Farnesoid X receptor regulates the growth of renal adenocarcinoma cells without affecting that of a normal renal cell-derived cell line

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ABSTRACT — The farnesoid X receptor (FXR) is a bile acid-activated nuclear receptor which is abundant in the liver, intestine, and kidney. FXR is a pivotal factor in cholesterol/bile acid homeostasis but is involved in the growth of hepatocellular carcinoma cells. In the present study, we investigated whether FXR is also involved in the growth of renal adenocarcinoma cells. The cell growth of renal adenocarcinoma cell line ACHN was inhibited by FXR knockdown and stimulated by FXR ligand, while that of a normal renal cell-derived cell line, HK-2, was not affected. The carcinoma-specific stimulation of cell growth by FXR was found to arise from down-regulation of p53 and p21/Cip1 mRNA expression. Our study showed that FXR stimulates proliferation of renal adenocarcinoma cells and that FXR knockdown is useful for growth suppression of renal adenocarcinoma without cytotoxicity to normal renal cells.

Key words: Farnesoid X receptor, Cyclin-dependent kinase inhibitors, Carcinoma-specific cytotoxicity, Renal adenocarcinoma cells

INTRODUCTION

The Farnesoid X receptor (FXR) is a bile acid-activated nuclear receptor (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999) which is abundant in the liver, intestine, kidney, and adrenal gland (Forman et al., 1995; Seol et al., 1995; Glass et al., 1994; Huber et al., 2002). FXR is a pivotal factor in cholesterol/bile acid homeostasis (Zhang and Edwards, 2008) but is also involved in the growth of hepatocellular carcinoma cells (Fujino et al., 2012). FXR down-regulates cyclin-dependent kinase (CDK) inhibitors p16/INK4a and p21/Cip1, resulting in the stimulated growth of hepatocellular carcinoma cells. In contrast, FXR neither affects CDK inhibitor expression nor growth of the normal hepatocyte-derived cell line. In the present study, we investigated whether FXR is also involved in the growth of renal adenocarcinoma cells. FXR knockdown in the renal adenocarcinoma cell line ACHN stimulated p21/Cip1 expression and inhibited cell growth, similar to its effect on hepatocellular carcinoma cells. In contrast, FXR knockdown in the normal renal cell-derived cell line HK-2 did not affect CDK inhibitor expression or cell growth. Our study showed that FXR knockdown is useful for the suppression of renal adenocarcinoma growth without cytotoxicity to normal renal cells.

MATERIALS AND METHODS

Materials

Antibodies (Abs) specific for β-actin (C-2), FXR (D-3), p21/Cip1 (H-164), and p16/INK4a (H-156) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. GW4064 was from TOCRIS bioscience, Ellisville, MO, USA. ECL™ anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep) and ECL™ anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey) were purchased from GE Healthcare, Buckinghamshire, UK.

Cell culture

ACHN is a renal adenocarcinoma cell line derived from a 22 year-old man (ATCC No. CRL-1611), 786-o is a renal adenocarcinoma cell line derived from a 58 year-old man (ATCC No. CRL-1670), and 786-o is a renal adenocarcinoma cell line derived from a 58 year-old man (ATCC No. CRL-1670).
old man (ATCC No. CRL-1932), and HK-2 is a proximal tubular cell line derived from normal human kidney. The cells were immortalized by transduction with human papilloma virus 16 (Ryan et al., 1994) (ATCC No. CRL-2190). These cell lines were obtained from ATCC and maintained in Dulbecco’s modified eagle medium (DMEM) containing 10% fetal calf serum (FCS), 50 units/mL penicillin G sodium salt, and 50 μg/mL streptomycin sulfate and cultured in a humidified atmosphere of 8.5% CO₂ at 37°C.

RNA interference experiments

Custom HP siRNA was purchased from Qiagen K.K. (Tokyo, Japan) and used to knockdown the expression of FXR as described below.

Based on the report by Sirvent et al. (2004), custom siRNA against FXR was prepared as follows. The mixture of the sense (5’-GUCGUGACUUUGCGACAAGTT-3’) and the anti-sense (5’-CUUGUCGCAAGUCAGCAGCT-3’) oligonucleotides were denaturated at 90°C and cooled down for annealing and used to knockdown FXR. In order to knock down endogenous FXR, cells were seeded on 60-mm dishes at a density of 2.0 × 10^5 cells (ACHN) or 4.0 × 10^5 cells (HK-2) per dish and transfected with siRNA against FXR (100 nM) using HiPerfect Transfection Reagent (Qiagen) according to the manufacturer’s instructions. After incubating for 24 hr, 48 hr, or 72 hr, the cell number was counted, and protein extracts for immunoblotting analyses or total RNA for real-time PCR analyses were prepared. In the RNA interference experiments, “Nonsilencing Control” siRNA (#1022076) from Qiagen was used as the control.

Immunoblotting

Cells were washed with PBS, and cell extracts were prepared using SDS sample buffer without loading dye. After normalization of protein content using the protein assay, the dye was added to the samples, followed by SDS-PAGE and immunoblotting analyses. For the detection of p16/INK4a, p21/Cip1, and β-actin, the membranes were incubated with the primary antibody (Santa Cruz Biotechnology) for 2 hr. Immune complexes on the PVDF membranes were visualized with enhanced chemiluminescence Western blotting reagent (Qiagen) according to the manufacturer’s instructions. After incubating for 24 hr, 48 hr, or 72 hr, the cell number was counted, and protein extracts for immunoblotting analyses or total RNA for real-time PCR analyses were prepared. In the RNA interference experiments, “Nonsilencing Control” siRNA (#1022076) from Qiagen was used as the control.

Quantification of mRNAs

mRNA was quantified by real-time PCR. Briefly, 5 μg of total RNA was reverse-transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Biosciences). The resultant cDNA was then subjected to real-time PCR analysis using a TaqMan Gene Expression Assay kit (Applied Biosystems, Tokyo, Japan). mRNA levels were determined using TaqMan assay mixtures as follows: FXR (Hs00231968), p16/INK4a (Hs99999189), p21/Cip1 (Hs01121172), and β-actin (4310881E). TaqMan assay mixture Hs00231968 can detect all of the four isoforms of FXR (Zhang et al., 2004). Amplification and quantification were performed using the PRISM 7000 Real-Time PCR System (Applied Biosystems). FXR, p16/INK4a, and p21/Cip1 mRNA levels were normalized to those of β-actin mRNA, which was the internal control. Data were analyzed using Student’s t-test.

RESULTS AND DISCUSSION

FXR stimulates the cell growth of renal adenocarcinoma cells without affecting that of the normal renal cell-derived cell line

In order to examine whether FXR is involved in the growth of renal adenocarcinoma cells, we performed FXR knockdown in the renal adenocarcinoma cell line ACHN and assessed cell growth. As shown in Fig. 1B, the cell growth of FXR-knocked down (Fig. 1A) cells was significantly lower than that of control cells, indicating that
FXR stimulates the cell proliferation of renal adenocarcinoma ACHN cells without affecting that of normal renal cell-derived HK-2 cells. A and B: Renal adenocarcinoma ACHN cells seeded at a density of $2.0 \times 10^5$ cells or normal renal-derived HK-2 cells at a density of $4.0 \times 10^5$ cells per 60-mm dish were transfected with a control siRNA or siRNA against human FXR and cultured for 72 hr. Total RNA extracted from ACHN and HK-2 cells was subjected to real-time RT-PCR analysis to quantify FXR mRNA levels. C and D: ACHN cells seeded at a density of $2.0 \times 10^5$ cells or HK-2 cells at a density of $4.0 \times 10^5$ cells per 60-mm dish were treated with DMSO as a control or 10 μM GW4064 for 72 hr. Cell number was counted at the indicated times. [methyl-$^3$H] thymidine incorporation in cells was measured from 48 hr to 72 hr. Data are shown as the mean ± S.D. of four determinations and analyzed by Student’s t-test.

Fig. 1. FXR regulates the growth of renal adenocarcinoma ACHN cells without affecting that of normal renal cell-derived HK-2 cells. A and B: Renal adenocarcinoma ACHN cells seeded at a density of $2.0 \times 10^5$ cells or normal renal-derived HK-2 cells at a density of $4.0 \times 10^5$ cells per 60-mm dish were transfected with a control siRNA or siRNA against human FXR and cultured for 72 hr. Total RNA extracted from ACHN and HK-2 cells was subjected to real-time RT-PCR analysis to quantify FXR mRNA levels. C and D: ACHN cells seeded at a density of $2.0 \times 10^5$ cells or HK-2 cells at a density of $4.0 \times 10^5$ cells per 60-mm dish were treated with DMSO as a control or 10 μM GW4064 for 72 hr. Cell number was counted at the indicated times. [methyl-$^3$H] thymidine incorporation in cells was measured from 48 hr to 72 hr. Data are shown as the mean ± S.D. of four determinations and analyzed by Student’s t-test.
FXR is involved in the growth of renal adenocarcinoma cells. FXR activation stimulated cell growth of ACHN cells (Fig. 1C), supporting the data on FXR-knocked down cells. All results obtained from ACHN cells were reproduced in an assay using renal adenocarcinoma cells 786-o (data not shown). In contrast to renal adenocarcinoma cells, neither FXR knockdown nor FXR activation affected the growth of the normal renal cell-derived cell line, HK-2 (Fig. 1B). Additionally, FXR stimulated the proliferation of renal adenocarcinoma cells, since FXR ligand stimulated the DNA synthesis of ACHN cells without affecting that of HK-2 cells (Fig. 1D). These results indicate that FXR stimulates the cell growth of renal adenocarcinoma cells without affecting the growth of the normal renal cell-derived cell line.

**FXR down-regulates the expression of CDK inhibitor p21/Cip1 in renal adenocarcinoma cells without altering that of the normal renal cell-derived cell line**

We previously reported that FXR stimulates the growth of hepatocellular carcinoma cells by down-regulating CDK inhibitors p16/INK4a and p21/Cip1 (Fujino et al., 2012) while it does not affect the growth of normal liver-derived cells (Fujino et al., 2015a). p16/INK4a and p21/Cip1 are also involved in the growth of renal cells because up-regulation of these CDK inhibitors in the renal proximal tubule-derived cell line, HK-2, inhibited cell growth (data not shown). Given that FXR stimulated the cell growth of renal adenocarcinoma cells without affecting that of the normal renal cell-derived cell line, we determined the expression of p16/INK4a and p21/Cip1 in these cell lines treated with siRNA for FXR or FXR ligand. FXR knockdown in renal adenocarcinoma ACHN cells caused an increase in mRNA (Fig. 2A) and protein (Fig. 2B) levels of p21/Cip1. In contrast, p21/Cip1 mRNA (Fig. 2A) and protein (Fig. 2B) levels in normal renal cell-derived HK-2 cells were not altered by FXR knockdown. As for p16/INK4a, we failed to detect p16/INK4a mRNA and protein of ACHN cells because of its marginal expression level, while p16/INK4a mRNA (Fig. 2A) and protein (Fig. 2B) levels in HK-2 cells were not affected by FXR knockdown. Consistent with the results from FXR knockdown experiments, FXR activation reduced mRNA (Fig. 2C) and protein (Fig. 2D) levels of p21/Cip1 in ACHN cells. In HK-2 cells, FXR activation did not affect p21/Cip1 mRNA (Fig. 2C) or protein (Fig. 2D) levels. Thus, FXR down-regulates the expression of p21/Cip1 and stimulates the growth of renal adenocarcinoma cells, while it does not alter the protein expression of CDK inhibitors or cell growth of normal renal cells.

**FXR down-regulates the expression of p53 in renal adenocarcinoma cells without altering that of normal renal cell-derived cell line**

Given that FXR down-regulates the expression of p21/Cip1 in renal adenocarcinoma cells without altering that in normal renal cell-derived cells, we examined the upstream factor for p21/Cip1 in order to reveal the mechanism that causes the different susceptibility of these cells to FXR ligand. A tumor suppressive factor, p53, is known to cause G1 arrest that inhibits cell growth by up-regulating expression of p21/Cip1 (el-Deiry et al., 1993). Indeed, we previously reported that natural compounds derived from *Digitalis purpurea* inhibited the growth of ACHN cells by up-regulating p53 expression (Fujino et al., 2015b). Thus, we determined the p53 level in FXR-activated ACHN and HK-2 cells. As shown in Fig. 3, p53 mRNA (Fig. 3A) and protein (Fig. 3B) levels of ACHN cells were decreased by the FXR ligand while that of HK-2 cells was not altered.

Overall, the growth of renal adenocarcinoma cells and normal renal cells are differently regulated by FXR. In renal adenocarcinoma cells, FXR activation down-regulates p53 expression, resulting in a reduction in p21/Cip1. In contrast, p53 and p21/Cip1 expression in normal renal cells is not altered by FXR activation. Thus, FXR stimulates the cell growth of renal adenocarcinoma cells only. In turn, FXR knockdown may be useful in the treatment of renal cancer as an agent which does not cause cytotoxicity to normal renal cells.

In hepatic cells, FXR causes a decrease in p21 mRNA in both normal liver-derived cells and hepatocellular carcinoma cells. While p21/Cip1 protein of hepatocellular carcinoma cells is decreased by the FXR ligand, down-regulation of p21/Cip1 mRNA by FXR ligand does not result in a decrease in p21/Cip1 protein in normal liver-derived cells. Thus, FXR ligand stimulates cell growth of hepatocellular carcinoma cells without affecting that of normal liver-derived cells (Fujino et al., 2015a). In contrast, FXR ligand exhibits carcinoma-specific effect on p21/Cip1 mRNA levels in renal adenocarcinoma cells (Fig. 2C). As shown in Fig. 3, carcinoma specificity probably arises from down-regulation of p53 expression in renal adenocarcinoma cells. It is known that p53 expression is negatively regulated by oncogenic miRNA miR-21 (Wu et al., 2016). Interestingly, we recently obtained preliminary data showing that FXR ligand up-regulates miR-21 expression in ACHN cells without affecting that in HK-2 cells (data not shown). Thus, the stimulation of growth of renal adenocarcinoma cells by FXR ligand may...
FXR down-regulates the expression of p21/Cip1 in renal adenocarcinoma cells without affecting that in normal renal cell-derived cells. A and B: ACHN cells seeded at a density of $2.0 \times 10^5$ cells or HK-2 cells at a density of $4.0 \times 10^5$ cells per 60-mm dish were transfected with a control siRNA or siRNA against human FXR and cultured for 72 hr. C and D: ACHN cells seeded at a density of $1.0 \times 10^5$ cells or HK-2 cells at a density of $2.0 \times 10^5$ cells per 60-mm dish were treated with DMSO as a control or $10 \mu$M GW4064 for 72 hr. Total RNA extracted from ACHN and HK-2 cells at the indicated times was subjected to real-time RT-PCR analysis to quantify p21/Cip1 and p16/INK4a mRNA levels (A and C). Total protein extracted from ACHN and HK-2 cells at the indicated times was subjected to immunoblotting analyses to detect p21/Cip1, p16/INK4a, and β-actin (B and D). Data are shown as the mean ± S.D. of four determinations and analyzed by Student’s t-test.

Fig. 2. FXR regulates the growth of renal adenocarcinoma cells without affecting that in normal renal cell-derived cells.
arise from the down-regulation of p53 translation in a miR-21-dependent manner.

There are several reports that state FXR regulates the proliferation of hepatocellular and other carcinoma cells (Zhang et al., 2012; He et al., 2015; Guo et al., 2015; Guan et al., 2013; Xie et al., 2016). Zhang et al. (2012) and He et al. (2015) have reported FXR inhibits the proliferation of hepatocellular carcinoma cell line HepG2 based on the data showing FXR ligand GW4064 reduced the value of MTT assay. However, we have obtained preliminary data showing the FXR ligand reduces the value of MTT assay although it stimulates DNA synthesis and increases the cell number of HepG2 and Huh7 cells (data not shown). Thus, in these cells treated with the FXR ligand, the value of MTT assay does not reflect proliferation. Guo et al. (2015) have also reported the FXR ligand inhibits the proliferation of HepG2 cells by showing cell cycle arrest caused by the FXR ligand, however, they performed the FXR ligand treatment in the medium containing very low concentration of FBS. Since our previous study in HepG2 cells (Fujino et al., 2012) and the present study have been performed in the medium containing 10% FCS sufficient for cell growth, our data is not comparable with the data obtained from Guo et al. Consistent
with our previous study (Fujino et al., 2012), Xie et al. (2016) have reported FXR ligand GW4064 stimulates cell proliferation of HepG2 cells by analyzing DNA synthesis. As similar to hepatocellular and renal carcinoma cells, FXR knockdown inhibits cell proliferation of esophageal cancer cells (Guan et al., 2013). They have shown Ki-67 expression, cell proliferation marker, is down-regulated by FXR knockdown.

This study is the first to show that FXR regulates the growth of renal adenocarcinoma cells. Given that the level of bile acid, a natural ligand for FXR, is supposed to be marginal in renal cells, the stimulation of growth of renal adenocarcinoma cells may be caused by an unknown ligand for FXR.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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