Retinoid X Receptor-specific Retinoids Inhibit the Ability of Retinoic Acid Receptor-specific Retinoids to Increase the Level of Insulin-like Growth Factor Binding Protein-3 in Human Ectocervical Epithelial Cells

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ABSTRACT

The hormones derived from vitamin A and related synthetic ligands (retinoids) are important regulators of differentiation and development and have been shown to be therapeutically useful in the treatment of cervical cancer. All-trans-retinoic acid exerts its effects by activation of retinoic acid receptor (RAR) and retinoid X receptor (RXR) heterodimers. These heterodimers bind to the retinoic acid response elements of target genes to regulate gene expression. RXR ligands act through RXR homodimers to regulate gene expression. In the present study, we describe the effects of RAR- and RXR-specific ligands on the regulation of insulin-like growth factor binding protein-3 (IGFBP-3) production and cell proliferation in human ectocervical epithelial (ECE) cell lines. Treatment of ECE16-1 cells with a RAR-specific ligand (TTNPB) or a ligand that interacts with both RAR and RXR receptors (9-cis-retinoic acid) increases IGFBP-3 levels and suppresses cell proliferation. In contrast, RXR-specific ligands (AGN191701, SR11217, and SR11237) do not regulate proliferation and slightly suppress the IGFBP-3 level. Cotreatment with increasing concentrations (0.01–1000 nM) of RXR-specific ligands antagonizes the growth suppressive and IGFBP-3-increasing effects of 1000 nM TTNPB. Similar results are observed in two other ECE cell lines, ECE16-D1 and ECE16-D2. These results indicate that RXR-specific ligands can antagonize RAR responses in these cell lines and suggest that a RAR-specific retinoid may be superior to one with mixed RAR/RXR binding activity for inhibiting cervical cancer cell proliferation. Moreover, the antagonism of RAR-dependent responses by RXR-specific ligands is consistent with a squelching model in which the RXR-specific ligand drives formation of RXR/RXR homodimers at the expense of the more active RAR/RXR heterodimers.

INTRODUCTION

Vitamin A and related ligands (retinoids) regulate cell function by interacting with members of a family of soluble nuclear receptor proteins (1, 2). This family includes six major proteins. The RARs,3 RARα, RARβ, and RARγ, selectively bind tRA (2–9), while the RXRs, RXRα, RXRβ, and RXRγ selectively bind 9-cRA (10, 11). The relative role of various members of this family in mediating retinoid effects is an area of intense interest, especially since “retinoids” may be natural cancer preventive agents and have been successfully used to treat cancer (12). Our laboratory is interested in the use of retinoids for the treatment of cervical cancer (13–21).

Our previous studies suggest that retinoids inhibit normal and HPV16-immortalized cell proliferation via several distinct mechanisms (18–20). An important mechanism is via modulation of the IGF signaling pathway (19, 21). IGFs are potent effectors of cell growth, differentiation, and metabolism (22). They are produced and secreted by mesenchymal cell types and produce autocrine and paracrine effects in target cells by interacting with a transmembrane type I receptor kinase (23). The concentration of free IGF in the extracellular environment is modulated by binding to the IGFBPs. The IGFBPs are a family of proteins, produced in a cell type-specific manner, that bind IGF-I and IGF-II with high affinity (24–26). Association with IGFBPs can either enhance or reduce IGF activity (27–33). IGFBP-3 is an important member of this family and is thought to reduce the effective IGF concentration by binding IGF and sequestering it away from the trans-membrane kinase receptor (31–33). IGFBP-3 may also act to directly regulate cell function by interacting with its own cell surface receptor (34).

Our previous studies show that IGFBP-3 is the major IGFBP produced by normal and HPV16-immortalized ECE cells (21). These studies also show that IGFBP-3 levels are increased by retinoids (19) and that this increase is correlated with a reduction in IGF-I-dependent proliferation (19, 21), suggesting that IGFBP-3 may play a role in mediating the growth-suppressive effects of retinoids.

In recent years, a great deal of new evidence has accumulated indicating that retinoids can be used in cancer treatment and prevention (12, 35–37). The promise of retinoids as cancer prevention and treatment agents has spurred efforts to design new retinoids that have an improved therapeutic index, i.e., high antitumor activity with minimal side effects (38). An important strategy in this effort is to design retinoids that interact only with a specific class of retinoid receptors (e.g., RAR-specific versus RXR-specific retinoids). In the present study, we compare the effects of a series of RAR-specific and RXR-specific retinoids on the regulation of IGFBP-3 expression in several HPV-immortalized human cervical epithelial cell lines. Our results indicate that RAR-specific ligands: (a) increase IGFBP-3 production; and (b) suppress cell proliferation. RXR-specific ligands do not effect proliferation and slightly suppress IGFBP-3 protein levels. However, when given simultaneously, the RXR-specific retinoids produce a concentration-dependent inhibition of the responses produced by RAR-specific ligands.

MATERIALS AND METHODS

Cell Culture and Growth Assays. HPV16-immortalized human ECE cell lines ECE16-1 (39), ECE16-D1, and ECE16-D2 (15, 20) were maintained in growth medium consisting of DMEM:F12 (3:1) supplemented with 5% FCS, 5 ng/ml insulin, 0.1 mM cholaerins, 5 μg/ml transferrin, 2 μM T3, 1 mg/ml EGF, 4 μM hydrocortisone, 0.18 mM adenine, 100 μM nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For routine maintenance, ECE16-D1 and ECE16-D2 cells were maintained on 3T3 feeder layers (20); however, feeders were removed prior to use of the cells.

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in experiments. For proliferation experiments, cells were seeded in 9.62-cm² dishes (10,000 cells/cm²) in growth medium and allowed to attach overnight. After attachment, the cells were transferred to DM consisting of DMEM:F12 (3:1) supplemented with 1 mg/ml BSA, 50 μg/ml transferrin, 1 mM TP, 0.18 mM adenine, and antibiotics. After 24 h, treatment was initiated by the addition of EGF and/or retinoid-supplemented DM; fresh treatment medium was added at 24-h intervals for 3 days. DM-EGF is DM containing 20 ng/ml EGF. The cells were harvested and fixed for Coulter counting as described previously (21). The conditioned medium from the last 24-h period was collected, centrifuged to remove cell debris, and stored at −20°C for subsequent assay of IGFBPs.

**RESULTS**

**Retinoid Regulation of ECE16-1 Cell Proliferation.** ECE16-1 cells grown in DM remain in a quiescent state, and treatment with EGF results in significant growth stimulation (Ref. 21; Fig. 2). Ligands that interact with RARs (TTNPB and AGN190168) or RARs and RXRs (tRA, 13-cRA, and 9-cRA) inhibit the EGF-stimulated proliferation. Although AGN190168 does not interact with RARs or RXRs, its acid metabolite AGN190299 binds strongly to RAR receptor subtypes (Fig. 1), suggesting that the acid metabolite is the active form in these cell lines. In contrast, RXR-specific ligands (AGN191701, SR11237, and SR11217) do not inhibit proliferation.

**Retinoid Regulation of ECE16-1 Cell IGFBP-3 Production.** The quantity and type of IGFBPs present in medium conditioned by cells treated with retinoids is shown in Fig. 3. As reported previously (21), IGFBP-3 is the predominant IGFBP produced by ECE16-1 cells, and the presence of EGF decreases IGFBP-3 production compared to treatment with TTNPB (100%) alone. In the converse experiment, treatment with varying concentrations of RXR-specific ligands again antagonize the effects of TTNPB (Fig. 4D).

**RXR-specific Retinoids Antagonize the Effects of RAR-specific Retinoids.** The results described above suggest that RAR-specific retinoids are active and RXR-specific retinoids are inactive in ECE16-1 cells. However, previous studies suggest that RXR-specific retinoids can modulate the activity of RAR-specific ligands (41, 43). To investigate this possibility, we simultaneously treated cells with RAR- and RXR-specific ligands. Dose-response curves show that maximal growth inhibition is attained with TTNPB concentrations of ≥10 nm (Fig. 4A), whereas SR11217 and AGN191701 did not significantly affect cell proliferation (Fig. 4B).

Treatment of cells with 1 μM AGN191701 or SR11217 partially reversed the TTNPB-dependent suppression of proliferation (Fig. 4C). In the presence of AGN191701 or SR11217, TTNPB was only able to maximally inhibit proliferation by 56 and 70%, respectively, compared to treatment with TTNPB (100%) alone. In the converse experiment, treatment with varying concentrations of RXR-specific ligand in the presence of a fixed concentration of 1 μM TTNPB, RXR ligands again antagonize the effects of TTNPB (Fig. 4D).

**RXR- and RAR-specific Retinoid Regulation of IGFBP Levels.** Dose-response studies show that the EGF-dependent reduction in IGFBP-3 is returned to control (DM) levels in the presence of 1.0 nm
Retinoid Regulation of Proliferation and IGFBP-3 Production in ECE16-D1 and ECE16-D2 Cells. To assess whether the response of ECE16-1 cells to RAR- and RXR-specific retinoids is typical, we examined the effects of these agents in two other HPV16-immortalized cell lines, ECE16-D1 and ECE16-D2 (20). As shown in Fig. 7, the proliferation response of ECE16-D1 and ECE16-D2 cells to treatment with TTNPB, AGN191701, or TTNPB + AGN191701 is very similar to that observed in ECE16-1 cells (i.e., TTNPB reduces cell number, AGN191701 is relatively inactive, and AGN191701 partially antagonizes the TTNPB-dependent suppression of proliferation).

Discussion

IGF and the Development of Cancer. Recent evidence suggests that the IGF-I receptor signaling pathway is required both for cell transformation and tumorigenesis (44). Therefore, treatments that interfere with this signaling pathway may be useful in the treatment of cancer. Our previous studies indicate that retinoids increase the level of IGFBP-3 produced and released by human ECE cells (19). We hypothesize that the increased IGFBP-3 binds IGF-I to reduce the concentration of free IGF-I available to stimulate cell responses (19, 21). Indeed, our studies show that an increased IGFBP-3 level is
IGFBP-3 can serve as a marker of retinoid action in cervical epithelial cells (19).

RAR-specific versus RXR-specific Retinoids. Retinoids have been successfully used in the treatment of skin diseases (45-47) and cancer (12, 37) and also appear to have potential as chemopreventive agents (35, 36). They have been shown to have efficacy in the treatment of head and neck cancer (48), skin cancer (35, 36, 48), and cervical cancer (12, 37). Retinoids interact with a family of receptors that includes six forms, RARα, RARβ, RARγ, RXRa, RXRβ, and RXRγ (49). These receptors, although similar in structure, are expressed in a tissue-specific manner, suggesting a specific role for each.

Correlated with a reduced response of ECE16-1 cells to IGF-I (21). In addition to the potential therapeutic importance of modulating IGFBP-3 levels, these studies indicate that IGFBP-3 protein and mRNA levels are extremely sensitive to the presence of retinoids and that...
family member (49). The RAR and RXR families are distinguished by the fact that they bind to different retinoid ligands and their effects are mediated via different DNA response elements. Thus, RARs and RXRs preferentially bind to 9-cRA and 9-cRXR, respectively (10, 11, 50). When interacting with retinoic acid response elements in DNA, RARs bind to DR2 and DR5 elements as RAR/RXR heterodimers, whereas RXRs bind to DR1 elements as RXR homodimers (51, 52). DR, or "direct repeat" elements, consist of the two direct repeats of the sequence AGGTCA separated by 1 to 5 bases (51, 52). RXRs are further distinguished from RARs by the observation that RXRs can additionally form heterodimers with thyroid hormone receptor, vitamin D receptor, and peroxisome proliferator activator receptor (49). Additional studies suggest that the predominant in vivo form of the retinoid receptor is the RAR/RXR heterodimer (49), but that RXR/RXR homodimers can be formed in the presence of high levels of an RXR-specific ligand (53).

**Regulation of Cell Function by Retinoids.** The results outlined above predict that RAR- and RXR-specific ligands should regulate different subsets of genes in cells, perhaps specifying completely different cell behavior. Most recent studies show that whereas RAR-specific ligands are highly active regulators of cell function (38, 41, 46, 47), RXR-specific ligands are relatively less active (41, 45, 54). However, one study suggests that cotreatment with RAR- and RXR-specific ligands can enhance biological response in ME180 cells (43).

**Regulation of ECE Cell Proliferation and IGFBP-3 Production by RAR- and RXR-Specific Ligands.** Our previous studies show that RAR-specific ligands suppress ECE16-1 cell proliferation and increase RARβ and cytokeratin K5 gene expression (39, 41). In contrast, RXR-specific ligands are less active regulators of gene expression and do not regulate cell proliferation (41). This is not because of a lack of receptors for these ligands, because ECE16-1 cells express mRNA encoding both RARs and RXRs (41). Previous studies also indicate that ECE16-1 cells express IGFBP-3 (21) and that IGFBP-3 levels are regulated by retinoids (19, 21). In the present study, we examine the effects of receptor-specific ligands on two other HPV16-immortalized cell lines, ECE16-D1 and ECE16-D2. Although the magnitude of the responses varied, the general trends were the same as observed in ECE16-1. The RAR-specific ligands inhibited proliferation and increased IGFBP-3 production, and these responses were antagonized by RXR-specific ligands. This suggests that this response is likely to be a common property of HPV-immortalized human cervical cell lines. These results suggest the possibility that a RAR-specific retinoid that cannot be metabolically converted to a RXR-binding form may be the optimal ligand for treatment of cervical disease.

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**REFERENCES**


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