Early in vitro passages of breast cancer cells are differentially susceptible to retinoids and differentially express RARβ isoforms

XINJIAN PENG1,2, ALBERT GREEN1, ANNE SHILKAITIS1, YONGHUA ZHU1, LAURA BRATESCU1 and KONSTANTIN CHRISTOV1

1Department of Surgery, University of Illinois at Chicago, Chicago, IL 60612; 2IIT Research Institute, Chicago, IL 60616, USA

Received February 25, 2011; Accepted April 18, 2011

DOI: 10.3892/ijo.2011.1070

Abstract. The effect of retinoids on breast cancer has been predominantly studied in vitro, on established cell lines, which in biology differ significantly from primary tumor cells. Little is known on whether early in vitro passages of breast cancer cells (EPBCCs) are differentially sensitive to retinoids and differentially express retinoic acid receptors (RARs) and retinoid X receptors (RXRs). We have previously identified a novel RARβ isoform (RARβ5) and hypothesized that it may serve as a potential target of retinoids in EPBCCs. Breast cancer cells isolated from primary tumors were cultured in vitro for 6-12 passages (EPBCCs) and their epithelial origin was confirmed by a cocktail of antibodies against cytokeratins. EPBCCs were treated for 4 days with 1.0 µM of all-trans retinoic acid (atRA), 9-cis retinoic acid (9cRA) or 4-hydroxy-phenylretinamide (4-HPR) and their viability determined by MTT assay. Among nine EPBCCs consistently grown in vitro, three were resistant to the above retinoids, five were susceptible to atRA, four to 4-HPR and two to 9cRA, suggesting that patients with breast carcinomas may differentially respond to various retinoids. All EPBCCs differentially expressed RARα, RARγ, RXRa, RXRβ proteins and RARβ5 and RARβ2 mRNAs. However, only one EPBCC (BCA-2) expressed RARβ5 at mRNA and protein level and it was resistant to retinoids, both in vitro and in a xenograft tumor assay. RARβ5 suppression by siRNA in BCA-2 cells increased their susceptibility to atRA. No correlation was found between sensitivity of EPBCCs to the above retinoids and RARβ5 and RARβ2 mRNA expression. atRA reduced RARβ expression in most EPBCCs suggesting that this retinoid receptor is most probably the prime target of retinoids in breast cancer. These data may have clinical implication in selecting patients with breast cancer that would benefit the most from clinical trials with retinoids.

Introduction

Most studies on breast cancer response to retinoids have been performed in vitro on established ERα and ERβ cell lines, which significantly differ in biology from primary tumor cells (1,2). Previously it was reported that ERα breast cancer cell lines are more sensitive to retinoids than ERβ cell lines (3,4) and that retinoids may affect tumor cells by receptor-dependent and -independent mechanisms (5,6). Retinoids are ligands of retinoic acid receptors (RARs α, β, γ) (7,8), whereas rexinoids preferentially affect retinoid X receptors (RXRs α, β, γ). Both RARs and RXRs are expressed in normal mammary epithelial cells (MECs), whereas RARβ and particularly its RARβ2 isoform is lacking in most breast carcinomas, suggesting its potential tumor suppressor role (9,11). The lack of RARβ2 in breast cancer has been related to hypermethylation of the P2 promoter and its dimethylation has been associated with increased cell sensitivity to retinoids (12,13). Transduction of RARβ2 to MCF-7 cells lacking the receptor has been associated with decreased proliferation activity and increased sensitivity to retinoids (14). A truncated RARβ isoform with a molecular mass of 40.6 kDa has also been identified in some breast cancer cell lines, and its transduction to MCF-7 cells has been associated with increased cell growth and resistance to retinoids (15,16).

We have recently identified a novel RARβ isoform (β5), which has its own promoter, P3, different from the previously known P1 and P2 promoters. RARβ5 was detected at the mRNA level in mammary premalignant MCF10AT cells and in some ERα breast cancer cell lines, all resistant to retinoids (17). The role of RARβ5 on malignant properties of breast tumor cells and on their sensitivity to retinoids is currently under investigation in our laboratory. In addition to RARβ5, short 5'-UTR RARβ2 transcripts were also detected in breast cancer cell lines and these transcripts may also serve as potential targets of retinoids (18). In this study, we characterized...
the susceptibility of EPBCC isolated from primary tumors to atRA, 9cRA, and 4-HPR and found 3 of 9 (33%) resistant to all three retinoids, whereas the other 6 more sensitive to atRA and 4-HPR than to 9cRA. All EPBCC differentially expressed RARβ5 and RARβ2 mRNA, as well as RARs α, γ and RXRs α, β with most significant decrease in RARα, which appears the principal target of retinoids in breast cancer.

Material and methods

*In vitro growth and establishment of EPBCC.* Tumor cells isolated from primary and metastatic breast carcinomas have been continuously cultured *in vitro* for 6-12 passages. UIC IRB approval was obtained prior to conducting this study, and informed consents were obtained from patients undergoing surgery for breast cancer prior to culturing of tumor cells *in vitro*. When cells from minced tissue formed a single layer in culture flasks, cells were passaged and cultured in MEM supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin and 10% FBS, 200 µM L-glutamine and 100 µM MEM non-essential amino acid. The epithelial origin of BCC was confirmed by immunocytochemistry (ICH) with a cocktail of antibodies against cytokeratins: 5, 8, 14 and 18 (data not shown). When the cells grown in culture reached 30-50% confluence, they were treated with retinoids or DMSO (solvent control). T47D cells, which serve as controls, were obtained from the American Cell Type Collection, Manassas, VA. The conditions for cell culturing are described in our recent publication (19).

*Retinoids.* atRA and 9cRA were purchased from Sigma, Inc., St. Louis, MO. 4-HPR was obtained from the repository of the National Cancer Institute (Bethesda, MD). Retinoids were dissolved in DMSO and added to the cell culture medium at 1.0 µM every other day for 4 days.

**MTT assay.** Effect of retinoids on cell growth and viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide) assay. Cells (1000 cells/well) were seeded in 96-well plates and cultured overnight. Then the cells were incubated with 1 µM of different retinoids. After a 4-day treatment, 10 µl MTT (5 mg/ml) was added to each well, mixed gently and incubated with the cells at 37°C for 1-3 h (19).

**RT-PCR.** Total RNA extraction and RT reaction were performed as described previously (17-19). Two RT reactions for each sample were pooled and diluted with an equal amount of DNase/RNase free water. For RT, a final volume of 20 µl with 2 µg total RNA and 100 units of MuLV reverse transcriptase (Invitrogen) was used. PCR was performed with 1 µl RT product using PCR Supermix (Invitrogen). PCR primer pairs are RARβ2 (475FP, GACGTGATGGATGTTCTGTCAG; 730RP, ATTTGTCTCGGCAAGCGAAGCA), RARβ5 (14FP, CTGGAAAGGTCTGACAGTGA; 343RP, GGACATTTCCCATTTCAAAGC), β-actin (FP, GTCAACAAGTGCAACA; RP, TGCCATCTCTTGTGCAAGA) was used as an internal control. Real-time PCR was performed with 1 µl RT product using 7900HT Sequence Detection System (ABI, Applied Biosystem) and ABI 2X SYBR Green PCR Master Mix (ABI# 4309155) according to ABI's recommended guidelines. Primer pairs for real-time PCR were RARβ2 (584FP, GATTTGACCAAACTGATGGCA GCA; 730RP) and RARβ5 (15FP, GGAAGTCTGACAC AGTGAATTCTCTGAG; RARβ2-730RP); real-time PCR data were analyzed using a software package (ABI Prism SDS2.1) provided with the instrumentation system.

*Inhibition of RARβ5 expression by siRNA.* Two siRNAs were designed to target the sequences 5'-AAA ATT CTG GAA GGT CGT ACA-3' and 5'-AAT TCT GGA AGG TCG TAC ACA-3', both of which are present in exon-6, a region unique to RARβ5. To suppress RARβ5 expression by siRNA, Ambion Inc. (Houston, TX) made the constructs and we transfected siRNA to BCA-2 cells. The efficacy of transfection was very high (>90%). We first determined the concentration of siPORT NeoFX transfection agent that was not toxic by using an MTT assay. Then we employed 3 concentrations of siPORT NeoFX, 0.3, 0.6 and 1.0 µl, and siRNA at 0.1, 0.5 and 1.0 nM. siRNA transfected (0.1 and 1.0 nM), vector-transfected, and control cells were treated with atRA, 1.0 µM for 2 days, and the effect of siRNA on RARβ5 expression was evaluated by Western blot. Another set of siRNA transfected, dsRNA transfected and control cells were treated with atRA, 1.0 µM for 3 days and their viability estimated by MTT assay (20).

*Western blot and immunocytochemistry (ICH).* Western blotting was used to detect RARβ5, RARβ2, RARα, RARγ, RXRα and RXRβ expression (RXRγ was not examined). Two antibodies were used to detect RARβ isoforms, one recognizing amino acids 430-447 in the C-terminus of RARβ2 (sc-552, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the other one recognizing amino acids 407-423 in the C-terminus.
of RARβ2 (Geneka Inc., Montreal, Quebec). Antibodies for RARα, RARγ, RXRα, and RXRβ were also purchased from Santa Cruz Biotechnology. For ICH, paraffin sections from tumor xenografts of BCA-2 cells that express RARβ5 protein and BCA-1 cells that do not express RARβ5 were deparaffinized and treated with 0.01 M citric buffer in a pressure cooker for 10 min. Next, the slides were blocked with mouse serum to eliminate non-specific staining and incubated with antibody for 2 h at room temperature. ABC elite kit and 3,3’-diaminobenzidine-tetrahydrochloride (DAB) were used to identify cells that expressed RARβ5. Parallel sections were not treated with antibody and served as negative controls.

**Nude mouse xenograft tumor assay.** For this study, BCA-1 cells (sensitive in vitro to atRA) and BCA-2 cells (resistant to atRA) were mixed with Matrigel (10^6 cells in 0.1 ml Matrigel) and injected in the mammary fat pad of 4-5 week old nude mice. The occurrence of tumor nodules was monitored weekly. Animals with palpable tumors (2-3 mm) were treated by gavage with atRA, 10.0 mg/kg body wt for 4 weeks. Control animals received a placebo (sesame oil). After sacrifice of the animals by CO2 asphyxiation, tumor nodules were removed, fixed in buffered, pH 7.4 formalin and embedded in paraffin. Samples from tumors were also frozen in liquid nitrogen for RNA and protein analysis. Mice were cared for in accordance with the procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

**Statistical analysis.** Statistical analysis of cell growth determined by MTT assay and the volume of control and atRA-treated tumor xenografts are given in the last paragraph of the Material and methods section. Three independent tests for cell viability (MTT assay) were performed and the mean ± standard deviation (SD) are presented. The difference with control (placebo treated) cells or treated tumors are calculated by two-sided Student’s t-test. Differences in p-values of <0.05 are considered significant.

**Results**

**EPBCC differ in their susceptibility to retinoids.** We examined 9 EPBCC (passage 6-12) out of 29 cell lines developed in our laboratory, to determine their susceptibility to retinoids. These EPBCC grew consistently in vitro and when transplanted in nude mice developed tumor xenografts, thus confirming their malignant properties. In a preliminary study, we found that it takes 3-4 days for retinoids at pharmacological doses to suppress the growth of breast normal and cancer cell lines (20). Tumor cells were treated for 4 days with 1.0 µM of atRA, 9cRA or 4-HPR, and MTT data for cell viability were compared with control (placebo-DMSO) cells (Fig. 1). Of nine EPBCC, five were susceptible to atRA (BCA-1, BCA-3, BCA-7, BCA-9 and BCA-11), four to 4-HPR (BCA-1, BCA-3, BCA-7 and BCA-10), and two to 9cRA (BCA-1 and BCA-3), indicating that EPBCC are differentially sensitive to retinoids. Interestingly, BCA-2, BCA-4 and BCA-8 were resistant to all three retinoids, suggesting that breast carcinomas could be divided into two groups, susceptible and resistant to retinoids.

**RARβ5 and RARβ2 isoforms are differentially expressed in EPBCC and differentially respond to retinoids.** Both RARβ isoforms were examined at mRNA and protein levels. As shown (Fig. 2A) RARβ5 and RARβ2 mRNA are differentially expressed in all EPBCC and differentially respond to atRA. For instance, the high level of RARβ5 mRNA expression in BCA-2 and BCA-8 cells correlated with their resistance to all three retinoids, whereas the low level in BCA-1, BCA-3, BCA-9, BCA-10, and BCA-11 cells correlated with their sensitivity to atRA. However, RARβ5 mRNA was barely detected in BCA-4 cells, but they were resistant to retinoids. To assess the effect of atRA on RARβ5 and RARβ2 expression, cells were treated with 1.0 µM atRA for 1, 2, or 4 days and mRNA and proteins levels examined (Fig. 2A). It is evident that atRA preferentially affected RARβ5 mRNA expression. However, when the data for alterations in both RARβ isoforms were compared with cell sensitivity to retinoids (Fig. 1), no correlation was found. At the protein level, EPBCC did not express RARβ2 (Fig. 2B), only one (BCA-2) expressed RARβ5 isoform, and the cells were resistant to retinoids both...
PENG et al.: SENSITIVITY OF BREAST CANCER CELLS TO RETINOIDS

in vitro and in a xenograft tumor assay (Fig. 3A-C). ICH detected RARβ5 protein only in BCA-2 cells, and it was mostly localized in the nucleus (Fig. 3C). Interestingly, in BCA-2 cells, RARβ2, RARα and RARγ proteins were also not detectable and their expression was not affected by atRA, suggesting that in a subset of breast carcinoma a disruption of all RARs may occur. The remaining EPBCC differentially expressed RARα, RARγ, RXRα and RXRβ protein (RXRγ was not examined) and differentially responded to atRA treatment (Fig. 2B). The most consistent decrease was found in RARα protein, which appears the principal target of retinoids in breast cancer cells.

Knock down of RARβ5 by siRNA increased cell sensitivity to atRA. To further characterize the potential role of RARβ5 expression on the sensitivity of breast cancer cells to retinoids, we knocked down RARβ5 expression by siRNA in BCA-2 cells (Fig. 4A). Ambion Inc. was provided with the RARβ5 sequences of sense- and anti-sense strand siRNA. They made the constructs and we transfected the corresponding siRNA to BCA-2 cells. Vector-transfected (VT), dsRNA, and siRNA (0.1 and 1.0 nM) transfected cells were treated with 1.0 µM atRA for 2 days and the effects of siRNA on RARβ5 expression was evaluated by Western blotting. Fig. 4A shows that siRNA significantly suppressed RARβ5 expression. However, the cells transfected with an empty vector also demonstrated limited sensitivity to atRA, suggesting that the empty vector may affect cellular targets of atRA not necessarily associated with RARβ isoforms. In a second set of experiments, RARβ5 and vector transfected cells were treated with atRA, 1.0 µM for 3 days, in 96-well plates and their viability estimated by MTT assay (Fig. 4B). The increase in atRA dose from 1.0 to 5.0 and 10 µM progressively suppressed cell growth, indicating that inhibition of RARβ5 expression by siRNA increases tumor cell sensitivity to retinoids.

RARβ5 expression in BCA-2 cells correlates with their in vivo resistance to atRA. To determine whether the resistance of BCA-2 cells to retinoids in vitro may persist in vivo, 10⁶ cells were mixed in Matrigel and transplanted in mammary fat pads of nude mice (6 mice per group). BCA-1 cells were used in a parallel experiment as a cell line sensitive to retinoids. When palpable tumors occurred (3-4 mm) the animals were randomized in control and atRA-treated groups. atRA was given by gavage at 40.0 mg/kg body weight for 4 weeks, 6 days a week. In a preliminary study, we found that atRA at
the above dose was not toxic and did not affect the body weight of the animals. As shown in Fig. 3B, atRA reduced the growth of sensitive BCA-1, but not that of resistant BCA-2 xenografts that expressed RARβ5.

Discussion

The main objective of this study was to assess the feasibility of EPBCC as a model system in testing the antitumor efficacy of retinoids and to determine the role of RARβ5 and RARβ2 isoforms as potential targets of retinoids. EPBCC appear closer in biology, ER status and genetic background to primary tumor cells than to established breast cancer cell lines (21). However, it is not easy to establish EPBCC consistently growing in vitro. It takes at least 3-5 passages of primary breast cancer cells to clear up the non-epithelial cell populations from the culture.

We confirmed the epithelial origin of EPBCC by treating cells with a cocktail of antibodies against cytokeratins (data not shown). atRA, 9cRA, and 4-HPR were employed because they differentially affect RARs and RXRs and in previous studies have shown efficacy in breast cancer preclinical and clinical studies (7,22,23). atRA is a ligand of RARs α, β, γ, 9cRA activates both, RARs and RXRs, and 4-HPR is a weak ligand of RARγ or does not need retinoid receptors to exert its antitumor effect (24).

We found that EPBCC differed in their sensitivity to the above retinoids; most (5 of 9) were sensitive to atRA and 4-HPR (4 of 9) but only two to 9cRA, suggesting that breast carcinomas are differentially susceptible to various retinoids. Surprisingly, 33% (3 of 9) of EPBCC were resistant to all three retinoids, suggesting that patients with these tumors may not benefit from clinical trials with retinoids. Several clinical studies with retinoids support our data. For instance, in a phase II breast cancer clinical trial with atRA, response has been observed in individual patients only (22). In a breast cancer prevention trial with 4-HPR that continued for more than 8 years, a 30% reduction in cancer development has been found in pre-menopausal women, data further supporting our results (23). Preclinical and clinical studies have shown that atRA and 9cRA are relatively toxic; therefore their clinical implication is limited.

Recently, rexinoids (LGD1069, LG10068, UAB30), which are ligands of RXRs, have shown low toxicity and promising efficacy in inhibiting mammary carcinogenesis in animal models (8,25,26). Some of the above rexinoids are currently employed in clinical trials for the treatment of breast and other types of cancer (27). Most studies on breast cancer cell lines, including ours, have shown that retinoids can suppress cell and tumor growth by inhibiting cell proliferation, inducing differentiation, cellular senescence and/or apoptosis (28,29). In mediating these cellular events, retinoids can modulate the expression of RARs and RXRs or employ receptor-independent mechanisms (30). As shown in Fig. 2, all EPBCC differentially expressed RARs, α, γ and RXRs, α, β, but not RARβ2 protein, suggesting previous studies indicating that RARβ2 is lacking in most breast cancers. Here, we provide data that in addition of RARβ2, RARβ5 mRNA was identified in all BCC. RARβ5 differs from RARβ2 and RARγ4 in exon organization (exon 5a) and in initiating point of translation (Fig. 5). Most importantly, RARβ5 has a distinct promoter, P3, different from the previously known P1 and P2 promoters of the RARβ1, RARβ2 and RARγ4 isoforms (31,32). Recently it was shown that RARβ2 expression in stroma cells may promote not suppress mammary carcinogenesis bringing a new level of complexity in assessing the efficacy of retinoids to suppress mammary carcinogenesis (33).

Since, as we have previously shown, RARβ5 is predominantly expressed in ER-negative breast cancer cell lines most of which are resistant to retinoids (18,19), we hypothesized that this isoform may predict the resistance/sensitivity of EPBCC to retinoids. The data generated in this study do not support this hypothesis. The lack of correlation between RARβ5 mRNA expression and the sensitivity of EPBCC to atRA suggests that other members of RARs and/or RXRs might be responsible for cell sensitivity to retinoids. RARγ2 should be also excluded, because its mRNA values do not correlate with cell sensitivity to retinoids. The data from Western blotting suggests that primary target of atRA in EPBCC is most probably RARα, the expression of which is suppressed by atRA in all but one EPBCC. Previous studies on MCF-cells support our data that RARα is the primer target of atRA (6). The decrease of RARα expression may affect RARβ2 transcription and further sensitivity of tumor cells to retinoids (7,34,35). Interestingly, BCA-2 cells constitutively expressed RARβ5, but not RARα, RARβ2, or RARγ1 proteins, suggesting significant post-translational defects in RAR signaling that correlated with cell resistance to retinoids both in vitro and in vivo. To further prove the potential role of RARβ5 in the resistance of BCA-2 cells to retinoids, we knocked down RARβ5 by siRNA; this was associated with increased cell susceptibility to atRA, suggesting that at least in
a subset of breast carcinomas, RARβ5 may serve as a potential biomarker of cell resistance to atRA and possibly to other retinoids. The response of breast cancer cells to retinoids is a complex phenomenon involving not only receptor-dependent but also receptor-independent mechanisms. In addition, there are co-activators and co-repressors of the RAR isoforms that might also affect the sensitivity of cells to retinoids (7,11). For instance, 4-HPR, which is known not to affect retinoid receptors or may weakly activate RARα (24), suppressed the growth of 4 of 9 EPBCC.

In conclusion, in this study we found that ~30% of EPBCC are resistant in vitro to retinoids, whereas the remaining are differentially sensitive to atRA, 9cRA, and 4-HPR. The response of EPBCC to retinoids is a complex phenomenon and do not depend on the expression levels of RARβ5 and RARβ2 receptors only. The data may help in selecting patients that benefit the most from clinical trials with retinoids.

Acknowledgements

This work was supported by Suzan G. Komen KG100509 grant (K.C.) and Illinois Department of Public Health Penny Stevens Breast and Cervical Cancer Research Fund (X.P.). We thank Dr Rajeshwari Mehta for her support and encouragement.

References


