Down-Regulation of Pregnane X Receptor Contributes to Cell Growth Inhibition and Apoptosis by Anticancer Agents in Endometrial Cancer Cells

Hisashi Masuyama, Hideki Nakatsukasa, Norio Takamoto, and Yuji Hiramatsu

Department of Obstetrics and Gynecology, Okayama University Graduate School of Medicine, Dentist, and Pharmaceutical Science, Okayama, Japan (H.M., H.N., N.T., Y.H.)

Received May 10, 2007; accepted July 17, 2007

ABSTRACT

Recent studies have revealed that pregnane X receptor (PXR) can function as a master regulator to control the expression of drug-metabolizing enzymes, cytochrome P450 3A (CYP3A) family, and members of the drug transporter family, including multiple drug resistance 1 (MDR1). We demonstrated previously that steroid/xenobiotic metabolism by tumor tissue through the PXR-CYP3A pathway might play an important role in endometrial cancer and that PXR ligands enhance PXR-mediated transcription in a ligand- and promoter-dependent fashion, leading to differential regulation of individual PXR targets, especially CYP3A4 and MDR1. In this study, we investigated the potential contribution of PXR down-regulation by RNA interference toward the augmentation of drug sensitivity and the overcoming of drug resistance. We observed the protein levels of both CYP3A4 and MDR1 in PXR small interfering RNA (siRNA)-transfected cells were not increased in the presence of PXR ligands, paclitaxel, cisplatin, estradiol, or me droxyprogesterone acetate (MPA) compared with control siRNA-transfected cells. There was no PXR-mediated transactivation or augmentation of transcription by coactivators in the presence of these ligands. We then found that PXR down-regulation caused a significant increase in cell growth inhibition and enhancement of apoptosis in the presence of the anticancer agents, paclitaxel, cisplatin, and MPA. Finally, we demonstrated that PXR overexpression caused a significant decrease in cell growth inhibition and inhibited apoptosis in the presence of paclitaxel or cisplatin. These data suggest that PXR down-regulation could be a novel therapeutic approach for the augmentation of sensitivity to anticancer agents, or to overcome resistance to them, in the treatment of endometrial cancer.

Pregnane X receptor (PXR), a new member of the steroid receptor superfamily, has been shown to mediate the genomic effects of several steroid hormones, including progesterone, pregnenolone, and estrogen, and those of xenobiotics, and to bind to specific DNA sequences, PXR-responsive elements (PXREs) in the mouse, rat, and human (Kliewer et al., 1998; Lehmann et al., 1998; Bertilsson et al., 1998; Blumberg et al., 1998; Schuetz et al., 1999; Pascussi et al., 1999; Zhang et al., 1999). These elements are found in the upstream regions of genes in the cytochrome P450 3A (CYP3A) family (Bertilsson et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Pascussi et al., 1999), which are monooxygenases responsible for the oxidative metabolism of certain endogenous substrates and xenobiotics (Ketter et al., 1995; de Wildt et al., 1999) and in the upstream region of the multiple drug resistance 1 (MDR1) gene (Geick A et al., 2001; Synold et al., 2001), which encodes P-glycoprotein, a multidrug transporter that has a major role in drug resistance (Ambudkar et al., 2003). Because the PXR pathway is activated by a large number of prescription drugs designed to treat infection, cancer, convulsion, and hypertension (Kliewer et al., 2002), PXR is believed to play roles in drug metabolism/efflux and drug-drug interactions. Recent research demonstrated that PXR regulates the metabolism of bile acid, which is essential for the elimination of excess cholesterol from the body and the transport of dietary lipids in the intestine (Staudinger et al., 2001). These data suggest that

This work was supported in part by research grants (17591739) from the Ministry of Education, Science, and Culture of Japan and the Okayama Medical Foundation (to H.M.)

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.


ABBREVIATIONS: PXR, pregnane X receptor; PXRE, pregnane X receptor-responsive element; MDR1, multiple drug resistance 1; SRC-1, steroid receptor coactivator-1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MPA, me droxyprogesterone acetate; estradiol, 17β-estradiol; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; CAT, chloramphenicol acetyl transferase; AIB1, amplified in breast cancer 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; DMSO, dimethyl sulfoxide; tk, thymidine kinase.
PXR regulates an entire program of genes in the liver and intestine that are involved in the metabolism of potentially toxic substrates and their elimination from the body (Kliwer et al., 2002). We demonstrated previously the expression of PXR in mouse reproductive tissues, uterine and ovarian, and in the liver and intestine, and we showed that the expression levels of PXR and CYP3A1 in the liver and ovary were significantly increased in parallel with the progression of hypersteroidemia evaluated toward term during pregnancy, suggesting that PXR may play a role in the regulation of steroid hormone metabolism during reproduction (Masuyama et al., 2001). In addition, our recent data suggested that steroid/xenobiotic metabolism by tumor tissue through the PXR-CYP3A pathway might play an important role in endometrial cancer, especially as an alternative gateway for gonadal hormone and EDC effects on endometrial cancers expressing low levels of estrogen receptor α (Masuyama et al., 2003). Moreover, we have reported that PXR ligands enhance PXR-mediated transcription in a ligand- and promoter-dependent fashion, leading to the differential regulation of the expression of individual PXR targets, especially CYP3A4 and MDR1, in endometrial cancer cells (Masuyama et al., 2005).

Endometrial cancer is a highly curable malignancy when it presents as uterine-confined disease, but the prognosis for metastatic or recurrent endometrial cancer is poor. Cytotoxic chemotherapy is indicated as the frontline treatment for the majority of women with metastatic or recurrent disease. Paclitaxel in combination with cisplatin and/or doxorubicin chemotherapy improves both the response rate and the median survival (Carey et al., 2006). Moreover, hormonal therapy may be a therapeutic option in a select population of patients with well differentiated and progesterone receptor-positive tumors, minimal symptoms, or non–life-threatening advanced or recurrent endometrial cancer (Carey et al., 2006).

The effectiveness of the chemotherapy is often limited by the drug resistance of tumors, and much effort has been expended to improve our understanding of the mechanisms of the drug resistance and determine an approach to overcome this resistance (Tsuruo et al., 2003). In this study, to investigate the potential contribution of PXR down-regulation toward the augmentation of drug sensitivity and the overcoming of drug resistance, we examined whether the down-regulation of PXR affected the expression of PXR targets in the presence of PXR ligands, anticancer agents and steroids, in endometrial cancer cells. We also analyzed whether the down-regulation of PXR affected PXR-mediated transcription and checked the effect of PXR knockdown on cell growth and apoptosis in the presence of PXR ligands. We then checked the effect of PXR overexpression on cell growth and apoptosis in the presence of PXR ligands. These data suggest that the down-regulation of PXR could be a novel therapeutic approach for the augmentation of sensitivity to anticancer drugs, or to overcome resistance to them, in the treatment of endometrial cancer.

Materials and Methods

Materials. MPA and 17β-estradiol (estradiol) were purchased from Sigma Co., Ltd. (St. Louis, MO). Carboplatin, cisplatin, doxorubicin, paclitaxel and docetaxel were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). HEC-1 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan).

Cell Culture and RNA Interference. HEC-1 cells were cultured in Dulbecco’s modified Eagle’s medium without phenol red, supplemented with 10% charcoal-stripped fetal bovine serum. The medium and serum were purchased from Invitrogen (Carlsbad, CA). The siRNA cocktail targeting human PXR was purchased from B-Bridge International Inc. (Mountain View, CA), which contains three siRNAs: first sequence, ggacaagcacacggau (sense) and uuacagggcuuguacg (antisense); second sequence, aggccagacgggaaa (sense) and uuacucgcuacggcu (anti-sense); and third sequence, ggccacagacggaga (sense) and ugucuccagucugc (antisense). Negative control cocktail, which consists of noncomplementary sequence of human, mouse, and rat, and liposome for siRNA transfection (siFECTOR) were also purchased from B-Bridge International Inc. Cells were transfected with PXR siRNA or control siRNA using siFECTOR according to the manufacturer’s protocol.

Western Blot Analysis. Whole-cell extracts were obtained from cells transfected with PXR siRNA or control siRNA using M-PER Mammalian Protein Extraction Reagents (Pierce Chemical Co., Rockford, IL) according to the manufacturer’s protocol and stored at −80°C until analysis. Protein content was determined using a bichoninic acid protein assay (Pierce Chemical Co.), and equivalent amounts of nuclear protein (25 µg/sample) from each extract were solubilized in SDS buffer (0.05M Tris-HCl, 2% SDS, 6% mercapto- ethanol, and 10% glycerol, pH 6.8) and analyzed by Western blot analysis as described previously (Masuyama and MacDonald, 1998). We used a goat polyclonal antibody for PXR (1:1000 dilution) and rabbit polyclonal antibodies for MDR-1 (1:500 dilution), β-actin (1:1000 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and CYP3A4 (1:1000) (PanVera Corporation, Madison, WI). The amount of each band was quantitated densitometrically using Image Scanner GT-9500 (Epson, Suwa, Japan) and BQ 2.0 software (Bio Image, Ann Arbor, MI).

Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from endometrial cancer cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Each sample was treated with DNase I to remove genomic DNA contamination. To generate first-strand DNA, 0.1 µg of total RNA was reverse-transcribed at 42°C for 20 min in 20 µl of reaction solution containing 1× PCR buffer, 5 mM MgCl₂, 1 mM dNTPs, 2.5 µM random 9-mer primer, 10 U RNase inhibitor, and 5 U avian myeloblastosis virus reverse transcriptase, using an RNA PCR kit (TAKARA Co., Ltd., Kyoto, Japan), according to the manufacturer’s protocol. The primers used to amplify human PXR and GAPDH have been described previously (Masuyama et al., 2003, 2005). Amplification of PXR and GAPDH was carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) as described previously (Masuyama et al., 2003, 2005). The number of PCR cycles resulting in PCR products in the linear logarithmic phase of the amplification curve was determined. PCR samples were electrophoresed on 3% agarose gels and visualized by staining with ethidium bromide. The housekeeping gene GAPDH was used to control for variations in RNA recoveries from each specimen.

Transient Transfection Studies. The pcSG5-PXR expression plasmid containing full-length human PXR cDNA was kindly provided by Dr. S. A. Kliwer (Kliwer et al., 1998). The (CYP3A4)³-kcat-chromophenicol acetyl transferase (CAT) vector was generated by insertion of three copies of a double-strand oligonucleotides containing the DR-3/CYP3A4 (5′-gggtcagcaagttca-3′), and the (MDR1)³-kcat vector was generated by insertion of three copies of a double-strand oligonucleotides containing the DR-4/MDR1 (5′-gggtcagcaagttca-3′) as described previously (Goodwin et al., 1999; Geick et al., 2001). The ABI1 cDNA, which was generated by reverse transcriptase-polymerase chain reaction (RT-PCR), and the steroid hormone receptor-1 (SRC-1) cDNA, which was a gift from Dr. M. J.
Tsai (Baylor College, Houston, TX), were subcloned into pcDNA3 expression vectors (Invitrogen). Ishikawa cells and HEC-1 cells were cotransfected with 1 μg of a reporter gene construct [(CYP3A4)]3-tk-CAT or (MDR1)]3-tk-CAT) or tk-CAT vector. For coactivator expression, 1 μg of pcDNA3-SRC-1 vector, pcDNA3-amplified in breast cancer 1 (AIB1) vector, or pcDNA3 vector alone was also transfected into the cells. In all transfections, liposome-mediated transfections were accomplished using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Transfected cells were treated either with vehicle alone or with the indicated concentrations of steroid hormones or anticancer agents for 24 h. Cell extracts were prepared and assayed for CAT activity. The amount of CAT was determined using a CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Co., Tokyo, Japan) according to the manufacturer’s instructions.

**Cell Growth and Apoptosis Assay.** Transfected cells (5 × 10^3 cells/well) were seeded and incubated in a 96-well culture plate for 0, 24, 48, 72, or 96 h. Cell proliferation was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay) according to the manufacturer’s protocol (Promega Corporation, Madison, WI). For the detection of apoptosis, the Cell Death Detection ELISA kit (Roche Diagnostics Co.) was used according to the manufacturer’s protocol.

**Statistical Analysis.** Statistical analysis was performed using one-way analysis of variance followed by Dunnett’s test, as shown in Figs. 1, 4, 5, 6, and 7. Data are the means ± S.D. *P* < 0.05 denotes the presence of a statistically significant difference.

**Results**

The Effect of PXR siRNA on the Expression of PXR, CYP3A, and MDR1 in the Presence of PXR Ligands. We used HEC-1 cells as an endometrial cancer cell line for this study because PXR is abundant in this cell line (Masuyama et al., 2003). To investigate the effect of PXR siRNA on the expression of PXR, CYP3A, and MDR1 in the presence of PXR ligands, we qualitatively examined the protein levels of CYP3A4, MDR1, and PXR in HEC-1 cells that had been exposed to steroids and anticancer drugs. In this study, we used known PXR ligands, estradiol, MPA, paclitaxel, and cisplatin, and other anticancer agents, docetaxel and carboplatin, which are not ligands for PXR (Masuyama et al., 2005), as control. First, we confirmed the efficacy of PXR siRNA for the knockdown of PXR mRNA in HEC-1 cells using RT-PCR and Western blotting. Neither PXR mRNA nor PXR protein was detected in HEC-1 cells transfected with PXR siRNA (Fig. 1A). In cells transfected with PXR siRNA, no significant increases were seen in CYP3A4 and MDR1 pro-

Fig. 1. The effect of PXR siRNA on the expression of PXR, CYP3A, and MDR1 in the presence of PXR ligands. A, HEC-1 cells were transfected with PXR or control siRNA. Whole-cell extracts were prepared as described under Materials and Methods. PXR and β-actin protein levels were determined by Western blotting using anti-PXR and anti-β-actin antibodies. Total RNA was also obtained from HEC-1 cells and analyzed for the expression of PXR and GAPDH mRNAs using RT-PCR. PCR products were separated on 3% agarose gels and visualized by ethidium bromide staining. B, HEC-1 cells were transfected with PXR or control siRNAs and treated with DMSO or various anticancer agents for 36 h. Whole-cell extracts were prepared, and CYP3A4, MDR1, and PXR protein levels were determined by Western blotting using antibodies against CYP3A4, MDR1, and PXR. As a loading control, β-actin protein levels were also examined using anti-β-actin antibody. Each bar represents the mean ± S.D. from three independent experiments (∗, p < 0.01 compared with control). C, PXR or control siRNA-transfected HEC-1 cells were treated with DMSO or 10^-6 M concentrations of various steroids for 36 h. Whole-cell extracts were prepared, and CYP3A4, MDR1, PXR, and β-actin protein levels were determined by Western blotting using the same antibodies. Each bar represents the mean ± S.D. from three independent experiments (∗, p < 0.01 compared with control siRNA-treated control). D, PXR or control siRNA-transfected HEC-1 cells were treated with DMSO or 10^-6 M paclitaxel for 36 or 72 h. Whole-cell extracts were prepared, and CYP3A4, MDR1, PXR, and β-actin protein levels were determined by Western blotting using the same antibodies. Each bar represents the mean ± S.D. from three independent experiments (∗, p < 0.01 compared with control siRNA-treated control).
tein levels in the presence of the PXR ligands paclitaxel and cisplatin compared with cells treated with control siRNA (Fig. 1B). The normally positive effects of steroids, estradiol, and MPA on CYP3A4 or MDR1 expression were not observed in PXR siRNA-transfected cells (Fig. 1C); the effects of paclitaxel on CYP3A4 or MDR1 expression were not observed in PXR siRNA-transfected cells for the incubations of 72 and 36 h (Fig. 1D). We observed no nonspecific effect by siRNA because there were no differences of the PXR expression and cell growth between the control-siRNA treated cells and no siRNA-treated cells. Moreover, we observed the same results of these experiments using other endometrial cancer cell line, Ishikawa cells, in which PXR is moderately expressed (Masuyama et al., 2003) (data not shown).

The Effect of PXR siRNA on PXR-Mediated Transcription through the PXR-Responsive Elements CYP3A4 or MDR1. Next, we examined the effect of PXR siRNA on PXR-mediated transcription through PXREs, CYP3A4 or MDR1. Three different reporter gene constructs, (CYP3A4)\(^3\)-tk-CAT, (MDR1)\(^3\)-tk-CAT, and tk-CAT, were introduced into HEC-1 cells. In control siRNA-transfected cells or untransfected cells using (CYP3A4)\(^3\)-tk-CAT, the steroids 17β-estradiol and MPA significantly activated native PXR-mediated transcription. The antitumor agents cisplatin and paclitaxel also significantly activated native PXR-mediated transcription, but the fold increases were lower compared with those in the presence of steroids in these HEC-1 cells. By contrast, cisplatin and paclitaxel had a stronger effect on native PXR-mediated transcription through the MDR1-responsive element compared with steroids. The other drugs we tested, carboplatin and docetaxel, had no effect on this transcription. In cells transfected with PXR siRNA, we observed no PXR-mediated transactivation in the presence of PXR ligands. No nonspecific effects by siRNA on the transcription were observed because there were no differences of the PXR-mediated transcription between the control siRNA-transfected cells and untransfected cells and no differences of basal transcription using the tk-CAT vector among PXR siRNA-, control siRNA-transfected cells, and untransfected cells (Fig. 2).

PXR siRNA Abolished the Effect of Overexpressed Coactivator on PXR-Mediated Transcription. We used a transient reporter expression assay in HEC-1 cells to examine whether PXR siRNA abolished the effect of an overexpressed coactivator on PXR-mediated transcription. As shown in Fig. 3, the coactivators tested here enhanced PXR-mediated transcription in the presence of PXR ligands, estradiol and paclitaxel, through both PXREs (CYP3A4 and MDR1). The effect of SRC-1 on PXR-mediated transcription in the presence of estradiol was more efficient than that in the presence of paclitaxel through the CYP3A4-responsive element. By contrast, the effect of AIB1 on PXR-mediated transcription in the presence of paclitaxel was significantly increased compared with that in the presence of estradiol through the MDR1-responsive element. In cells transfected with PXR siRNA, there was no augmentation of PXR-mediated transcription because of an overexpressed coactivator in the presence of these PXR ligands through either PXRE. We observed no nonspecific effects by siRNA on the augmentation of the transcription by an overexpressed coactivator because there were no differences of the PXR-mediated transcription between the control siRNA-transfected cells and untransfected cells and no differences of basal transcription using the tk-CAT vector among PXR siRNA-, control siRNA-transfected cells, and untransfected cells.

The Effect of PXR siRNA on Cell Proliferation in the Presence of Anticancer Agents. We then examined the effect of PXR down-regulation on cell proliferation in the presence of anticancer agents. Cell viability was determined by an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxypheno)yl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, and the effect of PXR siRNA on cell growth is shown in Fig. 4. We found that the down-regulation of PXR expression significantly enhanced cell growth inhibition in the presence of the anticancer agents paclitaxel and cisplatin, which are PXR ligands (Fig. 4, A and C). On the other hand, there was no difference in cell growth inhibition by other anticancer agents, docetaxel and carboplatin, between PXR siRNA- and control siRNA-transfected cells (Fig. 4, B and D). There was no difference in cell growth between control siRNA-transfected cells and untransfected cells.

The Effect of PXR siRNA on Apoptosis in the Presence of Anticancer Agents. To investigate whether the
effect of cell growth inhibition by PXR siRNA in the presence of anticancer agents is related to the induction of apoptosis, the effect of PXR siRNA on apoptotic cell death was examined. We observed that the down-regulation of PXR significantly enhanced apoptosis in PXR siRNA-transfected cells compared with cells transfected with control siRNA or without siRNA in the presence of paclitaxel or cisplatin (Fig. 5, A and C). On the other hand, there was no difference in apoptosis induced by other anticancer agents, docetaxel and carboplatin, between PXR siRNA- and control siRNA-transfected cells (Fig. 5, B and D). There was no difference in apoptosis between control siRNA-transfected cells and untransfected cells.

The Effect of PXR siRNA on Cell Proliferation and Apoptosis in the Presence of Steroids, Estradiol, or MPA. We also examined the effect of PXR down-regulation on cell proliferation and apoptosis in the presence of steroids, MPA, and estradiol. We found that the down-regulation of PXR expression significantly enhanced cell growth inhibition in the presence of MPA and the anticancer agents paclitaxel and cisplatin (Fig. 6A). On the other hand, estradiol induced a significant increase in cell growth in PXR siRNA-transfected cells compared with control siRNA-transfected cells (Fig. 6B). We also observed that the down-regulation of PXR significantly enhanced apoptosis in PXR siRNA-transfected cells compared with cells transfected with control siRNA or untransfected cells in the presence of MPA, paclitaxel, and cisplatin (Fig. 6C). However, there was no difference in apoptosis induced by estradiol between PXR siRNA- and control siRNA-transfected cells (Fig. 6D). There was no difference in cell growth and apoptosis between control siRNA-transfected cells and untransfected cells.

Fig. 3. PXR siRNA abolished the effect of an overexpressed coactivator on PXR-mediated transcription. HEC-1 cells were transfected with PXR siRNA or control siRNA, or without siRNA, and then transiently co-transfected with 1 μg of the (CYP3A4)₃-tk-CAT, (MDR1)₃-tk-CAT, or tk-CAT reporter gene constructs in addition to 1 μg of pcDNA3-AIB1, -SRC-1, or pcDNA3 expression vectors. Cells were treated with ethanol vehicle or 10⁻⁶ M estradiol or paclitaxel for 24 h. The amount of CAT was determined using a CAT ELISA kit. The results represent the means ± S.D. of three independent experiments.

Fig. 4. The effect of PXR siRNA on cell proliferation in the presence of anticancer agents. HEC-1 cells were transfected with PXR siRNA or control siRNA, or without siRNA, and were seeded and incubated with paclitaxel (A), docetaxel (B), cisplatin (C), or carboplatin (D) in 96-well culture plates for 0, 24, 48, or 72 h. Then cell proliferation was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay) according to the manufacturer’s protocol (Promega). The results represent the means ± S.D. of three independent experiments.
The Effect of Overexpressed PXR on Cell Proliferation and Apoptosis in the Presence of Anticancer Agents. Finally, we examined the effect of overexpressed PXR on cell proliferation and apoptosis in the presence of anticancer agents. We confirmed the overexpression of PXR in cells transfected with pSG5-PXR expression vector (Fig. 7A). We found that the overexpression of PXR significantly suppressed cell growth inhibition in the presence of paclitaxel or cisplatin after incubation for 72 and 96 h (Fig. 7, B and C). Moreover, we also observed that PXR overexpression significantly inhibited apoptosis in the presence of paclitaxel or cisplatin after incubation for 72 and 96 h (Fig. 7, D and E).

Fig. 5. The effect of PXR siRNA on apoptosis in the presence of anticancer agents. HEC-1 cells were transfected with PXR siRNA or control siRNA, or without siRNA, and were seeded and incubated with paclitaxel (A), docetaxel (B), cisplatin (C), or carboplatin (D) in 96-well culture plates for 0, 24, 48, or 72 h. Then, for the detection of apoptosis, a Cell Death Detection ELISA kit (Roche Diagnostics Co.) was used according to the manufacturer's protocol. The results represent the means ± S.D. of three independent experiments.

Fig. 6. The effect of PXR siRNA on cell proliferation and apoptosis in the presence of steroids, estradiol, or MPA. HEC-1 cells were transfected with PXR siRNA or control siRNA, or without siRNA, and were seeded and incubated with estradiol (A and C) or MPA (B and D) in 96-well culture plates for 0, 24, 48, or 72 h. Then cell proliferation was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay) according to the manufacturer's protocol (Promega). For the detection of apoptosis, a Cell Death Detection ELISA kit (Roche Diagnostics Co.) was used according to the manufacturer's protocol. The results represent the means ± S.D. of three independent experiments.
Discussion

We demonstrated previously that steroid/xenobiotic metabolism by tumor tissue through the PXR-CYP3A pathway might play an important role in endometrial cancer (Masuyama et al., 2003) and that PXR ligands enhance PXR-mediated transcription in a ligand- and promoter-dependent fashion, leading to the differential regulation of the expression of individual PXR targets, especially CYP3A4 and MDR1 (Masuyama et al., 2005). The CYP3A subfamily is involved in the metabolism of endogenous substrates such as steroid hormones and bile acids (Ketter et al., 1995; de Wildt et al., 1999). This subfamily also plays important roles in the metabolism of procarcinogens and pharmaceutical agents, including innumerable drugs, chemical carcinogens, mutagens, and other environmental contaminants (Ketter et al., 1995; de Wildt et al., 1999; Masuyama et al., 2000). MDR1 was originally identified because of its overexpression in cultured cancer cells associated with an acquired cross-resistance to multiple anticancer drugs and has been shown to be an ATP-dependent efflux pump of hydrophobic anticancer drugs (Ambudkar et al., 2003). It was reported recently that MDR1 is also regulated by PXR (Geick et al., 2001; Synold et al., 2001). Some endometrial carcinomas and normal endometrial controls from both proliferative and secretory phases of the menstrual cycle overexpress P-glycoprotein, which is encoded by the MDR1 gene (Schneider et al., 1993). Here, we show that the protein levels of neither CYP3A4 nor MDR1 were increased in cells transfected with PXR siRNA in the presence of PXR ligands, paclitaxel, carboplatin, estradiol, and MPA compared with cells treated with control siRNA. In addition, we observed no PXR-mediated transactivation or augmentation of transcription by coactivators in the presence of any PXR ligands. These data suggest that PXR ligands affect the expression of CYP3A4 and MDR1 genes through a PXR-mediated pathway and that the PXR-CYP3A4 and PXR-MDR1 pathways are blocked by PXR down-regulation in endometrial cancer cells.

A number of tumor cells, including endometrial cancer cells, have been reported to undergo apoptotic cell death when treated with chemotherapeutic agents such as Adriamycin, vincristine, etoposide, cisplatin, and paclitaxel (Bhalla, 2003; Tsuruo et al., 2003). Apoptosis and antiapoptosis pathways are related to drug sensitivity and resistance (Tsuruo et al., 2003). Next, we checked the effects of PXR knockdown on cell growth and apoptosis in the presence of PXR ligands. We found that down-regulation of PXR expression significantly enhanced cell growth inhibition and apoptosis in the presence of the anticancer agents paclitaxel and cisplatin, indicating that down-regulation of PXR in cancer cells might alter the effects of anticancer agents in the treatment of endometrial cancer. On the contrary, PXR overexpression caused a significant decrease in cell growth inhibition and inhibited apoptosis in the presence of paclitaxel or cisplatin. Because paclitaxel has been identified as a substrate of CYP3A4 and P-glycoprotein (Harris et al., 1994; Sparreboom et al., 1997) and the role of P-glycoprotein in mediating paclitaxel resistance in tumors has also been shown (Wu et al., 2003, Penson et al., 2004), PXR down-regulation might play a novel therapeutic role in the sensitivity of endometrial cancer cells to paclitaxel and their resistance to it through the inhibition of drug metabolism and/or transport/efflux of this drug. However, it is unclear whether MDR1-dependent protection is activated under cytotoxic conditions induced by cisplatin, which is not a substrate for MDR1 (Takara et al., 2002). Recent review indicated that cisplatin is a substrate for CYP3A4/5 (Harmsen et
suggesting that PXR down-regulation might inhibit the drug metabolism of cisplatin through PXR-CYP3A4 pathway, and the cells might become sensitive to cisplatin. Further analysis, including functional experiments tying the block in MDRI protein induction to a loss in cell surface efflux function or in drug metabolism, is required to clarify the mechanism underlying the enhancement of cell growth inhibition and apoptosis by PXR down-regulation in the presence of paclitaxel or cisplatin. A recent report showing that PXR levels are directly related to drug resistance in osteosarcoma cell lines also supports our data (Mensah-Osman et al., 2007). Although a number of clinical trials have attempted to alter P-glycoprotein activity and thus improve clinical outcomes, the majority of these studies showed no clearcut success (Dalton et al., 1995; Sonneveld et al., 2001; Leonard et al., 2003). Further analysis, including in vivo experiments, is required to show the potential of this PXR down-regulation for clinical use.

Hormonal therapy using MPA might be effective for the treatment of patients with endometrial cancer with well differentiated and progesterone receptor-positive tumors, minimal symptoms, or nonlife threatening advanced or recurrent endometrial cancer; however, trials to date have not been adequately powered to determine significant differences in survival (Carey et al., 2006). We demonstrated that down-regulation of PXR also enhances cell growth inhibition and apoptosis in the presence of MPA, suggesting that the down-regulation of PXR might enhance the effect of MPA on endometrial cancer cells. Because MPA is a substrate for CYP3A4 (Kobayashi et al., 2000), the PXR pathway might play an important role in mediating the metabolism/efflux of MPA in endometrial cancer cells. In addition, we observed that the down-regulation of PXR enhanced cell proliferation but did not affect apoptosis in the presence of estradiol, suggesting that endometrial cancer cells respond more effectively to estrogen under conditions of PXR down-regulation. Moreover, we have also demonstrated a significant inverse correlation between PXR and estrogen receptor in cases of endometrial cancer (Masuyama et al., 2003). Because estradiol is also a substrate for CYP3A4 (Tsuciya et al., 2005) and relatively high concentration of estradiol is required to enhance PXR-mediated transcription (Masuyama et al., 2003), PXR-mediated pathways might play an important role in the steroidogenesis of endometrial cancer cells under a local high-stEROid hormone condition, and the PXR level in tumors might affect the response to estrogen.

In summary, we examined whether the down-regulation of PXR affected the expression of PXR targets and PXR-mediated transcription in endometrial cancer cells. In cells transfected with PXR siRNA, neither CYP3A4 nor MDRI protein levels were increased in the presence of the PXR ligands, paclitaxel, cisplatin, estradiol, and MPA compared with cells treated with control siRNA. Moreover, we observed no PXR-mediated transactivation or augmentation of transcription by coactivators in the presence of PXR ligands. We also checked the effect of PXR knockdown on cell growth and apoptosis. We found that down-regulation of PXR expression caused a significant increase in cell growth inhibition and an enhancement of apoptosis in the presence of the anticancer agents paclitaxel and cisplatin. We also investigated the effects of PXR overexpression on cell growth and apoptosis.

Overexpression of PXR caused a significant decrease in cell growth inhibition and inhibited apoptosis in the presence of paclitaxel or cisplatin. These data suggest that down-regulation of PXR could be a novel therapeutic approach for the augmentation of sensitivity to anticancer agents, or to overcome resistance to them, in the treatment of endometrial cancer.

Acknowledgments

We gratefully thank Dr. Steven A. Kliewer for providing the CYP3A4 reporter vector.

References

Masuyama H and MacDonald PN (1998) Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the Af-2 domain of VDR. J Cell Biochem 71:429–440.


**Address correspondence to:** Dr. Hisashi Masuyama, 2-5-1, Shikata, Okayama, Okayama, 700-8558, Japan. E-mail: masuyama@cc.okayama-u.ac.jp