Abstract

Activation of farnesoid X receptor enhances osteoblastic differentiation

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Introduction : Farnesoid X receptor (FXR), a member of the nuclear receptor superfamily, functions as bile acid sensor in the control of bile acid homeostasis. While FXR has been shown to play an important role in metabolic diseases, including diabetes mellitus, dyslipidemia and atherosclerosis, the role of bile acids in bone remodeling is not clear.

Method : The role of FXR in the regulation of osteoblast differentiation was investigated using a series of bile acids. Endogenous FXR expression was compared between murine mesenchymal cells and pre-osteoblastic cells using RT-PCR. Finally, changes in alkaline phosphatase (ALP) activity were monitored during osteoblastic differentiation of mesenchymal cells after treatment with FXR agonists.

Results : FXR was expressed in mesenchymal stem cells (C3H10T1/2) and pre-osteoblastic cells (MC3T3.E1), and gradually increased during osteoblastic differentiation. ALP activity was increased in C3H10T1/2 cells cultured in osteogenic medium and treated with blie acids (6-ethylchenodeoxycholic acid (6-ECDCA) and chenodeoxycholic acid (CDCA)) or synthetic FXR agonists (GW4064 and fexaramine). In addition, mRNA expression levels of the COL1, ALP, BSP, Runx2 and Osx genes were significantly increased by treatment of C3H10T1/2 cells with bile acids (6-ECDCA or CDCA) or FXR agonists (GW4064 or fexaramine).

Conclusion : These results indicate that activation of FXR may enhance osteoblastic differentiation.

Keywords: Farnesoid X Receptor (FXR), bile acids, osteoblast, nuclear receptor

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Introduction

Farnesoid X receptor (FXR, also known as NR1H4), an “adopted” member of the nuclear receptor superfamily of ligand-activated transcription factors, was originally identified as a receptor activated by elevated concentrations of farnesol, an intermediate in the mevalonate pathway (1). Bile acids have been identified as physiological ligands of FXR, and potently activate the transcription of FXR target genes (2,3). Chenodeoxycholic acid (CDCA), a hydrophobic bile acid, is one of the strongest agonist of FXR, whereas the hydrophilic bile acids ursodeoxycholic acid (UDCA) and muricholic acid fail to activate this receptor (2). Increases in the intracellular concentration of bile acids lead to transcriptional activation of FXR via the formation of a heterodimer with the retinoid X receptor (RXR) and the subsequent binding of this heterodimer to the inverted repeat-1 (IR-1) DNA response element (4-7). This binding subsequently leads to regulation of the expression of genes encoding a number of biosynthetic enzymes involved in bile acid synthesis, detoxification and excretion (8). In this manner, FXR functions as a bile acid sensor to play a central coordinating role in the regulation of bile acid homeostasis. In addition, FXR also plays a crucial role in the regulation of glucose metabolism (9), insulin sensitivity (10) and lipid metabolism, as well as in atherosclerosis (11). As a result, modulators of FXR activity have been extensively studied as novel therapeutic targets for numerous metabolic disorders. A growing body of evidence shows that several nuclear receptors regulate skeletal homeostasis through their direct effects on osteoblasts or osteoclasts. Haploinsufficiency of the peroxisome

proliferator activated receptor-γ (PPARγ) results in increased bone mass by stimulating osteoblastogenesis in bone marrow progenitors (12) and our group has shown that targeted *in vivo* overexpression of PPARγ in osteoblast using the collagen type 1 promoter results in reduced bone mass gain in male mice and accelerated bone loss after ovariectomy in female mice (13). In addition, Remen et al. have demonstrated that activation of liver X receptor (LXR) inhibits receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclast differentiation (14). Furthermore, small heterodimer partner (SHP), encoded by one of the target genes of FXR, has been shown to stimulate bone formation by enhancing Runt-related transcription factor-2 (Runx2) transcriptional activity and augmenting osteoblast differentiation (15).

Given that FXR regulates diverse metabolic pathways, and that other nuclear receptors play important roles in the regulation of bone homeostasis, it can be reasonably speculated that FXR also plays a role in the regulation of bone metabolism. Although FXR is mainly expressed in classical bile acid target organs such as the liver and intestine, recent studies have shown that it is also expressed in the kidneys, adrenal glands (1,16), bone marrow stromal cells and SaOS2 osteoblast-like cells (17). Moreover, CDCA-activated FXR has been shown to regulate Runx2-stimulated osteoblastic differentiation and to inhibit adipogenesis in SaOS2 cells *in vitro*. In this study, I examined the endogenous expression of FXR in mesenchymal stem cells during osteogenic differentiation and investigated the effect of FXR agonists on osteoblastogenesis.

Materials and methods

Cell culture and differentiation

Murine embryonic mesenchymal C3H10T1/2 osteoblastic/stromal cells (American Type Culture Collection, Manassas, VA) are pluripotent cells that retain an immature appearance. C3H10T1/2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. During osteoblastic differentiation studies, cells were cultured at 37°C in DMEM containing 10% FBS supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate.

The MC3T3.E1 cell line (RIKEN cell bank, Tsukuba, Japan) is a clonal pre-osteoblastic cell line derived from newborn mouse calvaria. MC3T3.E1 cells were grown in DMEM with 10% FBS. After the cells reached confluence, they were cultured at 37°C in differentiation medium [DMEM with 10% FBS containing 50 μg/ml ascorbic acid and 10 mM β -glycerophosphate].

Reagents

The bile acid, chenodeoxycholic acid (CDCA) and 6-EDCA, a derivative of CDCA, were purchased from R&D system (Minneapolis, MN). The synthetic FXR agonists, GW4064 and fexaramine were purchased from Tocris Bioscience (Ellisville, MI). All reagents for quantitative PCR gene expression assays were from Applied Biosystems (Carlsbad, CA). Cell culture supplies

were obtained from Invitrogen (Carlsbad, CA) or Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Quantitative real-time PCR

Mesenchymal C3H10T1/2 and pre-osteoblastic MC3T3.E1 cells were cultured and washed three times with 1x PBS after which TRIzol was directly applied to the cells. In each case, RNA was isolated and quantified according to the manufacturer's protocol (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA using a Reverse Transcription System kit (Promega, Madison, WI). cDNA was amplified using the TaqMan Universal PCR Master Mix (Applied Biosystems) with TaqMan gene expression specific primer/probes (Applied Biosystems) for mouse FXR (Mm01240553_m1, FAM dye). Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) was used as an endogenous control. Amplification was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative quantification of data was carried out using the standard curve method or the comparative CT method (18). mRNA expression of osteoblast specific marker genes [Collagen type 1 (COL1)a1, Alkaline phosphatase (ALP), Bone sialoprotein (BSP), Runx2, and Osterix (Osx)] was quantified by PCR using the SYBR Green PCR technology using an ABI PRISM 7900 HT sequence detection system (Applied Biosystems). The primers used are listed in Table 1. PCR conditions were as follows: 10 sec at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 56°C, 30 sec at 72°C. Relative gene expression was measured by calculating expression levels relative to that of β-actin.

In vitro osteogenic differentiation study

For osteogenic differentiation studies, cells were plated in 12-well plates and mineralization was initiated at confluence with osteogenic medium (OM). For activation of FXR, natural bile acids (CDCA, 6-ECDCA) or synthetic agonists (GW4064, fexaramine) ligands were added with OM during differentiation. Medium was replaced every three other days and maintained for 10 days.

Alkaline phosphatase(ALP) assay

To assess ALP activity, cells were washed three times with ice-cold PBS (pH 7.4) and scraped immediately. Enzyme activity assays were performed in assay buffer (10 mM $MgCl_2$ and 0.1 M alkaline buffer, pH10.3) containing 10mM p-nitrophenylphosphate as a substrate. The absorbance was read at OD405. Relative ALP activity is defined as mmol of p-nitrophenolphosphate hydrolyzed per min per mg of total protein. ALP staining was carried out using an alkaline phosphatase kit according to the manufacturer's instructions (Promega,Southampton,U.K.).

Changes of FXR expression during osteogenic differentiation

C3H10T1/2 cells were maintained for 21 days in OM or control medium (CM). mRNA expression levels of FXR in the OM and CM groups were analyzed by real-time RT-PCR at 3, 7, 11, 14 and 21 days.

Statistical Analysis

All data are presented as mean ± S.E. Unpaired *t*-tests were performed using GraphPad Instat Software (GraphPad Software, San Diego, CA). A significance level of 5% was assigned to all tests.

Table 1. Sequences of PCR primers used to amplify each of the genes in RT-PCR

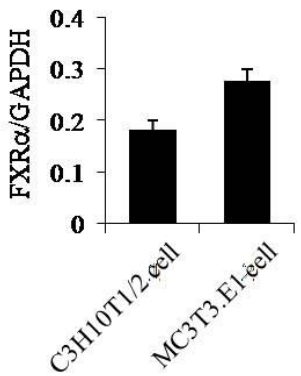
Gene		Primer Sequence (5'->3')
FXR	Forward	GAGAGATGGGGATGTTGGC
	Reverse	GAGTTCCGTTTTCTCCCTGC
β-actin	Forward	TGGGTATGGAATCCTGTGGC
	Reverse	CCAGACAGCACTGTGTTGGC
Osteoblast marker		
Runx2	Forward	CTGTGGTAACCGTCATGGCC
	Reverse	GGAGCTCGGCGGAGTAGTTC
Osterix (Osx)	Forward	ATTGAATTTGGAGGAATGGT
	Reverse	CTTGAAGTACGTGTAACGTG
Alkaline phosphatase (ALP)	Forward	TCAGGGCAATGAGGTCACATC
	Reverse	CACAATGCCCACGGACTTC
Collagen type 1 (COL1)a1	Forward	GCGAAGGCAACAGTCGCT
	Reverse	CTTGGTGGTTTTGTATTCGATGAC

Results

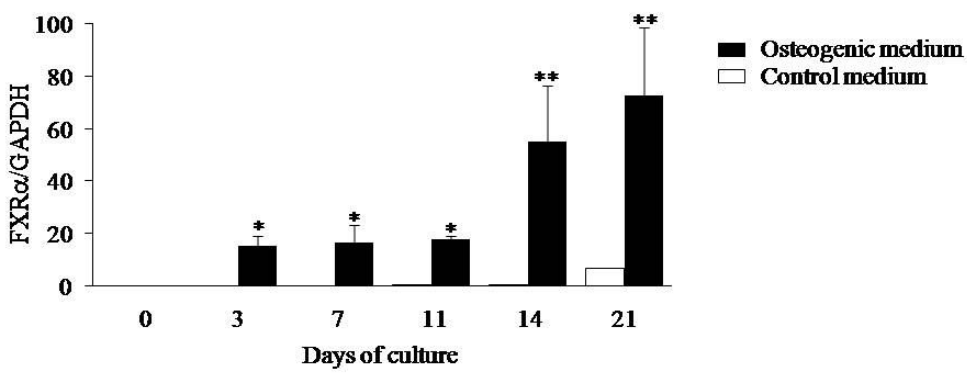
Expression of endogenous FXR in bone cells and their precursors (Figure 1)

To assess the functional role of FXR in bone metabolism, endogenous expression of FXR was examined in progenitor cells by real-time PCR. As shown in Fig. 1A, FXR mRNA was expressed in mesenchymal C3H10T1/2 cells and at higher levels in osteoblastic MC3T3.E1 cells. FXR mRNA was Induced in a time-dependent manner (0, 3, 7, 11, 14 and 21 days of culture) in response to osteoblastic differentiation of C3H10T1/2 cells (Fig. 1B).

Figure 1. Endogenous expression of FXR in C3H10T1/2 and preosteoblastic MC3T3.E1 cells.



A



B

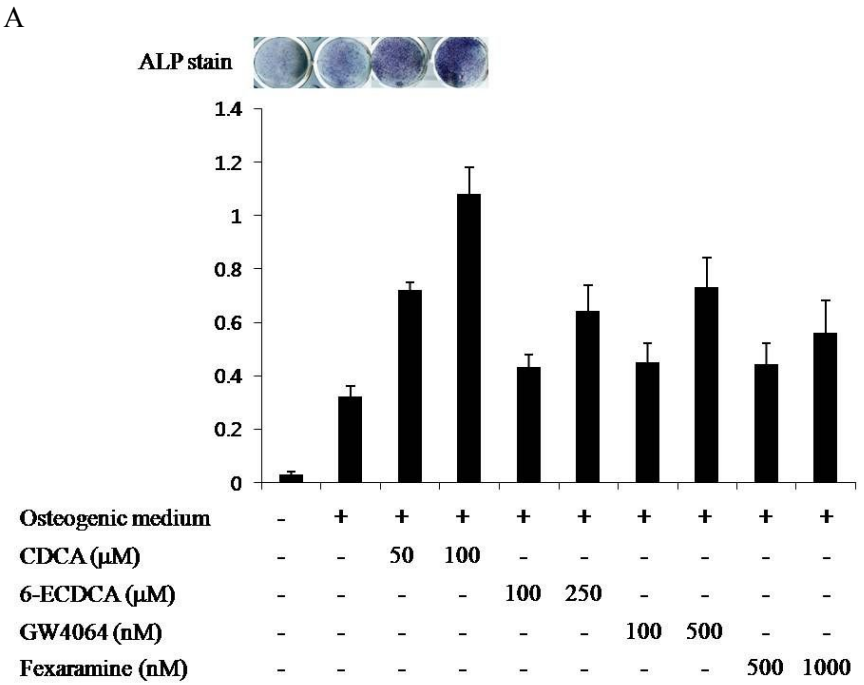
Endogenous expression of FXR was measured by real time PCR in (A) murine mesenchymal C3H10T1/2 and preosteoblastic MC3T3.E1 cells. (B) Changes in mRNA expression of FXR during osteogenic differentiation of C3H10T1/2 cells. $^{*}p<0.05$, $^{**}p<0.01$ control medium.

Bile acids lead to activated osteoblastogenesis (Figure 2)

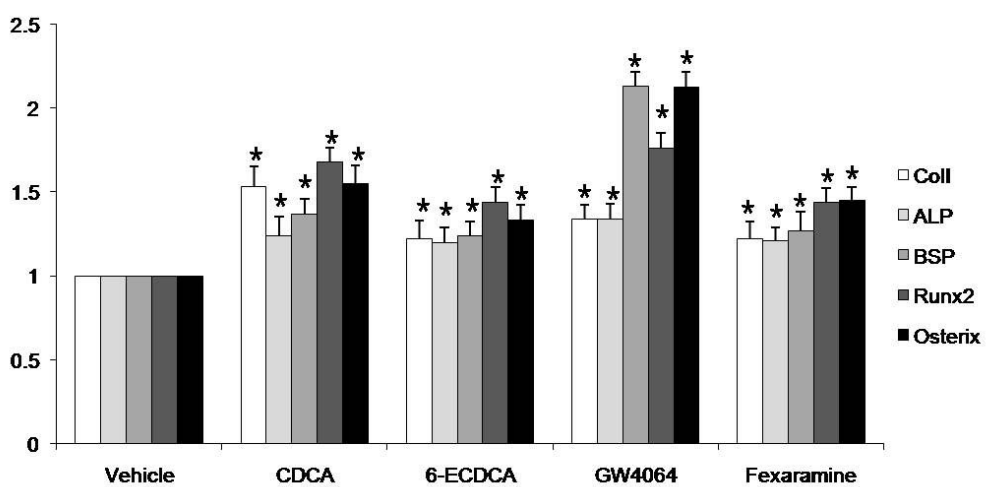
We next investigated the effect of bile acids and FXR agonists on osteoblastic differentiation of mesenchymal progenitors. ALP activity was induced 10-fold relative to control cells in C3H10T1/2 cells cultured in OM for 10 days. Dose-dependent increases in ALP activity were observed in cells treated with the bile acid, CDCA, the semisynthetic bile acid, 6-ECDCA and the synthetic FXR agonists, GW4064 and fexaramine (Fig.2A). Consistent with increased ALP activity, increased intensity in ALP staining was also observed in cells treated with these compounds. Furthermore, mRNA expression of osteoblast-specific genes, such as Collagen type 1 (COL1)a1, ALP, bone sialoprotein (BSP), Runx2, and osterix (Osx) were also significantly increased by treatment for 5 days with each compound (Fig. 2B).

Figure 2. Effects of FXR on osteoblastic differentiation *in vitro*.

A



B



(A) Alkaline phosphatase (ALP) activity was determined and normalized to

protein content. C3H10T1/2 cells were cultured with OM in the absence or presence of CDCA (50 or 100 μM), 6-ECDCA (100 or 250 μM), GW4064 (100 or 500 nM), or fexaramine (500 or 1000 nM) for 10 days as indicated.

*$p<0.05$ vs. vehicle

(B) Total RNA was extracted from the cells grown for 5 days in the presence of bile acids or FXR agonists as indicated. The amount of mRNA for osteoblast products was determined by real-time quantitative PCR and is expressed as the mRNA abundance relative to vehicle treatment. *$p<0.05$ vs. vehicle.

Discussion

In this study, we showed that FXR is expressed during osteogenesis in cultured bone progenitor cells. In addition, activation of FXR with bile acids and FXR agonists potently stimulated osteogenic differentiation in murine pluripotent cell cultures.

Although the expression of FXR in osteoblast has only recently been documented (17), uptake and accumulation of the secondary bile acid salt, sodium deoxycholate (DC), was previously identified in MG63 osteosarcoma cell lines (19). In the latter study, the authors demonstrated that bone cell-derived DC is initially released from liver before being taken up by osteoblasts, suggesting a possible link between bile acids and bone metabolism.

Interestingly, we found that the expression level of FXR was higher in preosteoblastic MC3T3-E1 cells compared with mesenchymal C3H10T1/2 cells. In addition, culturing of C3H10T1/2 cells with OM resulted in a gradual induction of FXR expression. These results strongly suggest that FXR may play a role in the osteoblastogenic differentiation of mesenchymal cells.

Evidence presented in this report has clinical implications in that FXR may be utilized as a therapeutic target in the treatment of osteoporosis. Indeed, FXR has become an attractive target for drug development in diseases of the metabolic syndrome such as diabetes mellitus (20), dyslipidemia and atherosclerosis. Moreover, selective bile acid receptor modulators have been extensively studied as a potential drug for metabolic diseases (6,21). With

the addition of the data in this study, there is a strong argument for including osteoporosis as one of the target metabolic diseases in which modulation of FXR has therapeutic potential. That said, several limitations remain to be addressed in current drug development strategies for FXR. Firstly, since the molecular mechanisms underlying the FXR metabolic actions are very complex and differ between individual pathways in different tissues (reviewed in ref. (21)), the systemic use of simple FXR agonists or antagonists could have undesired adverse effects. Another major issue is the existence of species-specific differences in FXR biology. For instance, guggulsterone, a natural FXR antagonist derived from a tree resin, increases HDL-cholesterol levels and decreases serum TG levels in rats (22,23), but has no effect in dyslipidemic patients (24).

In conclusion, this study has demonstrated that activation of FXR enhances osteoblastic differentiation *in vitro,* and suggests a potential use of bile acids and FXR agonists for the treatment of osteoporosis

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요약 (국문 초록)

서론: Farnesoid X receptor(FXR) 은 핵수용체 상과 중에 하나로, 담즙산의 항상성을 조절하는 센서로서의 역할을 한다. FXR 은 당뇨나 이상지질혈증, 동맥경화증 등의 대사 질환에서 중요한 역할을 하는 것으로 보이나 아직까지 골대사에 어떤 역할을 하는지는 잘 알려져 있지 않다.

방법: 우리는 담즙산 및 FXR 효현제를 사용하여 FXR이 조골 세포 분화를 조절하는데 있어서 어떤 역할을 하는지에 대해 조사해보고자 하였다.

결과: 중간엽 줄기 세포인 C3H10T1/2 세포와 조골세포 전구세포인 MC3T3.E1 을 골형성 배지 (50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate) 에서 조골세포로 분화시키는 동안에 FXR 의 내인성 표현이 점차 증가하는 것을 RT-PCR 을 사용하여 확인하였다. 또한 C3H10T1/2 세포를 골형성 배지에서 분화시키면서 6-Ethylchenodeoxycholic acid (6-ECDCA), chenodeoxycholic acid (CDCA) 혹은 FXR 효현제인 GW4064, fexaramine을 첨가하였을 때, 의미있게 ALP 활성이 증가하였으며 그 첨가량을 증량함에 따라 ALP 활성도 증가하였다. 또한, C3H10T1/2 세포에 6-ECDCA, CDCA, GW4064, fexaramine을 첨가한 경우, 조골세포 특이 표지 유전자들 (Col1, ALP, BSP, Runx2 and osterix)의 mRNA 표현이 증가하였다.

결론: 상기 결과로 보았을 때, FXR이 활성화 될수록 조골세포로의 분화를 강화시키는 것으로 추정된다.

주요어: Farnesoid X Receptor(FXR), 담즙산, 조골세포, 핵수용체

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