treated with IGFBP-3 antiserum compared to control cells treated with non-immune serum. Thus, depletion of IGFBP-3 rendered these RA-resistant BCCs more sensitive to the growth-inhibitory effects of atRA. Treatment of cells with IGFBP-3 antiserum alone had no effect on cell growth. There was no statistically significant difference in the atRA-responsive of these cells in the absence or presence of non-immune serum.

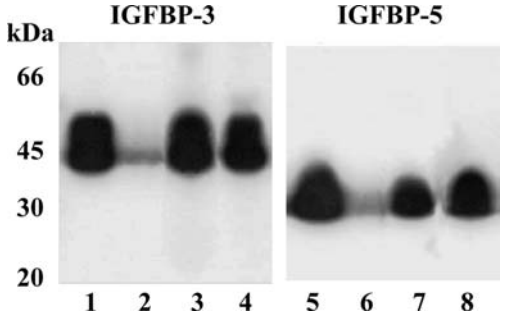

Fig. 1. Interaction between IGFBP-3 or IGFBP-5 and the retinoid receptors. IGFBP-3 or IGFBP-5 (1 μg) was incubated with GST (lanes 2 and 6, respectively), GST-RXR-α (lanes 3 and 7, respectively) or GST-RAR-α (lanes 4 and 8, respectively) immobilized on glutathione–Sepharose beads. The bound IGFBPs were detected by IGF-I ligand blotting and compared to 0.5 μg IGFBP-3 (lane 1) or IGFBP-5 (lane 5) standards. The positions of molecular mass markers are indicated.

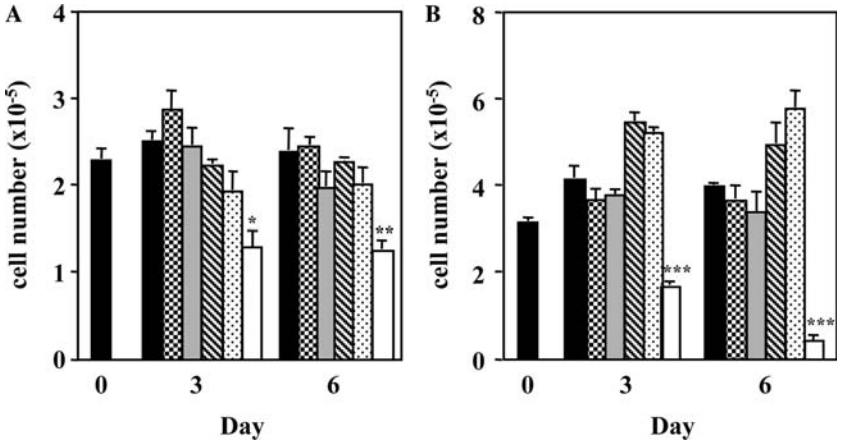

Fig. 2. IGFBP-3 modulates the atRA-sensitivity in BCCs. The atRA-resistant Hs578T (A) and MDA-MB-231 (B) human BCC lines were treated with vehicle (■), atRA alone (▨) or atRA plus affinity-purified IGFBP-3 antiserum (□) or atRA plus affinity-purified non-immune serum (▩). These atRA-resistant BCC lines were also treated with non-immune serum (▩) or IGFBP-3 antiserum (▩) alone as controls. Viable cell numbers were determined at days 0, 3, and 6. Each experimental condition was performed in triplicate and repeated twice. Values are expressed as means ± SD. *$p = 0.01$; **$p = 0.002$; and ***$p < 0.0001$ for atRA-treated cells: IGFBP-3 depleted cells compared to cells treated with non-immune serum.

IGFBP-3 prevents ligand-induced transactivation of the RARE in BCCs

The ability of IGFBP-3 to modulate ligand-induced transactivation of the RARE-DR5 element was investigated in BCCs. Hs578T cells transiently co-transfected with DR5-TK-luciferase (expressing the firefly luciferase gene) and pRL-TK vector (expressing the *Renilla* luciferase gene) were treated with IGFBP-3 antiserum or non-immune serum prior to stimulation with atRA for 24 h, and the cell lysates were assayed for luciferase activity. In control Hs578T cells treated with non-immune serum there was no significant increase in reporter gene expression following stimulation with atRA compared to vehicle treated cells ($p = 0.2$) (Fig. 3). However, depletion of IGFBP-3 from Hs578T cells caused a 7-fold increase in firefly luciferase gene expression in response to atRA.

IGFBP-3 decreases retinoid receptor availability

If IGFBP-3 sequestered RXR and/or RAR and blocked the formation of RAR:RXR heterodimers, then this could explain the ability of IGFBP-3 to modulate the atRA-response in BCCs. To determine this in vitro, immobilized GST-RXR-α was pre-incubated with or without IGFBP-3 and then His_6-RAR-α was added to each reaction. The amount of His_6-RAR-α that was able to bind RXR-α was determined by Western immunoblotting (Fig. 4A). In the absence of IGFBP-3, RAR-α was co-precipitated with RXR-α, demonstrating that a heterodimer was formed. However, in presence of IGFBP-3 the amount of RAR-α that bound to RXR-α was significantly reduced. Quantitation of data from three similar experiments showed that IGFBP-3 reduced the amount of RAR-α co-precipitating with RXR-α by 72% (Fig. 4B).

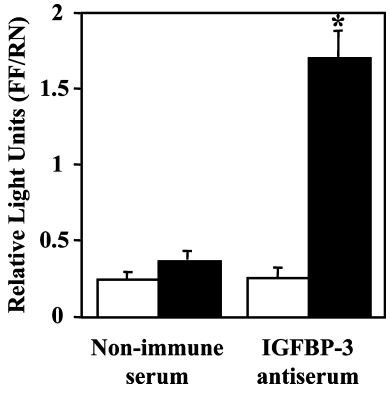

Fig. 3. IGFBP-3 prevents ligand-induced transactivation of the RARE in BCCs. Hs578T cells were transiently co-transfected with the reporter plasmid, DR5-TK-luciferase, and the internal control plasmid, pRL-TK. Cells were treated with IGFBP-3 antiserum or non-immune serum prior to stimulation with vehicle (□) or atRA (■). Cell lysates were prepared 24 h after treatment with atRA. The luciferase activity is shown as the relative light units obtained for firefly luciferase normalized for transfection efficiency using the relative light units obtained for *Renilla* luciferase. Each experimental condition was performed in triplicate and applied in two independent experiments. Values are expressed as means ± SD. *$p < 0.0001$ for atRA-treated cells: IGFBP-3 depleted cells compared to cells treated with non-immune serum.

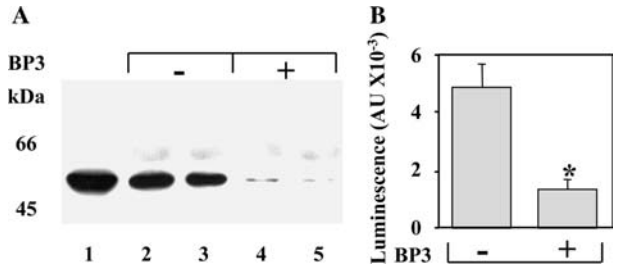

Fig. 4. Effect of IGFBP-3 on RAR:RXR heterodimerization. GST-RXR-α was immobilized on glutathione beads and pre-incubated without (lanes 2 and 3: duplicate samples) or with 7.5 μg IGFBP-3 (lanes 4 and 5: duplicate samples) (A). Equimolar amounts of His_6-RAR-α were then added to each reaction and bound His_6-RAR-α was analyzed by Western immunoblotting and compared to His_6-RAR-α run as standard (lane 1). The positions of molecular mass markers are indicated. Data from three experiments were quantified as described in Material and methods (B). Values are expressed as means ± SD. *$p < 0.02$.

Effect of IGFBP-3 on ligand binding to retinoid receptors

To investigate whether IGFBP-3 was able to modulate atRA binding to RAR-α, immobilized GST-RAR:GST-RXR heterodimers were incubated with or without IGFBP-3 in the presence of radiolabeled atRA. Specific binding of atRA was demonstrated by competing with increasing concentrations of unlabeled atRA. Scatchard analysis showed that IGFBP-3 did not alter the affinity of atRA for the RAR:RXR heterodimer ($K_d = 1.5$ nM, in good agreement with published values [20]), nor the number of atRA binding sites on RAR-α (Fig. 5A). To determine whether IGFBP-3 could dissociate RAR:RXR heterodimers and reduce the amount of ligand-activated receptor complex, we carried out a similar study using His_6-RAR:GST-RXR heterodimers immobilized on glutathione beads (Fig. 5B). Because atRA is a specific ligand for RAR-α, a reduction in the number of binding sites for atRA in the presence of IGFBP-3 would suggest that IGFBP-3 is capable of displacing His_6-tagged RAR-α from GST-RXR-α. We found that the presence of IGFBP-3 substantially decreased the binding of atRA to RAR:RXR heterodimers. Scatchard analysis of these data showed that the affinity of atRA for this form of heterodimer was not altered by the presence of IGFBP-3 ($K_d = 1.3$ nM), however, IGFBP-3 decreased the number of atRA binding sites by 66%, from 1.45 to 0.95 nM.

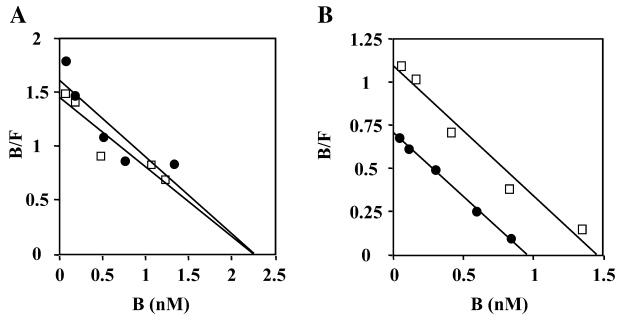

Fig. 5. IGFBP-3 reduces the amount of ligand-activated receptor complex. (A) Pre-formed GST-RAR:GST-RXR heterodimers were immobilized and incubated without (□) or with (●) IGFBP-3 in the presence of [^{3}H]atRA and increasing amounts of unlabeled atRA. The amount of bound [^{3}H]atRA was determined by scintillation counting and the results were subjected to Scatchard analysis. (B) Similar ligand binding assays were carried out using heterodimers formed between GST-RXR-α and His_6-tagged RAR-α. Scatchard analysis was used to determine the affinity of atRA for this form of heterodimer and the concentration of atRA binding sites on RAR-α. Graphs are representative of three independent experiments.

Discussion

This study is the first to demonstrate an interaction between IGFBP-3 and RAR-α and between IGFBP-5 and any retinoid receptor. We have also shown that the presence of IGFBP-3 can modulate the sensitivity of BCCs to the growth-inhibitory effects of atRA and block ligand-induced transactivation of the RARE, and that IGFBP-3 can limit the formation of active RAR:RXR heterodimers in vitro.

Previous work has shown that prolonged exposure of RA-sensitive ER+ve MCF7 and ZR-75-1 BCCs to atRA generated a population of cells that were fully RA-resistant and partially estrogen-insensitive [23]. Interestingly, the basal level of IGFBP-3, which was undetectable in the parental MCF7 cells, was elevated in the RA-resistant population. This was also true for the ZR-75-1 cells, although the rise in IGFBP-3 expression in the RA-resistant population was not as marked. This increase in basal IGFBP-3 expression is similar to that seen when breast cancers spontaneously progress from ER+ve/RA-sensitive to ER−ve/RA-resistant phenotypes in vivo [13–15]. We hypothesize that in parental MCF7 and ZR-75-1 cells, which do not express IGFBP-3 prior to stimulation with atRA, active RAR:RXR heterodimers were present to transactivate the initial atRA signal. However, when these cells switched to expressing IGFBP-3 constitutively, IGFBP-3 was present to sequester RXR and/or RAR, and attenuate the atRA signal. To determine whether the constitutive expression of IGFBP-3 observed in the more aggressive ER−ve BCCs was related to their atRA-resistant phenotype, we decreased the level of intracellular IGFBP-3 in the atRA-resistant Hs578T and MDA-MB-231 BCC lines. We hypothesized this would increase the bioavailability of active RAR:RXR heterodimers and enhance the atRA-responsiveness of these cells. As predicted, our results showed that depletion of IGFBP-3 from these cells increased their sensitivity to the growth-inhibitory effects of atRA.

The observation that IGFBP-3 can bind the retinoid receptors and modulate the atRA-response in BCCs suggests a role for intracellular IGFBP-3 in preventing the formation of RAR:RXR heterodimers. We have shown that IGFBP-3 can diminish the interaction between RXR-α and RAR-α and can dissociate preformed RAR:RXR heterodimers in vitro. The disruption of the ligand-inducible receptor complex by IGFBP-3 has the potential to reduce RARE-mediated transactivation of target genes. These findings are consistent with the observation that IGFBP-3 attenuates RARE-mediated signaling [16]. Although the region(s) in the retinoid receptors that interacts with IGFBP-3 are unknown, these findings suggest that IGFBP-3 blocks heterodimerization either by binding at or close to the dimerization surface (direct competition), or by an allosteric mechanism. The ability of IGFBP-3 to bind to RXR and compete with its natural heterodimerization partners suggests an important role for IGFBP-3 in regulating nuclear hormone activity. In this way, intracellular IGFBP-3 could attenuate the ligand-induced activity of RAR, and possibly also that of other nuclear receptors such as VDR, TR, and PPAR.

The results of this study suggest that when IGFBP-3 is located in the nucleus it prevents the formation of RAR:RXR heterodimers and blocks the cellular response to retinoic acid. We have shown that this effect can be reversed by depleting the intracellular pool of IGFBP-3 in atRA-resistant BCCs. Retinoids are effective chemopreventative agents against a number of cancers, including breast cancer [24]. However, clinical trials of retinoids in patients with advanced breast cancer have not been successful. If IGFBP-3 is responsible for blocking retinoid-signaling in resistant tumors, then targeted down-regulation of IGFBP-3 expression in combination with the administration of retinoid receptor agonists may prove effective against this subclass of breast tumors.

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