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A novel osteogenic helioxanthin-derivative acts in a BMP-dependent manner

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Abstract

To effectively treat serious bone defects using bone regenerative medicine, there is a need for the development of a small chemical compound that potently induces bone formation. We now report a novel osteogenic helioxanthin-derivative, TH. TH induced osteogenic differentiation in MC3T3-E1 cells, mouse primary osteoblasts, and mouse embryonic stem cells. The combination of TH and bone morphogenetic protein (BMP) 2 induced the mRNA expression of osteoblast marker genes and calcification in primary fibroblasts. The TH induced the mRNA of the inhibitor of DNA-binding 1 (Id-1), and its osteogenic effect was inhibited by Smad6 or Noggin. Furthermore, TH induced the mRNA expression of Bmp4 and Bmp6. These data suggest that TH exerts its potent osteogenic effect in a BMP-dependent manner by enhancing the effects of the existing BMPs and/or increasing the expression of Bmp4 and Bmp6. TH may help establish a more efficient bone regeneration system.

Keywords: Bone regeneration; Small compound; Osteogenic differentiation; Osteoblast; Fibroblast; ES cell; BMP

The treatment of serious bone defects remains a great challenge, and bone regenerative medicine has been drawing attention for the treatment of such defects [1]. The local transplantation of autologous multipotent cells has been widely used for bone regeneration [1,2]. For sufficient and timely bone formation, however, the current system requires the addition of exogenous signaling factors including BMPs [3], Hedgehogs [4], Runx2 [5], transforming growth factors (TGFs) [6], fibroblast growth factors (FGFs) [7], and vascular endothelial growth factors (VEGFs) [8]. These factors were locally applied using direct protein delivery or viral gene delivery.

Direct protein delivery, however, can suffer from protein instability and inadequate post-translational modifications of the recombinant proteins [9]. For example, although the use of BMPs has been extensively studied for bone regeneration, a large amount of BMP is required, and BMP-containing devices fail in a certain percentage of cases, raising concerns over costs and safety [10–12]. As Franceschi et al. pointed out, the reason may be due to its short biological half-life [13]. As for viral gene delivery, their clinical use is severely limited due to the potential risk of immunogenic responses and the difficulty in manipulation and mass production.

Thus, there is a clear need for the development of a small chemical compound that directly or indirectly induces bone formation. Despite recent successes with drugs inhibiting bone resorption, there is a limited number of reports on such anabolic agents that effectively increase bone formation. Statins [14], isoflavone derivatives [15,16], and TAK-778 [17] were reported to stimulate osteogenic differentiation, but their osteogenic activity was shown

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only in specific cell types including osteoblastic cells and stem cells. We hypothesize that, if we are able to identify a small compound that potently induces osteogenic differentiation, we may be able to broaden the cell sources for cell transplantation and thereby establish a more efficient bone regeneration system.

Through screening the small compound library of Takeda Chemical Industries, 4-(4-methoxyphenyl)pyrido[4′,3′:4,5]thieno[2,3-*b*]pyridine-2-carboxamide (TH) was found to induce alkaline phosphatase (ALP) activity in MC3T3-E1 cells. This study was aimed to investigate the osteogenic effect of TH and its molecular mechanism using an *in vitro* culture system.

Materials and methods

Reagents and vectors. TH (Fig. 1A) was synthesized at Takeda Chemical Industries (Osaka, Japan). The recombinant human (rh) BMP2 was provided by Astellas Pharma, Inc. (Tokyo, Japan). Noggin/Fc chimera was purchased from Sigma–Aldrich (St. Louis, MO); the anti-phospho-Smad1/5/8 antibody and anti-Smad1 antibody from Cell Signaling Technology (Beverly, MA); the anti-Runx2 antibody from MBL (Nagoya, Japan); the anti-actin antibody from Sigma–Aldrich; and the HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG from Promega (Madison, WI). The adenoviral vectors expressing Smad6 (AxSmad6), plasmids expressing mouse Runx2 and 12xGCCG-luc were generous gifts from K. Miyazono (the University of Tokyo); the plasmids expressing Cbfb were from T. Komori (Nagasaki University); and the 1050Oc-luc from G.S. Stein (University of Massachusetts Medical School).

Cell culture. The MC3T3-E1 cells and NIH3T3 cells were obtained from the Riken Cell Bank (Tsukuba, Japan); and the human dermal fibroblasts (hDFs) from Cambrex (East Rutherford, NJ). The heterozygous Runx2-null (Runx2+/−) mice were kindly provided by Dr. M.J. Owen (Wales College of Medicine). The homozygous Runx2-null (Runx2−/−) and wild-type (WT) mESs were isolated de novo as already described [18]. The primary osteoblasts (POBs) were isolated from the WT C57BL/6N mice as already described [19]. Isolation of the mouse DFs (mDFs) was performed as already described [20]. The NIH3T3 cells, mDFs, and hDFs were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) containing 10% fetal bovine serum (FBS, Sigma–Aldrich) and 1% penicillin/streptomycin (Sigma–Aldrich) (10% FBS/DMEM); the MC3T3-E1 cells and POBs were maintained in αMEM containing 10% FBS and 1% penicillin/streptomycin (10% FBS/αMEM). For the osteogenic culture, these cells were cultured in 10% FBS/DMEM containing 50 μg/mL ascorbic acid phosphate (AsAP), 10 mM β-glycerophosphate (β-GP), and 0.1 μM dexamethasone (Dex) (osteogenic medium). For the serum-free osteogenic culture, the hDFs were cultured in DMEM containing ITS+1 (Sigma–Aldrich), 50 μg/mL AsAP, 10 mM β-GP, and 0.1 μM Dex (serum-free osteogenic medium). Alkaline phosphatase (ALP) and von Kossa stainings were performed as already described [21]. Maintenance of the isolated ES cells and induction of their subsequent differentiation were performed as already described [22].

Real-time RT-PCR analysis. The total RNA was extracted using an ISOGEN Kit (Wako Pure Chemicals Industry, Ltd., Tokyo, Japan) and treated with DNase I (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After reverse-transcription using Takara RNA PCR Kit (AMV) version 2.1 (Takara Shuzo Co., Shiga, Japan), PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and QuantiTect SYBR Green PCR Master Mix (Qiagen). All reactions were run in triplicate and the mRNA copy number of a specific gene in the total RNA was calculated as already described [21]. All data were expressed as means ± SDs of triplicate wells. The primer sequences are available upon request.

Immunoblot analysis. For preparation of the whole cell lysates, the cells were lysed using the M-PER Kit (Pierce Chemical Co., Rockford, IL). The separated extraction of the cytoplasmic and nuclear proteins was performed using the NE-PER Kit (Pierce Chemical Co.). Immunoblotting

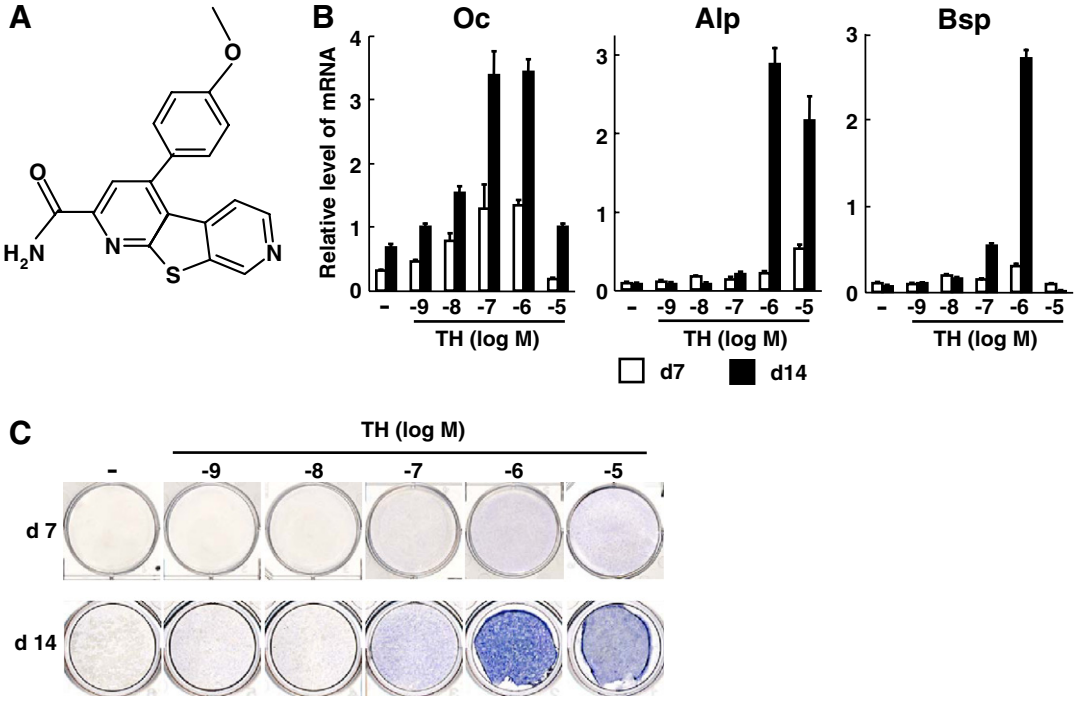

Fig. 1. Osteogenic induction of MC3T3-E1 cells by TH. (A) Chemical structure of TH. (B) mRNA expression of osteoblast marker genes in MC3T3-E1 cells treated with TH determined by real-time RT-PCR analysis. Oc, osteocalcin; Alp, alkaline phosphatase; Bsp, bone sialoprotein. (C) ALP staining of MC3T3-E1 cells treated with TH.