Vitamin K2 Suppresses Proliferation and Motility of Hepatocellular Carcinoma Cells by Activating Steroid and Xenobiotic Receptor

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Abstract. Vitamin K2, known as a cofactor for γ-carboxylase, also serves as a ligand of a nuclear receptor, Steroid and Xenobiotic Receptor (SXR). Several clinical trials revealed that vitamin K2 reduced de novo formation and recurrence of hepatocellular carcinoma (HCC). To examine the role of SXR in HCC as a receptor activated by vitamin K2, the cells stably overexpressing SXR were established using a HCC cell line, HuH7. Overexpression of SXR resulted in reduced proliferation and motility of the cells. Further suppression of proliferation and motility was observed when SXR overexpressing clones were treated with vitamin K2. These results suggest that the activation of SXR could contribute to tumor suppressive effects of vitamin K2 on HCC cells.

Key words: Vitamin K2, Hepatocellular carcinoma, Steroid and xenobiotic receptor

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UGT1A1, and MDR1, which are involved in drug metabolism and excretion, are known as SXR target genes reflecting its function as a xenobiotic sensor [9]. We showed vitamin K2 binds to SXR and induced SXR target genes [8]. Moreover, tsukushi, one of the vitamin K2-dependent SXR target genes in osteoblasts was involved in collagen accumulation, suggesting that vitamin K2 contributes to bone strength partially through the activation of SXR [13].

In the present study, we hypothesized vitamin K2 improves prognosis of HCC through the activation of SXR. Therefore, we analyzed the effects of SXR overexpression and stimulation with vitamin K2 on the HCC cells. We here demonstrate vitamin K2 suppressed proliferation and motility of HuH7 HCC cells, and these effects required the expression of SXR.

Materials and Methods

Plasmids

FLAG-tagged human SXR (FLAG-SXR) and tk-(Cyp3A4)₃-Luc containing three-copy SXR responsive elements from human cytochrome P-450 (Cyp) 3A4 promoter were described previously [13]. FLAG-HA-tagged human SXR was generated by site directed mutagenesis using primers (forward: 5'-p-GAGGTGA GCCCAAAGAAAGCTGG-3', reverse: 5'-CTCTGA CACTTCCGGCTAGTGCGACGCCTAGGG TATCCTCCATCGAGCTTG-3') and FLAG-SXR as a template.

Cell culture and transfection

HuH7 is obtained from RIKEN Cell Bank (Tsukuba, Japan). HepG2 and LS180 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). HuH7, HepG2, and LS180 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS), at 37°C under 5% CO₂. HuH7 and HepG2 cells were cultured in phenol red-free DMEM with 5% charcoal/dextran-treated FCS for at least 24 h prior to rifampicin (Nakalai Tesque, Kyoto, Japan), MK-4 (gifted by Eisai Co., Ltd., Tokyo, Japan), or vehicle (0.1% ethanol) treatment. Transfection was performed using FuGENE 6 (Roche, Castle Hill, Australia). To establish stable transfectants, HuH7 clones were selected using G418 (SIGMA, St. Louis, MO, USA) at a concentration of 250 µg/mL.

Reverse-transcription PCR analysis

Total RNA was isolated using the TOTALLY RNA kit (Ambion, Austin, TX, USA). First strand cDNA was generated by using PrimeScript RT reagent Kit (TAKARA, Kyoto, Japan). For PCR amplification, the primer sequences are as follows: human SXR (forward: 5’-CCTTTGACACTACCTTCTCC-3’, reverse: 5’-GTTGACACAGCTCGAAAGCG-3’); human 18S rRNA: (forward: 5’-AAGCATTTGCCAAGAATGTTT-3’, reverse: 5’-TTAAGTTTCAGCTTTGCAACCA-3’). mRNAs were quantified by real-time PCR using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) and the 7500 Fast Real-Time PCR system (Applied Biosystems). The evaluation of relative differences of PCR product amounts among the treatment groups was carried out using 18S rRNA as an internal control.

Luciferase Assay

Luciferase assay was performed using HuH7 cells (1 x 10⁶ cells/well on 24-well plates) transfected with 200 ng of tk-(Cyp3A4)₃-Luc, 200 ng of pRL-CMV (Promega, Madison, WI, USA), and 200 ng of FLAG-HA-pcDNA3 or FLAG-HA-tagged SXR plasmids using the FuGENE 6 reagent (Roche). When ligand stimulation was done, cells were treated with 10 µM rifampicin (Nakalai Tesque), MK-4 (Eisai Co., Ltd.), or vehicle (0.1% ethanol) for 1 h prior to transfection. Luciferase activities were determined by a Mithras LB 940 microplate luminometer (Berthold Technologies; Bad Wildbad, Germany) using the dual-luciferase assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase, which was used as a transfection control.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 1,000 cells/well. The viable cell number was quantified by tetrazolium salt, WST-8 (Nakalai Tesque) that could be converted to a water-soluble formazan by metabolically active cells. Spectrophotometric absorbance for formazan dye was measured at 450 nm, with absorbance at 655 nm as reference using microplate reader (BIO-RAD, Hercules, CA, USA).
is shown as a positive control. These results indicate that endogenous SXR is expressed in HCC cell lines, like in some of colorectal carcinoma cell lines, though the level of expression varies among cell lines.

**Cell migration assay**

The cell migration assay was performed as previously described [14]. Briefly, the number of HuH7 cells migrating through a polyethylene terephthalate (PET) filter with 8-µm pores (Beckton Dickinson, Franklin Lake, NJ, USA) in 24 h was counted under microscopic examination.

**Statistical analyses**

Differences between the mean values were analyzed using the Student’s t-test.

**Results**

**Detection of endogenous expression of SXR in HCC cell lines**

To test the hypothesis that vitamin K2 affect the character of HCC cells by regulating SXR induced genes, the expression of SXR in HCC cells were evaluated by Reverse Transcription-PCR analysis. Messenger RNA of SXR was detected in HepG2 and HuH7, HCC cells, though expression level of SXR in HuH7 was relatively low (Fig. 1). Expression level of SXR mRNA in a colorectal carcinoma cell line, LS180, is shown as a positive control. These results indicate that endogenous SXR is expressed in HCC cell lines, like in some of colorectal carcinoma cell lines, though the level of expression varies among cell lines.

**Ligand dependent promoter activation of exogenous SXR in HuH7 cells**

To analyze SXR function in HCC cells, HuH7 cells, which have relatively low expression of SXR were transfected with SXR expression vector and the effect of vitamin K2 (Menatetrenone; MK-4) was evaluated with dual luciferase reporter assay. The effect of SXR agonist, rifampicin (RIF) was also examined. SXR responsive element in the promoter of Cyp3A4 gene, a known SXR target gene, was used as reporter plasmid. In SXR transfected cells, basal activity of promoter was increased even with vehicle stimulation (Fig. 2). The significant enhancement of promoter activity was observed with rifampicin or MK-4 stimulation in SXR transfected cells (Fig. 2). Activation of endogenous SXR was not detected in this method (Fig. 2).

**Growth suppression in SXR overexpressing cells**

To examine the role of SXR in HCC cells, the clone stably overexpressing SXR was established. Relative expression of SXR in the clone overexpressing exog-
Suppression of motility of SXR overexpressing cells

We then focused on the effects of SXR on motility of HuH7 cells. The SXR overexpressing clone (HuH7-SXR) and the control clone (HuH7-vec) were seeded on collagen coated filter with 8 μm pores in DMEM with 10% FCS. The cell growth was assayed using WST-8 tetrazolium salt. The number of migrating cells of the SXR overexpressing clone was significantly smaller than that of vector clone (Fig. 4A). Then migration assay was performed in the presence of rifampicin or MK-4. Phenol red free DMEM with charcoal/dextran-treated FCS (5%) were used to eliminate potential ligands contained in the medium. The SXR overexpressing clone showed decreased motility than the vector clone (HuH7-vec) in the vehicle treatment (Fig. 4B). Stimulation of cells with rifampicin or MK-4 further suppressed migration in the SXR overexpressing clone, while rifampicin or MK-4 did not significantly affect the motility of the vector clone (Fig. 4B).

We then evaluated the effect of rifampicin and MK-4 on the motility of HepG2 cells where relatively much amount of endogenous SXR was expressed. Both of the SXR ligand, rifampicin and MK-4, sup-
VITAMIN K AFFECTS HCC CELLS VIA SXR

Discussion

In the present study, we showed expression and activation of steroid and xenobiotic receptor SXR suppressed proliferation and motility of hepatocellular carcinoma HuH7 cells. Our results support the hypothesis that a novel mechanism of vitamin K2, activation of SXR, contributes to efficacy of first and second prevention of HCC.

Liver is one of the organs in which SXR is mainly expressed. However, whether SXR is expressed in HCC cells has not been studied so far. We demonstrated in this study that expression of SXR was detectable by RT-PCR and that the level of expression varied among cell lines. Considering HCC arises from hepatocytes, it is reasonable that some HCC cells inherit expression pattern of hepatocytes. The activity of endogenous SXR was not detected in HuH7 cells by luciferase assay using tk-(Cyp3A4)_Luc. Together with the results of vector clones in growth assay and motility assay, amount of SXR molecules in HuH7 cells we used may be not enough to cause visible effect. HepG2 cells, in which we could detect higher amount of endogenous SXR mRNA compared with HuH7 cells (Fig. 1), showed suppressed motility with SXR ligand stimulation. We could speculate that the response to MK-4 in HCC cells may depend on the expression level of SXR. This result also suggested that effects of MK-4 through SXR activation can be observed in HCC cells other than HuH7 cells.

In our study, we employed the MK-4 concentration mainly at 10 μM. Plasma concentration of single administration of pharmacological dose (15 mg) of menatetrenone in healthy male volunteers was reported to be approximately 1 μM [15]. In the experiment using rat, menatetrenone was shown to accumulate in liver at more than 10 times concentration than plasma concentration after oral administration [16]. Therefore, 10 μM is considered to be an adequate concentration...
to evaluate pharmacological effect of menatetrenone.

As for the mechanism of anti-tumor effect of vitamin K2, several theories were proposed. Otsuka et al. reported that vitamin K2 inhibited the growth and motility of HCC by activation of protein kinase A and by inhibition of Rho kinase [17]. Suzuki et al. showed Des-gamma-carboxyl prothrombin (DCP), also known as PIVKAII, induced proliferation of HCC [18]. Some of the mechanisms are explained by the function of vitamin K2 as a co-enzyme for γ-carboxylase. Besides these mechanisms of vitamin K2 action, we propose another mechanism through the activation of SXR. Considering the weaker effect of vitamin K2 on the vector clone compared with SXR overexpressing clone in the present study, we assume that vitamin K2 affects growth and motility of HCC mainly by activating SXR in some conditions. This notion is further supported by the results indicating rifampicin, an SXR agonist, also suppressed the motility of HuH7-SXR and HepG2. Rifampicin also tended to suppress the growth of HuH7-SXR, though this effect was not statistically significant. We assume, unlike vitamin K2 specific γ-carboxylase dependent mechanism, SXR dependent mechanism is not specific for vitamin K2 and other potential ligands can trigger this effect.

As for the downstream of SXR, we consider that the function of SXR would be mediated by interaction with other transcription factors, or by induction of SXR responsive genes. For example, Ozaki et al. recently reported that vitamin K2 inhibited HCC growth through inhibition of NF-κB activation [19]. On the other hand, Zhou et al. showed mutual repressive relationship between SXR and NF-κB [20]. Therefore, it is tempting to speculate that the inhibition of NF-κB by vitamin K2 could be mediated by activation of SXR.

It is noteworthy that function of SXR might be regulated not only by its ligands. Our result of luciferase assay showed that promoter activity was elevated only with SXR expression even in the absence of ligands and further enhanced with ligand treatment. In the present study, phenol red-free DMEM and charcoal/dextran-treated FCS were used to minimize the effect of potential ligands contained in the medium. Although suppression of proliferation and motility in HuH-SXR compared with HuH-vec even without ligand stimulation can be due to residual ligands in the medium, it is also possible that other factors such as modification of SXR molecule affect the transcriptional activity or interaction with other transcription factors.

In conclusion, we showed that activation of SXR suppresses proliferation and motility of HCC cells. These results suggest that vitamin K2 prevents occurrence and recurrence of HCC not only by activating γ-carboxylase pathway but also by a novel mechanism, activating SXR. Interacting molecules and target genes of SXR should be clarified to understand detailed mechanism of SXR functions and to find novel therapeutic targets of HCC.

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References


