The Farnesoid X Receptor Is Expressed in Breast Cancer and Regulates Apoptosis and Aromatase Expression

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Abstract

Bile acids are present at high concentrations in breast cysts and in the plasma of postmenopausal women with breast cancer. The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that regulates bile acid homeostasis. FXR was detected in normal and tumor breast tissue, with a high level of expression in ductal epithelial cells of normal breast and infiltrating ductal carcinoma cells. FXR was also present in the human breast carcinoma cells, MCF-7 and MDA-MB-468. Activation of FXR by high concentrations of ligands induced MCF-7 and MDA-MB-468 apoptosis. At lower concentrations that had no direct effect on viability, the FXR agonist GW4064 induced expression of mRNA for the FXR target genes, small heterodimer partner (SHP), intestinal bile acid binding protein, and multidrug resistance–associated protein 2 (MRP-2), and repressed the expression of the SHP target gene aromatase. In contrast to MRP-2, mRNA for the breast cancer target genes MDR-3, MRP-1, and solute carrier transporter 7A5 were decreased. Although multidrug resistance transporters were regulated and are known FXR target genes, GW4064 had no effect on the cell death induced by the anticancer drug paclitaxel. Our findings show for the first time that FXR is expressed in breast cancer tissue and has multiple properties that could be used for the treatment of breast cancer. (Cancer Res 2006; 66(20): 10120-6)

Introduction

The farnesoid X receptor (FXR; NR1H4) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that forms a heterodimer with the retinoid X receptor (1). Originally identified through its activation by high levels of farnesol, an isoprenoid cholesterol synthesis intermediate, FXR has since been characterized as a bile acid receptor (1–3). Individual bile acids differ in their efficiencies as activators of FXR, with the primary bile acid chenodeoxycholic acid (CDCA) being the most potent (3). Bile acids are the major product of cholesterol metabolism in the liver and aid lipid absorption in the intestine (1). In addition to acting as FXR ligands, bile acids have direct chemical activities, can activate alternative nuclear receptor targets (e.g., pregnane X receptor, vitamin D receptor; refs. 4, 5), and have a recently identified G-protein linked receptor (TGR5; ref. 6).

FXR activation results in the expression of distinct target genes. In the liver, FXR activation prevents bile acid synthesis by repressing cytochrome P450 7A1 (CYP7A1; ref. 2), induces bile acid efflux transporters such as bile salt export pump (BSEP; ref. 7) as well as multidrug resistance–associated protein 2 (MRP2; ref. 8) and down-regulates bile acid uptake transporters, including the apical sodium-dependent bile salt transporter (ASBT; ref. 9). FXR also limits free bile acids in the circulation by inducing ileal bile acid binding protein (IBABP; ref. 2) and multidrug resistance protein 3 (MDR3; ref. 10). FXR can down-regulate genes by inducing the small heterodimer partner (SHP), a nuclear receptor that lacks a DNA binding domain (11, 12). SHP interacts with other receptors, including the liver receptor homologue-1 (LRH-1; ref. 11) and estrogen receptor (13), preventing their activation. It is this FXR-SHP-LRH-1 cascade that down-regulates CYP7A1 and ASBT (11). FXR is, however, not limited to the regulation of bile acids, as it also regulates lipid (14) and carbohydrate (15) homeostasis, and has roles in amino acid transport (16), liver regeneration (17), and gastrointestinal defense (18).

FXR is expressed in nonenterohepatic tissues, including high levels in the kidneys and adrenal gland, which are “nonclassic” bile acid targets (1), and low levels in the heart, vascular tissue, thymus, ovary, spleen, and testes (19, 20). The functions of FXR in these nonenterohepatic tissues are poorly understood, particularly within humans.

Breast cancer is the most common form of cancer in women in the Western world. Its incidence is epidemiologically linked to high-fat diets (21), which increase the amounts of bile acids in the body (22). High levels of the plasma bile acid deoxycholic acid (DCA) are found in postmenopausal women newly diagnosed with breast cancer, whereas CDCA is present at high μmol/L levels in breast cyst fluid (23, 24). Moreover, CDCA can inhibit the growth of the breast carcinoma cell line MCF-7, whereas glycol-CDCA causes proliferation (25). Here, we show the expression of FXR in normal breast ductal epithelial cells and in the cells of infiltrating ductal carcinoma of the breast. In breast cancer cell lines, FXR activation down-regulates the breast cancer target genes; local estrogen producer aromatase and the transporters MDR3, MRP-1, solute carrier transporter 7A5 (SLC7A5); and inhibits cell proliferation. It also induces the expression of the known FXR target genes SHP, IBABP, and MRP2. We propose that endogenous bile acids through FXR play a protective role in breast cancer development, and that FXR is an antineoplastic target for drug development in breast cancer.
Materials and Methods

Cell culture. MCF-7 (estrogen receptor positive) and MDA-MB-468 (estrogen receptor negative) breast carcinoma cell lines and HepG2 hepatoma cell line were grown and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1× antibiotics and antimycotics. MCF-7 cells were from the American Type Culture Collection (Manassas, VA). MDA-MB-468 cells were a gift of Dr. John Marshall. HepG2 cells were from European Collection of Animal Cell Cultures (Salisbury, United Kingdom).

Breast tissue samples. Samples of human infiltrating ductal breast carcinoma and near tumor normal samples were obtained from 10 patients after receiving ethical approval and individual patient consent. Protein was extracted using Cytobuster (Novagen, Merck Biosciences, Nottingham, United Kingdom). Breast carcinoma, matched tumor, and normal adjacent tissue microarrays were purchased from Folio Biosciences, Inc. (Columbus, OH). Immunohistochemistry was done using standard techniques as previously described (19).

Western blot analysis. Protein was extracted from cell lines, and Western blot analysis was done as previously described (19), using the rabbit anti-FXR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250, or a mouse monoclonal anti-β-actin antibody (Sigma, Poole, United Kingdom) at a dilution of 1:500.

Measurement of MCF-7 and MDA-MB-468 cell death. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (26). Cells were serum starved for 24 hours before use. In serum-free medium, cells were treated with test compounds or vehicle (0.3% DMSO) for 48 hours. Apoptosis was measured by nuclear morphology after Hoechst staining (27) following 48-hour treatment with compounds. In some experiments, the caspase-3 inhibitor ZVAD.fmk (30 µmol/L; ref. 27) was given 1 hour before GW4064 treatment. In other experiments, GW4064 (3 µmol/L) was given 24 hours before treatment with paclitaxel (48 hours; Calbiochem, Merck Biosciences, Nottingham, United Kingdom).

FXR reporter gene. FXR reporter gene activity was measured as essentially previously described (28), using the IR-1 luciferase reporter gene, cotransfected with pcDNA-rFXR (28), into MCF-7 cells using Novafector (Venn Nova, Pompano Beach, FL); additional FXR was required due to the low transfection efficiency of the system (Supplementary Data). Cells were treated with FXR ligands for 24 hours, and then lysed for measurement of luciferase activity.

Figure 1. FXR is expressed in human breast tissue. A, Western blot analysis for FXR in six matched near tumor “normal” (N) and infiltrating ductal breast carcinoma (T) tissue samples. β-Actin expression was measured on the same membrane after FXR antibody stripping. Representative of n = 10 paired samples. B, FXR immunohistochemistry on representative examples of paired normal (Normal 1-4) and ductal carcinoma (Tumor 1-4) breast tissue, from a breast cancer tissue array. No staining was observed when the primary antibody was omitted (represented by controls matching section 4; normal and tumor 4 controls). Positive immunoreactive protein (brown immunoperoxidase) was found at high levels in the cells lining the ducts in normal breast tissue and in the tumor cells of infiltrating ductal carcinoma, with low-level expression in other cells. All magnifications are ×200. These panels are representative of n = 40 paired samples. FXR is expressed in breast cancer cell lines MCF-7 and MDA-MB-468: C, RT-PCR for human FXR. FXR was observed as a band at 582 bp in MCF-7 and MDA-MB-468 cells. D, Western blot analysis for FXR in MCF-7 and MDA-MB-468 cells seen as a band at ~56 kDa; HepG2 was used as a positive control (19).
Inhibition of FXR activity by FXR dominant-negative overexpression.

The effect of dominant-negative FXR (FXR-DN; ref. 28) on GW4064-induced cell death in living cells was measured indirectly using green fluorescent protein (GFP) as a marker, as described previously (27). Twenty-four hours after transfection, the cells were treated with GW4064 (30 μmol/L) or vehicle (0.3% DMSO) in serum-free medium. After 24 hours, the number of cells expressing GFP was assessed by fluorescence microscopy.

Immunofluorescence for aromatase. Immunofluorescence was as previously described (19) using rabbit anti-CYP19 (aromatase) polyclonal antibody (Immunesystems, Ltd., Paignton, United Kingdom) at 1:50 dilution.

Reverse transcription-PCR analysis. Total RNA was extracted from the cells by Trizol reagent (Invitrogen, Paisley, United Kingdom). Reverse transcription-PCR (RT-PCR) was done by using standard techniques. Primers for human FXR (19), SHP, aromatase, LRH-1 (29), IBABP (30), MRP2 (31), MDR3, MRP2, OATP8, SLC21A6; 57°C for 2 minutes, followed by 35 cycles (aromatase 40 cycles; β-actin 25 cycles), followed by 10 minutes at 72°C. For all target genes, each cycle consisted of 30 seconds at the annealing temperatures described below, and 1 minute at 72°C. The annealing temperatures used were as follows: 55°C aromatase, ASBT, BSEP, LRH-1, MDR3, MRP2, OATP8, SLC21A6; 57°C FXR, 58°C ABCG2, β-actin, IBABP, LRH, MRP1, SLC7A5, and 60°C SHP. PCR products were size fractionated with a 1.5% agarose gel and the bands were visualized with ethidium bromide. In parallel reactions, where Moloney murine leukemia virus reverse transcriptase was omitted, no bands were visible (data not shown).

For quantification, bands were analyzed using ImageJ (NIH, Bethesda, MD) and presented as a ratio to β-actin. MCF-7 and MDA-MB-468 cells were incubated with vehicle (0.03% DMSO), GW4064 (3 μmol/L), or phorbol-12-myristate-13-acetate (PMA; 4 mol/L; Sigma) alone or in combination with 3 μmol/L GW4064 for 24 hours in medium containing 10% FBS. GW4064 was given 1 hour before the addition of PMA. HepG2 RNA was used as a control for FXR and aromatase RT-PCR.

Results

Analysis of FXR Expression in Human Breast Cancer

FXR was present in both the infiltrating ductal carcinoma and the paired adjacent normal breast tissue samples from n = 10 separate patients (Fig. 1A). Breast tissue array analysis (Fig. 1B) showed high levels of FXR staining present both in infiltrating tumor cells (39 of 40 tissue sections) and in cells lining the normal ducts (Fig. 1B). FXR expression correlated with the level of ductal cells (which express FXR), either normal or tumor, within the sample. No positive staining was observed when the primary antibody was omitted (Fig. 1B, controls).

FXR mRNA (measured by RT-PCR as a 362-bp band; Fig. 1C) and protein (as a band of ~56 kDa; Fig. 1D) were also found in MCF-7 and MDA-MB-468 ductal epithelial breast cancer cell lines; HepG2 cells were used as a positive control for FXR expression (19).

FXR Agonists Induce Breast Cancer Cell Death by Apoptosis

CDCA and GW4064 induced cell death in a concentration-dependent manner in both MCF-7 (Fig. 2A) and MDA-MB-468 (Fig. 2B) cells, effects that also correlated with their ability to...
activate FXR in MCF-7 cells (Fig. 2C). Glycol-CDCA had no effect on MCF-7 or MDA-MB-468 cell growth (Fig. 2A and B), and had no activity on the FXR reporter gene in MCF-7 cells (Fig. 2C). As high concentrations of ligands were required to induce cell death, we tested the FXR dependency of the system using FXR-DN. MCF-7 cells were transiently transfected with either FXR-DN or control empty vector (pcDNA3.1). Similar to the results of the cell viability assays, GW4064 reduced the number of control GFP-expressing MCF-7 cells. In contrast, GW4064-induced cell death was significantly reduced in FXR-DN–expressing cells (Fig. 2D). Similar results were also found in MDA-MB-468 cells using small interfering RNA (siRNA) knockdown of FXR (Supplementary Data). MCF-7 and MDA-MB-468 cell death induced by GW4064 was characterized as apoptosis, morphologically by nuclear condensation (Fig. 3A) and by inhibition using the broad-spectrum caspase inhibitor ZVAD.fmk (Fig. 3B).

FXR Ligands Regulate FXR and Breast Cancer Target Genes

**FXR target genes.** GW4064 used at a concentration (3 μmol/L) that had minimal effects on cell viability significantly induced the mRNA expression of known FXR target genes SHP (Fig. 4), MRP2 (Fig. 4), and IBABP (Fig. 6A) in each of the samples tested.

Aromatase. A low level of aromatase (CYP19) was detected by RT-PCR (Fig. 5A) and immunofluorescence (Fig. 5B) in MDA-MB-468 and MCF-7 cells (Supplementary Data), which was sensitive to induction by PMA (Fig. 5). GW4064 (3 μmol/L) alone had no significant effect on the basal expression of aromatase but reduced the induction of aromatase by PMA (Fig. 5). A similar inhibition by GW4064 of aromatase induction by PMA was observed in MCF-7 cells (Supplementary Data). Aromatase is induced in response to PMA in a LRH-1–dependent manner (40). SHP inhibits the action of the receptor LRH-1 (11). LRH-1 mRNA expression was detected at low levels in both MCF-7 and MDA-MB-468 cell lines by RT-PCR (Fig. 4A). Consistent with an SHP-LRH-1–mediated effect, the LRH-1–regulated growth arrest and DNA damage–inducible protein (GADD) 45β (41) was also significantly repressed by the FXR ligand GW4064 (Supplementary Data).

**Multidrug resistance proteins and transporters.** Known FXR target genes include a number of transporters and proteins of the P-glycoprotein class that act as multidrug resistance proteins, which are known limiters of the long-term efficacy of a number of chemotherapy drugs. Although semiquantitative RT-PCR has its limitations, with a robust induction profile of FXR target genes, we looked at the regulation of alternative FXR and breast cancer target genes in these samples. The expression of MDR3, MRPI, and SLCT7A5 compared with β-actin was significantly reduced by GW4064 (Fig. 6A), whereas LRP and breast cancer resistance protein ABCG2 expressions were unchanged (Fig. 6A). Additional FXR target genes, SLC21A6, OATP8, BSEP, and ASBT mRNA, were undetectable in MCF-7 cells (data not shown). To test whether FXR activation had an effect on multidrug resistance, we tested the effects of a 24-hour GW4064 (3 μmol/L) preincubation on the anticancer drug (paclitaxel)–induced cell death. GW4064 had no effect on the ability of paclitaxel to kill MCF-7 cells in a concentration-dependent manner (Fig. 6B).
Discussion
Breast cancer is the most common cause of cancer-related mortality in women. Many of the hormone-based therapeutic drugs currently in use are limited by their effect on nonbreast tissues, such as bone, and alternatives are much needed. Here, we show that FXR is expressed in both adjacent normal and infiltrating ductal carcinoma tissue samples. Immunohistochemical analysis identified the expression of FXR in the ductal epithelial cells of normal breast tissue, which are the origin of ~98% of malignant proliferations in the breast (42). In accordance with this, FXR was present at high levels in infiltrating ductal carcinoma cells.

The FXR activators CDCA and GW4064 induced apoptosis in the FXRexpressing breast cancer cell lines MCF-7 and MDA-MB-468 irrespective of estrogen receptor status. Previously, CDCA has been shown to inhibit the growth of MCF-7 cells and the bile acid glycol-CDCA to be proproliferative (25). In line with its inability to activate FXR, glycol-CDCA had no effect on cell proliferation in our hands. Although higher concentrations of GW4064 (up to 30 \( \mu \text{mol/L} \)) were required to induce cell death than to induce gene expression, the FXR dependency of this cell death was confirmed using both FXR-DN and siRNA. These results do, however, contrast to a recent report suggesting that bile acids promote the growth and metastasis of breast cancer through FXR (43). In that study, DCA at low concentrations (10 \( \mu \text{mol/L} \)) was shown to promote the survival and migration of MDA-MB-231 cells (43). However, the authors state that higher concentrations (100-150 \( \mu \text{mol/L} \)) of DCA caused apoptosis in the MDA-MB-231 cells, in line with both our findings and the reported EC\(_{50}\) for DCA on FXR (100 \( \mu \text{mol/L}; \) refs. 3, 43).

High levels of circulating bile acids are found in postmenopausal women with breast cancer and in breast cyst fluid (23, 24). Given the expression of FXR in the ductal epithelial cells and its known role in regulation of bile acid transport in the liver, we examined...
the effects of FXR ligands on the expression of transporters known to be FXR target genes in the liver and intestine. The FXR ligand GW4064 induced the expression of IBAPB and MR2. IBAPB is a cytosolic fatty acid binding protein that binds bile salts and so may play a role in the transport of bile acids in breast cancer (2). MR2 is a multispecific anion transporter in the bile canalicular membrane of hepatocytes. MR2 is also expressed in breast cancer (44), but does not seem to have a major role in chemotherapy resistance (44). Many of the other known FXR target genes were not expressed in the MCF-7 cells irrespective of treatment, including BSEP, ASBT, OATP8, and SLC21A6 (7, 9, 45, 46). The expression of the FXR liver inducible gene MDR3 was surprisingly reduced by GW4064 (10). This differential regulation between liver and breast may be due to numerous tissue-specific differences, including coactivator/corepressor balance or availability. Given the key role of FXR in regulating the expression of transporters in the liver and the restricted high-level expression of FXR in normal ductal epithelial cells, the effects of FXR ligand on the expression of other transporters, known to be expressed in breast, were examined (37, 47). Both MR1 and SLC7A5 were down-regulated by FXR ligand in MCF-7 cells. The reduction in MR1 may be beneficial in prevention of drug resistance to chemotherapeutic drugs. However, MR1 may also inhibit accumulation in milk of xenobiotics present in the breast, so it remains to be seen whether FXR regulates these transporters in the normal breast tissue and what effect that may have on normal breast functions. SLC7A5 transports large neutral amino acids and is involved in cellular amino acid uptake (47). It has been suggested that SLC7A5 plays an important role in tumor cell proliferation by providing certain essential amino acids, so its down-regulation may contribute to the antiproliferative effects of FXR ligands (47). Interestingly, another SLC7 family member is also down-regulated by FXR ligands in primary mouse hepatocytes (16). The expression of breast cancer resistance protein ABCG2 and the major vault protein LRP were reduced by GW4064 (10). This differential regulation between liver and breast may be due to numerous tissue-specific differences, including coactivator/corepressor balance or availability. Given the key role of FXR in regulating the expression of transporters in the liver and the restricted high-level expression of FXR in normal ductal epithelial cells, the effects of FXR ligand on the expression of other transporters, known to be expressed in breast, were examined (37, 47). Both MR1 and SLC7A5 were down-regulated by FXR ligand in MCF-7 cells. The reduction in MR1 may be beneficial in prevention of drug resistance to chemotherapeutic drugs. However, MR1 may also inhibit accumulation in milk of xenobiotics present in the breast, so it remains to be seen whether FXR regulates these transporters in the normal breast tissue and what effect that may have on normal breast functions. SLC7A5 transports large neutral amino acids and is involved in cellular amino acid uptake (47). It has been suggested that SLC7A5 plays an important role in tumor cell proliferation by providing certain essential amino acids, so its down-regulation may contribute to the antiproliferative effects of FXR ligands (47). Interestingly, another SLC7 family member is also down-regulated by FXR ligands in primary mouse hepatocytes (16). The expression of breast cancer resistance protein ABCG2 and the major vault protein LRP were unchanged, demonstrating that although not exhaustive, FXR regulates distinct transporters within the breast and is unlikely to induce the major transporters involved in the development of drug resistance in breast cancer. Accordingly, FXR activation had no effect on the anticancer agent paclitaxel–induced MCF-7 cell death, a drug often susceptible to resistance.

One of the well-characterized mechanisms by which FXR down-regulates gene expression in the liver and intestine is through induction of SHP (11). SHP is an atypical nuclear receptor lacking both a DNA-binding domain and the NH2-terminal ligand-independent activation domain (12). This receptor down-regulates genes by interacting with other nuclear receptors, including LRH-1 and that SHP was induced by treatment with FXR ligand GW4064. Aromatase expression induced by PMA treatment (mimicking prostaglandin E2) was inhibited at both mRNA and protein levels by the FXR ligand GW4064, suggesting that the FXR-SHP-LRH-1 cascade is active in the breast cancer cell lines and can be used to inhibit aromatase expression. Thus, FXR is a promising target to inhibit aromatase in breast cancer therapy, particularly as it targets the alternative promoter II, which is functional in breast tissue but not in other tissues, such as bone (29).

A role for LRH-1 in the control of proliferative processes has also been proposed in several tissues (41), including the initiation of intestinal tumorigenesis (49), estrogen-induced proliferation of MCF-7 (50), and in the hepatocellular carcinoma cell line BEL-7402 (41) associated with a down-regulation of antiapoptotic gene GADD45β. Similarly, in MCF-7 cells, GW4064 suppresses GADD45β. It is, therefore, tempting to speculate from the inhibition of aromatase and GADD45β that this FXR-SHP-LRH-1 pathway is functionally active in breast cancer and may represent a tool to repress proliferation.

Our results clearly show that FXR is expressed in breast, a tissue not generally considered to be a bile acid target. The high level of expression of FXR in the duc tal epithelial cells of normal breast suggests that FXR is involved in the regulation of transport from the stroma into the milk or vice versa. Bile acids can accumulate in plasma during breast cancer and in breast cyst fluid. Whether these bile acids are the endogenous ligands for FXR, what their roles are, and whether FXR is responsible for their accumulation in the breast remains to be seen. We show here that in breast cancer cells, FXR regulates the expression of genes involved in the transport of bile acids, amino acids, and xenobiotics in the breast. Here, we find that FXR is an antiproliferative therapeutic target, mediating apoptosis in breast cancer cells and reducing the expression of aromatase, the local source of proproliferative estrogen. The function of FXR in breast cancer, via direct target gene interaction and/or SHP, promotes an antiproliferative and neutral drug-resistant state. FXR, therefore, represents both a novel endogenous system for which bile acids may limit breast cancer and a promising new therapeutic target for ductal breast cancer.

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