Bexarotene Induces Cellular Senescence in MMTV-Neu Mouse Model of Mammary Carcinogenesis

Anne Shilkaitis, Laura Bratescu, Albert Green, Tohru Yamada, and Konstantin Christov

Abstract

Previous studies have shown that retinoids and rexinoids can prevent breast cancer in animal models and in women with increased risk of developing the disease. The cellular effects of these vitamin A analogues have been primarily associated with induction of differentiation and inhibition of proliferation. In this study, we tested the hypothesis that bexarotene (LGD1069, Targretin), a rexinoid, can not only inhibit cell proliferation but also induce cellular senescence in mammary epithelial cells, premalignant lesions, and tumors of the MMTV-Neu model of mammary carcinogenesis, which develops estrogen receptor–negative tumors. Mice with palpable mammary tumors were treated for 4 weeks with bexarotene at 80 or 40 mg/kg body weight, and senescent cells were determined by SA-β-Gal assay. Bexarotene decreased in a dose-dependent manner the multiplicity of premalignant lesions and tumors, and this was associated with inhibition of cell proliferation and induction of cellular senescence and apoptosis. By double labeling of senescent cells, first by SA-β-Gal and then by antibodies against genes related to cellular senescence, we found that p21, p16, and RARβ, but not p53, were upregulated by bexarotene in mammary tumors and in breast cancer cell lines, suggesting involvement of multiple signaling pathways in mediating the senescence program of rexinoids. These findings indicate that, in addition to cell proliferation and apoptosis, cellular senescence could be used as a potential biomarker of response in breast cancer prevention and therapy studies with rexinoids and possibly with other antitumor agents. Cancer Prev Res; 1–10. ©2013 AACR.

Introduction

Over the last 20 years, significant progress has been made in preventing and treating estrogen receptor–positive (ER+) breast cancer by tamoxifen and aromatase inhibitors (1, 2). Little has been achieved for estrogen receptor–negative (ER-) breast cancer, which comprises about 30% of all breast carcinomas, most of them with unfavorable prognosis (3). For ER+ breast cancer, retinoids and rexinoids have been considered potential alternatives because they suppress the growth of both ER+ and ER- breast cancer cell lines, relevant animal models of mammary carcinogenesis, as well as breast cancer development in women (4–7). Retinoids appear less toxic than retinoids; therefore, they have advantage in clinical studies (8, 9). Retinoids are ligands of retinoid X receptors α, β, γ (RXRs α, β, γ) and exert their biologic effects by receptor-dependent and -independent mechanisms (10–12). The cellular effects of retinoids and rexinoids are primarily associated with inhibition of proliferation and induction of differentiation (13–16). To the best of our knowledge, no data are available about whether rexinoids, particularly bexarotene, can induce cellular senescence (CS) in ER+ models of mammary carcinogenesis. Most previous and recent studies on cellular senescence have been conducted in vitro on mouse embryo fibroblasts and on various normal and tumor cell lines treated with cytotoxic agents or irradiation that induce DNA damage and gene instability, both leading to p53 and/or p21 upregulation (17–19). RAS, MYC, and BRAF oncogenes, when activated, can also induce cellular senescence, which appears also associated with DNA alterations (20, 21). Previously, we found that all-trans retinoic acid (atRA) is an efficacious inducer of cellular senescence in ER+ MCF-7 breast cancer cells, and we developed an in vitro assay to discriminate quiescent cells from senescent cells (SC; ref. 22). By using the SA-β-Gal assay, we showed in animal carcinogenesis models that 2 other retinoids, 9-cis-retinoic acid (9cRA) and 4-hydroxyphenilretinamide (4-HPR, Fenretinide), can also induce cellular senescence in mammary premalignant lesions and tumors (23). Recently, we found that 9cRA suppressed prostate carcinogenesis in p27-deficient mice, and this was associated with inhibition of cell proliferation and induction of cellular senescence (24). Gene analysis studies on MCF-7 cells treated with atRA (25) and on normal human breast epithelial cells treated with bexarotene (26) revealed up- and downregulation of hundred of genes, most of them associated with cell-cycle

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regulation, intercellular matrix proteins, cell differentiation, retinoid metabolism, various transcription factors, and secretion of cytokines that may affect surrounding and distant cells and tissues. However, the affected genes in both studies, except for RARβ transcription, were not directly associated with cellular senescence (26).

Here, we used the MMTV-Neu mammary carcinogenesis model in mice and breast cancer lines to determine potential target genes associated with the bexarotene-induced cellular senescence. Previous studies have shown that bexarotene suppressed mammary carcinogenesis in MMTV-Neu mice by a dose-dependent manner, and this was associated with no changes in ErbB2 expression, but with inhibition of cell proliferation (12, 16, 27). The combination of bexarotene with the Cox-2 inhibitor celecoxib further increased inhibitory effects on mammary carcinogenesis (28). Those authors also showed that bexarotene is superior in p53-negative mammary tumor cell lines, as compared with the tyrosine kinase inhibitor gefitinib (29). The combination of another retinoid (LG100268) with triterpenoids or selective estrogen receptor modulators (SERM) was also more efficacious in inhibiting mammary carcinogenesis than both agents given separately (13, 15, 30). In the above in vivo studies, the decreased cell proliferation in mammary epithelial cells (MEC) and tumors was the principal biomarker of response to retinoids. Here, we showed that bexarotene in addition to inhibiting cell proliferation also induces cellular senescence in normal MEC, premalignant lesions, and tumors of MMTV-Neu mice and that p21, p16, and RARβ are over-expressed in senescent cells.

Material and Methods

Mice and tumors
MMTV-Neu female mice at the age of 4 weeks were purchased from the Jackson Laboratory. Animals were fed a controlled MIN-76–purified mouse diet from Harlan Teklad. When palpable tumors (about 2–3 mm/diameter) occurred, usually at the age of 6 months, mice were randomized in placebo (sesame oil) and bexarotene-treated groups. A separate group of animals with mammary tumors were also treated for 7 days with doxorubicin at 3.0 mg/kg bw and given by gavage 6 days per week for 4 weeks. atRA was purchased from Sigma, Inc., and 4-HPR was obtained from the NIH Repository. Doxorubicin was purchased from Ben Venue Laboratories, Inc.

Rexinoids and retinoids
Bexarotene (LGD1069, Tarantino) was provided from the NCI repository. Bexarotene at 80 or 40 mg/kg bw was mixed in sesame oil (0.1 mL) and given by gavage 6 days per week for 4 weeks. atRA was purchased from Sigma, Inc., and 4-HPR was obtained from the NIH Repository. Doxorubicin was purchased from Ben Venue Laboratories, Inc.

Histology and ICH
For ICH, 4-μm paraffin sections were deparaffinized, endogenous peroxidase was blocked by 3.0% hydrogen peroxide, and sections were transferred to a pressure cooker for 6 minutes with 0.01 mol/L citric buffer. Then slides were treated for 20 minutes with corresponding blocking serum, followed by specific antibodies against: p21 (AB-5, 1:100, Lab Vision), p16 (M-156, 1:100, Santa Cruz Biotechnology, Inc.), phospho-pRb (AB-7, 1:40, Lab Vision), p53 (DO-7, sc-47698, 1:50, Santa Cruz Biotechnology, Inc.), RXRα (D-20, sc-553, 1:50, Santa Cruz Biotechnology, Inc.), and RARβ (Abcam). ABC kit and 3,3′-diaminobenzidine (DAB) from Vector Laboratories were used to identify the above antigens.

Cell proliferation
Proliferating cells in mammary tumors and tissues were determined by bromodeoxyuridine (BrdUrd) labeling, as we described previously (31).

Apoptosis
Cells in apoptosis were evaluated by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, as recommended by the ApopTag In Situ Hybridization Detection Kit (Oncor, Co.; ref. 31).

Senescent cells
SA-β-Gal activity assay (Cell Signaling Technology, Inc.) was used to identify senescent cells in mammary tissues and tumors and in breast cancer cell lines (32). In brief, frozen sections (4- to 6-μm thick) from control and bexarotene-treated animals were fixed in 3.0% formaldehyde for 5 minutes, washed in PBS, and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) Kit at pH 5.5 to 6.0 for 24 hours at 37°C. To determine senescent cells in vitro, cells grown on coverslips were treated for 7 days with bexarotene at 1.0 or 5.0 μmol/L, and the percentage of cellular senescence was determined by SA-β-Gal staining. To confirm whether senescent cells overexpress p53, p21, p16, pRb, and RARβ, double-staining protocols were developed in which frozen sections were first stained by SA-β-Gal overnight to detect senescent cells and then the same slides were treated for 2 hours by one of the above antibodies. Because SA-β-Gal stains the cytoplasm, whereas the above biomarkers are expressed in the nucleus, it was possible with relative high accuracy to determine the level of their nuclear expression in senescent cells and nonsenescent cells. A progressive dilution (from 1:40 to 1:500) of the above antibodies was used to discriminate cells with high and low levels of gene/protein expression. A 4-point grading
system (0–3) was introduced to evaluate semiquantitatively the expression level of the above biomarkers in senescent cells and nonsenescent cells with: 0 = no expression (similar to the background level); 1 = weak expression; 2 = moderate expression; and 3 = strong expression. The percentage of senescent cells with no (0 score) or low levels of expression (scores of 1 and 2) of the above biomarkers were compared with those with high expression (score 3) in control and bexarotene-treated animals. Two pathologists (A. Green and K. Christov) independently evaluated the slides in a blind fashion, and the scores were in the range of 15% to 20% difference.

**Cell lines and Western blotting**

To determine the effects of bexarotene on cellular senescence in vitro, ERα, ERβ, MCF-7, T47D, and BT474 and ERβ, MDA-MB-231 breast cancer cell lines were used. The above cell lines were obtained from American Type Culture Collection. For Western blotting, cells were cultured in 75 cm² flasks, treated for 24 or 72 hours with 0.0, 1.0, or 5.0 μmol/L bexarotene, atRA, or 4-HPR; the total protein was isolated by lysis buffer (Roche), subjected to electrophoreses, and the gels were transblotted to Immobilon-P membranes (Millipore). Blots were probed with primary antibody and correspondent secondary antibody.

**Statistical analysis**

Data for multiplicity of premalignant lesions and tumors, as well as for cellular senescence, cell proliferation, apoptosis, RARβ, and RXRα, in control and bexarotene-treated animals and cells were compared using Student t test.

**Results**

**Bexarotene inhibits multiplicity of mammary premalignant lesions and tumors**

To assess the effect of bexarotene on the development and progression of mammary premalignant lesions and tumors, animals with the first palpable mammary tumor detected were treated for 4 weeks with placebo (sesame oil) or bexarotene given at 2 doses: 40 mg/kg bw (low dose, L) or 80 mg/kg bw (high dose, H). At the beginning of the experiment, we used 100 mg/kg bw bexarotene as a high dose, but some animals lost weight and developed signs of toxicity; therefore, we decreased the high dose to 80 mg/kg bw. In addition to tumors, lesions with characteristics of AH and MIN were also identified [Fig. 1A (a) and (b)]. As shown in Fig. 1B, 13 control animals (placebo-treated) with 13 palpable tumors at the start of experiment 4 weeks later developed 3.1 tumors per animal, as determined by histologic examination of all mammary glands. Bexarotene decreased tumor multiplicity in a dose-dependent manner, from 3.1 ± 1.1 per animal in the placebo group to 2.2 ± 1.0 (*, P < 0.05) in the low-dose group and further to 1.7 ± 0.8 (**, P < 0.001) in the high-dose group. In one animal treated with high-dose bexarotene, the palpable tumor totally regressed; in 2 other animals, tumors were almost totally replaced by inflammatory and death cells. As shown in Fig. 1A (d), bexarotene at 80 mg/kg bw induced disintegration of tumor cells in peripheral areas (arrows) where proliferating cells predominate. In addition to mammary tumors, three animals developed salivary gland tumors and 2 developed uterine tumors. MIN was detected in 8 of 13 placebo-treated animals, and its number progressively decreased, from 1.2 ± 0.4 per animal in the control group to 0.9 ± 0.6 in low-dose and to 0.6 ± 0.4 (*, P < 0.05) in high-dose group (Fig. 1B). Lesions with characteristics of AH were identified in only a small number of animals, and their number decreased from 1.5 ± 0.9 in placebo animals (n = 7) to
0.8 ± 0.4 low-dose bexarotene-treated animals (n = 4; P < 0.1) and further to 0.6 ± 0.3 in high-dose animals (n = 5; **, P < 0.05; Fig. 1B).

**Bexarotene decreased cell proliferation and induced apoptosis in MECs, MINs, and tumors**

As shown in Table 1, BrdUrd-labeled (proliferating) cells progressively increased in the course of mammary carcinogenesis: from 2.6% ± 0.8% in MECs to 4.4% ± 1.0% (*, P < 0.005) in AH, to 8.3% ± 1.5% (**, P < 0.001) in MINs, and to 9.6% ± 2.6% (**, ***, P < 0.001) in tumors. Bexarotene decreased proliferating cells in MECs, AH, MINs, and tumors in a dose-dependent manner from 2.6% ± 0.8% in placebo, to 1.8% ± 0.7% in low-dose (*, P < 0.02), and to 1.5% ± 0.5% in high-dose–treated animals (**, P < 0.02). Similar decreases in cell proliferation occurred in AH (**, P < 0.02) for high-dose group; in MIN for low- (**, P < 0.05) and high-dose (**, P < 0.001) groups; and in tumors for low- (**, P < 0.05) and high-dose (**, P < 0.005) groups (Table 1). In addition to a decrease in cell proliferation, bexarotene induced apoptosis in MECs, MINs, and tumors (Fig. 2A and B). Apoptosis was rare in MECs, MINs, and tumors of placebo-treated animals [Fig. 2A (a) and (c) brown-stained cells] and increased in bexarotene-treated animals [Fig. 2A (b and d) and B]: for MEC (***, P < 0.02; ***, P < 0.005), MIN (***, P < 0.001; ***, P < 0.002), and tumors (**, P < 0.001) for low- and high-dose groups, respectively. Note the relatively high number of cells in apoptosis among MEC of lobules [Fig. 2A (b)] and in tumors [Fig. 2A (d)] of bexarotene-treated animals.

**Bexarotene induced cellular senescence in MECs, MINs, and tumors**

To determine cellular senescence, frozen tissue and tumor sections were stained overnight by SA-β-Gal reaction, which has been consistently used for identification of senescent cells, both in vitro and in vivo (32). As shown in Fig. 3A (a), single senescent cells (blue-stained) were identified among MECs of lobules (arrows) and in AH (arrow) of a control animal. Single senescent cells were also detected in tumors (T) and in surrounding stromal cells [Fig. 3A (b), arrows] of control animals. Treatment of animals with bexarotene significantly increased senescent cells in ducts (D), lobules (L, arrows), and terminal end buds (TEB, arrow), which are considered places of origin of mammary tumors [Fig. 3A (c), arrows]. Note that, in some lobules, almost all epithelial cells are SA-β-Gal–positive (L, arrows). Senescent cells also increased in tumors [Fig. 3A (d), blue-stained cells]. In animals treated with bexarotene, senescent cells usually formed clusters [Fig. 3A (d)] or were preferentially localized in differentiated structures [acinar or papillary formations; Fig. 3A (c)].

**Figure 2.** Bexarotene induces apoptosis in MECs, MINs, and tumors. A (a), cells in apoptosis identified by TUNEL assay. Note single cells in apoptosis (brown-stained, arrows) among ductal cells of a control animal. The slide is counterstained by methyl green. ×200; b, increased cells in apoptosis (arrows) are presented among lobular cells of animal treated for 4 weeks with high dose of bexarotene. ×200. C, several cells in apoptosis (arrows) in mammary tumor of a control animal. ×200. D, sharp increase in apoptotic cells (brown-stained) in mammary tumor of animal treated with high dose of bexarotene. ×200. B, percentage of cells in apoptosis in MECs, MINs, and tumors. A dose-dependent increase in apoptosis was found in MECs (***, P < 0.02; ***, P < 0.005), MINs (**, P < 0.01; ***, P < 0.02), and tumors (**, P < 0.001; ***, P < 0.001), respectively, for low and high doses. The number of animals examined is given inside the columns.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg/kg bw</th>
<th>MECs No X ± SD</th>
<th>AH No X ± SD</th>
<th>MIN No X ± SD</th>
<th>Tumors No X ± SD</th>
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<td>Placebo</td>
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<td>4.4 ± 1.0</td>
<td>8.3 ± 1.5</td>
<td>26 9.6 ± 2.6</td>
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<tr>
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<td>11 1.8 ± 0.7</td>
<td>6.4 ± 1.4</td>
<td>6.4 ± 1.4</td>
<td>18 8.0 ± 2.5</td>
</tr>
<tr>
<td>Bexarotene</td>
<td>80</td>
<td>5 1.5 ± 0.5</td>
<td>5 2.8 ± 0.8</td>
<td>5 3.7 ± 0.9</td>
<td>15 6.6 ± 2.8</td>
</tr>
</tbody>
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*a1, P < 0.02; a2, P < 0.02; b1, P < 0.05; b2, P < 0.01; c1, P < 0.05; c2, P < 0.005, **, P < 0.001.
Role of Cellular Senescence in Cancer Prevention

Figure 3. Bexarotene induces cellular senescence in mammary tissues and tumors. A(a), SA-\(\beta\)-Gal-positive cells (blue-stained) were identified in normal lobules (arrows) and in AH of a control 6-month-old mice. The slide is counterstained by neutral fast red. \(\times\)200. b, single SA-\(\beta\)-Gal-positive cells were detected among tumor cells (arrow) and among fibroblast surrounding tumor (arrows) of a control animal. \(\times\)400. c, plenty of SA-\(\beta\)-Gal-positive cells (blue-stained) among epithelial cells of ducts (D), lobules (L) and terminal end buds (TEB) of a 5-month-old mice treated with bexarotene, 80 mg/kg bw. \(\times\)200. d, high number of SA-\(\beta\)-Gal-positive cells (blue-stained) among tumor parenchyma of animal treated with bexarotene for 4 weeks. \(\times\)400. B, dose-dependent increase of senescent cells among MECs among MECs (\(\times\), \(P < 0.05\); **, \(P < 0.001\)), low and high doses, respectively), MINs (\(P < 0.02\) and \(P < 0.001\)), bexarotene-treated animals; C, control animals; L, treated with low-dose bexarotene; H, treated with high-dose bexarotene; D, treated with doxorubicin. Most significant increase in senescent cells was observed in doxorubicin-treated animals (**, \(P < 0.001\)).

Figure 4. Senescent cells in mammary tumors overexpressed p21 and p16. A (a), representative picture of mammary tumor from an animal treated with bexarotene; p53 is expressed at low level in almost all tumor cells. Note the lack of p53 overexpression in SA-\(\beta\)-Gal–positive cells. \(\times\)200. b, parallel section from the same tumor as in (a), the slide is double stained by SA-\(\beta\)-Gal and p21 antibody. In most senescent cells, p21 is overexpressed (dark-stained nuclei). \(\times\)200. c, double staining of tumor tissue section by SA-\(\beta\)-Gal and p16 antibody from animal treated with high dose of bexarotene. Most tumor cells expressed p16 (brown-stained). However, p16 was overexpressed in most senescent cells (arrows). \(\times\)400. d, representative picture of pRB overexpression in the nucleus of senescent cells (arrows). \(\times\)400. B (a), the values of senescent cells that overexpress p53, p21, p16, and pRb are presented. C, control animals; H, animals treated with high-dose of bexarotene. There is no difference in p53 expression between senescent cells and nonsenescent cells (\(P > 0.5\)). However, p21 (**, \(P < 0.001\)) and p16 (\(*, P < 0.01\)) are overexpressed in senescent cells of tumors from animals treated with high dose of bexarotene. No difference was also found in pRb expression between senescent cells and nonsenescent cells (\(P > 0.5\)). b, p21 expression in breast cancer cell lines after treatment for 24 hours with bexarotene, 1.0 \(\mu\)mol/L. p21 increased in MDA-MB-231 and BT474 cells did not change in T47D cells and decreased in MCF-7 cells. c, bexarotene at 1.0 and 5.0 \(\mu\)mol/L induced pan-cytokeratin and p21 expression in MDA-MB-231 and BT-474 cells.
Bexarotene-induced cellular senescence is associated with increased p21 and p16 expression

Most studies on cellular senescence have been conducted in vitro with genotoxic agents, which upregulate p53-p21 expression (17–19, 33, 34). Here, we asked whether bexarotene, a cell differentiation agent, can also induce cellular senescence by affecting the above biomarkers in mammary tumors. To answer this question, we first examined by ICH p53, p21, p16, and pRb protein expression in tumor cells of control and bexarotene-treated animals. In formalin-fixed, paraffin-embedded tissue sections, all the above biomarkers were detected in the nucleus of almost all (>90%) tumor cells, and it was difficult to find any difference in the level of their expression between placebo- and bexarotene-treated animals. To determine whether some of the above biomarkers are overexpressed in senescent cells, double labeling of senescent cells was conducted, as indicated in Materials and Methods. In frozen sections, p53 was detected at low level in all tumor cells, and its expression did not increase in senescent cells of control and bexarotene-treated animals. As shown in Fig. 4A (a), a weak p53 nuclear expression in tumor cells of animals treated with bexarotene is evident, but it did not increase in senescent cells (blue-stained). However, a parallel frozen section from the same tumor showed clear p21 overexpression in senescent cells [Fig. 4A (b), arrows]. Because p21 was overexpressed in single senescent cells of control animals as well, we compared senescent cells with p21 overexpression in control (C) and bexarotene-treated (high-dose) animals. As shown in Fig. 4B (a), bexarotene significantly increased the percentage of senescent cells that overexpress p21 in mammary tumors: 36.2% ± 6.5% in bexarotene-treated (high-dose) versus 12.3% ± 3.3% (*, P < 0.001) in placebo-treated animals. The same approach was also used for p16 and pRb expression in senescent cells [Fig. 4A (c) and (d)]. Bexarotene induced p16 expression in senescent cells of tumors, as evident in Fig. 4A (c) (arrows) and in Fig. 4B (a), with 21.2% ± 5.8% in bexarotene-treated versus 40.1% ± 12.2% (H, **, P < 0.01) in placebo-treated animals. pRb protein [Fig. 4A (d), arrows] was also overexpressed in the nucleus of some senescent cells, but no difference was found in the values between control and bexarotene-treated animals [Fig. 4B (a)].

Bexarotene induces differentiation and p21 expression in breast cancer cell lines

To make sure that p21 is one of the bexarotene targets, as was shown above in mammary tumors, MDA-MB-231, BT474, MCF-7, and T47D cells, which differentially express p21 and differ in ER, PR, and Her2/Neu status, were treated for 24 hours with 1.0 μmol/L bexarotene, and p21 was examined by Western blotting. As shown in Fig. 4B (b), bexarotene induced p21 expression in MDA-MB-231 and BT-474 cells, had no effect in T47D cells, and decreased its expression in MCF-7 cells. To confirm that bexarotene induces cell differentiation, MDA-MB-231 and BT474 cells were treated for 24 and 72 hours with 1.0 or 5.0 μmol/L, and cytokeratin (pan-cytokeratin) expression was determined [Fig. 4B (c)]. Bexarotene increased pan-cytokeratin expression in MDA-MB-231 cells within the first 24 hours with similar effect after 3 days of treatment. The cytokeratin induction in the above cell lines by bexarotene correlated with a p21 increase, suggesting that the latter is a consequence of cell differentiation. Cytokeratin was also upregulated in BT474 cells treated for 3 days with bexarotene [Fig. 4B (c)].

Bexarotene differentially affect RARβ and RXRα expression in MEC and tumors

RARβ expression was detected by ICH in MECs of ducts and lobules and in tumors of control and bexarotene-treated animals. As shown in Fig. 5A (a), only single RARβ-positive cells were found in tumors (T). Bexarotene induced RARβ in MECs: from 7.2% ± 1.6% in placebo to 15.3% ± 3.8% (*, P < 0.05) in bexarotene-treated animals; and in 3 of 8 tumors examined, with variability in the values in individual tumors between 1.2% and 15.4% positive cells [Fig. 5A (b)]. To determine whether RARβ is overexpressed in senescent cells, frozen sections were first treated by SA-β-Gal agents and then with anti-RARβ antibody from Abcam. No RARβ + SA-β-Gal cells were identified among placebo and bexarotene-treated animals. We also found that RXRα was expressed in 82.2% ± 18.8% of MECs and in 75.5% ± 10.3% of tumor cells of control animals, with no significant difference between the values [Fig. 5A (c)]. Treatment of animals with high doses of bexarotene did not affect RXRα-positive MECs, whereas the high dose decreased receptor expression in tumor cells [Fig. 5A (d)] from 75.5% ± 10.3% in placebo to 60.2% ± 12.8% [Fig. 5B (b); *, P < 0.02]. To assess in vitro the effect of bexarotene on RARβ and RXRα expression, MDA-MB-231 cells were treated for 24 hours with 1.0 and 5.0 μmol/L of 4-HPR, bexarotene, and atRA (Fig. 5C); the nuclear protein was isolated, and Western blotting was conducted. As shown in Fig. 5C, 4-HPR, a ligand of RARγ, did not affect RARβ2 expression, whereas both bexarotene at 1.0 μmol/L and atRA at 1.0 and 5.0 μmol/L induced RARβ2 expression. Bexarotene also decreased RXRα expression in MDA-MB-231 cells but had no effect in BT474 cells (Fig. 5C).

Discussion

In this study, we found that bexarotene not only inhibited cell proliferation but also induced cellular senescence and apoptosis in MECs, MINs, and tumors of the MMTV-Neu model of mammary carcinogenesis. Our data confirm previous studies of Brown’s group, which found that bexarotene suppressed mammary carcinogenesis and tumor growth in MMTV-Neu mice and that this was associated with inhibition of cell proliferation (12, 16). For assessment of apoptosis, we used the TUNEL assay, whereas Brown’s group used the caspase-3 assay that may explain the
Figure 5. Bexarotene induces RARβ in MECs and decreases RXRα expression in mammary tumors. A (a) RARβ expression in MECs of lobules and ducts (arrows) of a control animal. In tumor (T), there is lack of RARβ-positive cells. RARβ is predominantly expressed in MECs of lobules and ducts (arrows). The slide is counterstained by hematoxylin. ×200. b, RARβ expression (brown-stained) among tumor cells of an animal treated with bexarotene. RARβ is localized in the nucleus only. c, RXRα expression in a duct (D) and in 2 tumors (T) of a control animal. There is a clear decrease in RXRα expression in tumors as compared with ductal MECs. ×200. d, RXRα expression is decreased in mammary tumor of animal treated with bexarotene, as compared with its expression in tumors of control animals. ×200. B, left, percentage of RARβPositive cells among MECs of ducts and lobules and in tumors (T). Bexarotene induced RARβ expression among MECs (P < 0.01) and in 3 of 9 mammary tumors. However, in 6 tumors, only single RARβ-positive cells were detected. Right, RXRα decreased in mammary tumors of bexarotene-treated animals (P < 0.02) but not among MEC of ducts and lobules. C, top, effects of bexarotene on RARβ2 expression in MDA-MB-231 cells. Cells were treated for 24 hours with 1.0 or 5.0 μmol/L 4-HPR, bexarotene (Bx), and atRA. The alterations in RARβ2 expression as compared with the values of control cells are given below the line of differences in the percentage of cells in apoptosis between their and our study. It is generally believed that apoptosis induced by retinoids is a consequence of oxidative species, which, depending on the cell system and the dose used, may also cause cellular senescence (35). Here, we tried to distinguish the effect of bexarotene on cell proliferation and cellular senescence from apoptosis by using 2 doses of bexarotene (80 and 40 mg/kg bw), assuming that the high dose will preferentially induce apoptosis, whereas the low dose will suppress cell proliferation and eventually induce cellular senescence. We found a dose-dependent increase in senescent cells concomitantly with increase in apoptosis and decrease in proliferation of MECs, MINs, and tumors. Potential implementation of much lower doses of bexarotene (20, 10 mg/kg bw) may distinguish cellular senescence from apoptosis and cell proliferation, as has been reported for in vitro studies. Bexarotene was highly efficacious in inducing cellular senescence in TEBs, which have high proliferation activity and are considered places of origin of most mammary tumors, thus supporting our data and those of others that retinoids and retinoid are more efficacious in inhibiting early than late (MIN, tumors) stages of mammary carcinogenesis (16, 23).

Increased cellular senescence and apoptosis in MECs of lobules and ducts suggest that bexarotene, in addition to inhibition of carcinogenesis, can also alter mammary gland architecture, as has been reported for 4-HPR in earlier studies (6). Bexarotene, like other retinoids and retinoid, is a cell differentiation agent (4, 9, 10, 36), as we also confirmed in this study by cytokeratin expression in MDA-MB-231 and BT474 tumor cells [Fig. 4B (c)], suggesting involvement of distinctive signaling pathways different from those induced by cytotoxic agents. For instance, we found that p21 and p16, but not p53, were overexpressed in senescent cells of mammary tumors of bexarotene-treated animals [Fig. 4A (b) and (c)], contrasting the data from cytotoxic agents which primarily induce DNA damage leading to p53 transcription and overexpression (25, 34). Colateral in vitro data provided in this study support the in vitro data and showed increased p21 expression in MDA-MB-231 and BT474 cells treated with 1.0 μmol/L bexarotene [Fig. 4B (b)]. Surprisingly, in ER+ MCF-7 and T47D cells, bexarotene had the opposite effect; it suppressed p21 expression, suggesting involvement of different molecular mechanisms in cell and tumor growth inhibition by retinoids in ER+ and ER breast carcinomas (37, 38). In addition to p21, p16 was also overexpressed in senescent cells of mammary tumors of bexarotene-treated animals [Fig. 4A (c)]. p16Ink4a is considered a tumor suppressor gene, and its activation by bexarotene or by other antitumor agents is associated with inhibition of cell proliferation and induction of cellular senescence (33, 39, 40). Because, p16 cooperates with pRb, the latter...
was also evaluated in mammary tumors of control and bexarotene-treated animals. Although in some senescent cells, a clear pRb overexpression was found, no significant difference was established when the values were compared with those in nonsenescent cells. Another alternative for bexarotene-induced cellular senescence is through upregulation of RARβ. Previous studies have shown that RARβ and particularly its RARβ2 isoform are increased in human breast epithelial cells, which after continuous (>20 passages) in vitro culturing develop senescent phenotype (41). In breast premalignant lesions and tumors, RARβ2 is lost, suggesting its tumor suppressor role (42, 43). Transfection of RARβ2 in tumor cells lacking the receptor was associated with cell growth inhibition and induction of apoptosis (44). Our previous data obtained from the promoter luciferase activity assay (45) and those of others obtained by gene analysis of human breast epithelial cells (26) revealed that bexarotene, in addition to RXRs, can also induce RARβ transcription and thus promotes cellular senescence. Here, we showed that atRA is a more powerful inducer of RARβ than bexarotene, which correlates well with differences in their binding affinity to RARs (38, 46). RXRα expression in mammary tumors was found to be lower than in normal MECs, most probably as a consequence of overall RARs and RXRs decrease in tumor development and progression. Bexarotene also decreased RXRα expression in MDA-MB-231 and BT474 cells, suggesting receptor protein degradation, as has been reported for RXRs in MCF-7 cells treated with atRA (47). The role of RARβ and RXRα in mediating antitumor potential of retinoids and rexinoids seems to be overestimated, as reported by gene profiling as well (25, 26). Previous studies (15, 22, 25) and our data in this study showed that retinoids and rexinoids can, in addition to modulating RARs and RXRs activity, also affect expression of p21, cyclins D1-A, cdk2/4, pRb, E2F, and other genes/proteins and thus facilitate retinoid receptor–independent cell and tumor growth inhibition as well as development of cellular senescence. In support of these data, we recently found that about 30% of early passages (<12 passages) of breast cancer cells did not respond to atRA, 9cRA, and 4-HPR and that the expression of both RARβ isoforms (β2 and β5) did not predict cell sensitivity to retinoids (48, 49). In addition, senescent cells induced by bexarotene may secrete cytokines, which may stimulate proliferation of fibroblasts or activate microphages and tumor suppressor lymphocytes and thus by collateral mechanisms inhibit mammary carcinogenesis (50). Thus, our data and those of others suggest that the modulation of one or more of the above genes may prevent tumor cells to senesce and thus increase their resistance to rexinoids and other antitumor agents. However, bexarotene is not a pure RXR agonist but also induces RAR expression that increases its cytotoxicity and thus its long-term clinical implication in cancer prevention and treatment trials remains questionable (8, 12). Senescent cells with their permanent growth arrest can give additional information about the long-term efficacy and cellular mechanisms of cancer prevention agents. Cellular senescence also opens new avenues in developing drugs that selectively induce cellular senescence and thus suppress tumor development and progression. This study strongly supports the notion that cellular senescence, in addition to inhibition of cell proliferation, can bring additional information about the efficacy and molecular mechanisms of rexinoids in breast cancer prevention and therapy studies. Findings also showed that bexarotene-induced cellular senescence is a consequence of multiple signaling pathways involving p21, p16, and RARβ expression. Clinical studies need to confirm whether cellular senescence, alone or in combination with other biomarkers, could be used in assessing the efficacy of bexarotene and other agents in preventing and treating breast and other types of cancer.

Disclosure of Potential Conflict of Interest
No potential conflicts of interest were disclosed.

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References
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