Bone 51 (2012) 158–167

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Interaction of Tmem119 and the bone morphogenetic protein pathway in the commitment of myoblastic into osteoblastic cells

Ken-ichiro Tanaka [a,b], Yoshifumi Inoue [a], Geoffrey N. Hendy [c,d,e,f], Lucie Canaff [c,d,e,f], Takenobu Katagiri [g], Riko Kitazawa [h], Toshihisa Komori [i], Toshitsugu Sugimoto [b], Susumu Seino [a,j], Hiroshi Kaji [a,j,k,*]

[a] Division of Diabetes, and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
[b] Internal Medicine 1, Shimane University Faculty of Medicine, Izumo 693-8501, Japan
[c] Department of Medicine, Montréal, Québec, Canada H3A 1A1
[d] Department of Physiology, McGill University, Montréal, Québec, Canada H3A 1A1
[e] Department of Human Genetics, McGill University, Montréal, Québec, Canada H3A 1A1
[f] Calcium Research Laboratory and Hormones and Cancer Research Unit, Royal Victoria Hospital, Montréal, Québec, Canada H3A 1A1
[g] Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, Hidaka, Saitama 350-1241, Japan
[h] Division of Diagnostic Molecular Pathology, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
[i] Department of Cell Biology, Unit of Basic Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8501, Japan
[j] Division of Cellular and Molecular Medicine, Department of Physiology and Cellular Biology, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
[k] Department of Physiology and Regenerative Medicine, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan

ARTICLE INFO

Article history:
Received 6 October 2011
Revised 23 January 2012
Accepted 15 April 2012
Available online 2 May 2012

Edited by: M. Noda

Keywords:
Tmem119
Bone morphogenetic protein
Osteoblast
Myoblast
Fibrodysplasia ossificans progressiva
Muscle ossification

ABSTRACT

Bone morphogenetic proteins (BMPs) are critical for bone regeneration and induce ectopic bone formation in vivo. The constitutively activating mutation (R206H) of the BMP type 1 receptor, activin A type 1 receptor/activin-like kinase 2 (ACVR1/ALK2), underlies the molecular pathogenesis of fibrodysplasia ossificans progressiva (FOP) in which heterotopic ossification occurs in muscle tissue. In the present study, we performed a comparative DNA microarray analysis between stable empty vector- and ALK2(R206H)-transfected mouse myoblastic C2C12 cells. Forty genes were identified whose expression was increased > 3.5 times in the experimental group versus the control. The bone formation-related factor, Tmem119, was included in this group. Osteoblast differentiation markers and mineralization were enhanced in C2C12 cells stably expressing Tmem119. Differentiation of myoblastic cells into myotubes was suppressed but differentiation into chondrocytes was little affected. Transcriptional activity of the BMP-2 signaling molecules, Smad1/5, was increased even in the absence of exogenous BMP-2. Endogenous BMP-2 levels positively correlated with Tmem119 levels. A BMP-2/4 neutralizing antibody and dorsomorphin, an ALK2 inhibitor, antagonized Tmem119-enhanced alkaline phosphatase (ALP) levels. Tmem119 siRNA antagonized the BMP-2-induced ALP and osteocalcin, but not Runx2 and Osterix, mRNAs, in C2C12 cells. In conclusion, Tmem119 levels were increased by the FOP-associated constitutively activating ALK2 mutation in myoblasts. The data show that Tmem119 promotes the differentiation of myoblasts into osteoblasts and the interaction with the BMP signaling pathway likely occurs downstream of Runx2 and Osterix in myoblasts. Tmem119 may play a critical role in the commitment of myoprogenitor cells to the osteoblast lineage.

Introduction

Fibrodysplasia ossificans progressiva (FOP; MIM#135100) is a rare autosomal dominant disorder of skeletal malformations and progressive extraskeletal ossification [1]. A heterozygous constitutively activating mutation (c.617G>A; p.R206H) in a bone morphogenetic protein (BMP) type I receptor, the activin receptor type I (ACVR1; MIM#102576)/activin-like kinase 2 (ALK2), is found in patients having the classic form of FOP [2]. Constitutive activation of the BMP signaling molecule Smad1 or Smad5 induces ectopic bone formation in FOP [3]. A recent study has revealed that the mild activation of the BMP signaling pathways by the ALK2(R206H) mutant in the absence of exogenous BMP is due to its impaired binding to FKBP1A/FKBP-12, a molecule that is known to safeguard against the leakage of TGF-β or BMP signaling, and an altered subcellular distribution such that the ALK2(R206H) mutant is mainly at the plasma membrane regardless of stimulation with BMP-2 [4]. The ALK2(R206H)

* Corresponding author at: Department of Physiology and Regenerative Medicine, Kinki University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. Fax: +81 72 366 2853.
E-mail addresses: ken1nai@med.shimane-u.ac.jp (K. Tanaka), ino4423@med.kobe-u.ac.jp (Y. Inoue), geoffrey.hendy@mcgill.ca (G.N. Hendy), lucie.canaff@gmail.com (L. Canaff), katagiri@saitamamed.ac.jp (T. Katagiri), riko@med.kobe-u.ac.jp (R. Kitazawa), komorit@nagasaki-u.ac.jp (T. Komori), sugimoto@med.shimane-u.ac.jp (T. Sugimoto), seino@med.kobe-u.ac.jp (S. Seino), hkaji@med.kindai.ac.jp (H. Kaji).

8756-3282/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2012.04.017

mutant also sensitizes mesenchymal cells to BMP-induced osteoblast differentiation and bone formation [5]. Yu et al. reported that a selective inhibitor of ALK2 reduces activation of Smad1, Smad5 and Smad8 in mouse tissues expressing constitutively active ALK2 induced by injection of adenovirus-driven Cre recombinase [6]. However, the details of the heterotopic ossification of muscle in FOP remain to be fully elucidated.

BMP-2 was originally identified as a polypeptide that induces ectopic bone and cartilage formation in extraskeletal tissues in vivo [7,8]. Extensive studies have demonstrated that BMP-2 plays important roles in bone formation and bone cell differentiation [9–12]. BMPs are the only factors that induce bone regeneration and ectopic bone formation in vivo. BMPs act through a complex of type I and type II transmembrane serine/threonine kinase receptors. The ligand bound type II receptor phosphorylates the type I receptor, which activates Smad1/5/8. Activated Smad1/5/8 molecules form a complex with the common Smad, Smad4. The complex translocates to the nucleus and regulates transcription of genes important for controlling the induction of bone formation.

Mouse Tmem119 is a 280 amino acid single pass type 1a membrane protein with a signal peptide. Tmem119 was detected in perichondrium, trabecular bone and stratum osteogenicum in periosteum in mouse embryos [13]. Tmem119 mRNA is expressed in several tissues including blood, bone, brain, intestine, kidney, liver, lung, skin and uterus in mice (IGID: 2703244: Unigene Mm.41681). We previously showed that inhibition of ERK1/2 enhanced Smad3-induced bone anabolic action in osteoblasts [14]. Tmem119 was identified in a comparative DNA microarray analysis between empty vector-transfected and ERK1/2 inhibitor-treated stably Smad3-overexpressing mouse osteoblastic MC3T3-E1 cells [15]. We studied Tmem119 further because of its rapid induction by parathyroid hormone (PTH) independent of later increases in endogenous TGF-β. Although Tmem119 promotes osteoblast differentiation [15,16], the precise mechanisms by which Tmem119 induces the differentiation and how it affects the commitment of undifferentiated mesenchymal stem cells into osteoblasts, chondrocytes and myocytes are not known.

In the present study, we performed a comparative DNA microarray analysis between empty vector-transfected and ALK2 with the R206H mutation [ALK2(R206H)]-transfected mouse myoblastic C2C12 cells. Tmem119 was identified as one of the factors which were enhanced by ALK2(R206H) and detected in muscle cells. Here, we investigated its role in the differentiation of myoblastic cells into osteoblasts, chondrocytes and myocytes.

Materials and methods

Materials

Human (h) recombinant bone morphogenetic protein-2 (BMP-2), anti-β-actin, anti-troponin T antibodies, actinomycin D and cycloheximide were obtained from Sigma-Aldrich Corp (St. Louis, MO). Anti-Runx2, anti-alkaline phosphatase (ALP), anti-phosphorylated Smad1/5/8, anti-Smad1, anti-Smad5, anti-BMP-2, anti-Smad6, anti-Smad7 antibodies, BMP-2 siRNA (m), Tmem119 siRNA (m) and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). The pcDEF3-ALK2(R206H) V5-tagged construct was previously described [3]. Anti-osterix antibody was from Abcam Inc. (Cambridge, MA). Anti-V5, anti-noggin, and anti-human BMP-2/4 neutralizing antibody were from Invitrogen (San Diego, CA), AlphaGenix (West Lafayette, IN), and R&D Systems, respectively. Dorsomorphin dihydrochloride was from Tocris Cookson Ltd. (Bristol, UK). All other chemicals used were of analytical grade.

Cell culture

Mouse myoblastic C2C12 (ATCC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen). The medium was changed twice a week.

Transient and stable transfection

Each vector was transfected into C2C12 cells with Lipofectamine (Invitrogen Life Technologies, Inc., Grand Island, NY), as previously described [15]. Six hours later, the cells were fed fresh DMEM containing 10% FBS. Forty-eight hours later, the transiently transfected cells were used for experiments. To generate stably transfected C2C12 cells, after incubation in DMEM containing 10% FBS for 48 h, the cells were passaged, and clones were selected in DMEM supplemented with G418 (0.3 mg/ml; Invitrogen Life Technologies, Inc.) and 10% FBS. Twenty-four clones were picked after 3 weeks of culture in G418. Several clones were selected after Western blotting with anti-Tmem119 or anti-V5 antibody and semi-quantitative RT-PCR. At least three independent clones for each stable transfection were characterized to rule out the possibility of clonal variation. Empty vector-transfected cells were used as control.

Protein extraction and Western blot analysis

Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonylfluoride, complete protease inhibitor mixture (Roche Applied Science, Tokyo, Japan), 1% Triton X-100, and 1 mM sodium orthovanadate. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride. Blots were blocked with 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20, and 3% dried milk powder. The membranes were immunoblotted with each primary antibody. The antigen–antibody complexes were visualized using the appropriate secondary antibodies (Sigma-Aldrich Corp.) and the enhanced chemiluminescence detection system, as recommended by the manufacturer (Amersham Biosciences, Arlington Heights, IL). The results depicted in each figure are representative of at least three independent cell preparations.

RNA extraction and real-time PCR

Total RNA was prepared from cells using TRIZOL reagent. cDNA was synthesized using the Superscript™ First-Strand Synthesis System for RT-PCR (GIBCO BRL). Specific mRNA was quantified by real-time PCR using a 7500 Real-time PCR system (Applied Biosystems, Rotkreuz, Switzerland) with SYBR Premix Ex Taq™ II (Perfect Real Time) kits (TaKaRa) according to the manufacturer's standard protocol. The mRNA value for each gene was normalized relative to the mouse GAPDH mRNA levels in RNA samples. Primer sequences (forward and reverse) were as follows: GAPDH, 5′-GTGTACATGGTTCCAGTATGAGTCC-3′ and 5′-AGTGAGTTGTCATATTTCTCGTGGT-3′; Tmem119, 5′-TGGTTCCTCCTGTCTCTGCT-3′ and 5′-ATGATCCCTTCCAGGAGGTT-3′; osteocalcin (OCN), 5′-CCTGAGTCTGACAAAGCCTTCA-3′ and 5′-GCCGGAGTCTGTTCACTACCTT-3′; Runx2, 5′-AAATGCCTCCGCTGTTATGAA-3′ and 5′-GCTCCGGCCCACAAATCT-3′; ALP, 5′-ATCTTTGGTCTGGCTCCCATG-3′ and 5′-TTTCCCGTTCACCGTCCAC-3′; Osterix, 5′-AGCGACCACTTGAGCAAACAT-3′ and 5′-GCGGCTGATTGGCTTCTTCT-3′; Type I collagen, 5′-ATGCCTGGTGAACGTGGT-3′ and 5′-AGGAGAGCCATCAGCACCT-3′; Type II collagen, 5′-CCTCCGTCTACTGTCCACTGA-3′ and 5′-ATTGGAGCCCTGGATGAGCA-3′; Type X collagen, 5′-GCCAAGCAGTCATGCCTGAT-3′ and 5′-GACACGGGCATACCTGTTACC-3′; BMP-2, 5′-GGTCACAGATAAGGCATTGC-3′ and 5′-GCTTCCGCTGTTTGTGTTTG-3′; MyoD, 5′-GACGGCTCTCTCTGCTCCTT-3′ and 5′-AGTAGAGAAGTGTGCGTGCT-3′; Myogenin, 5′-GCTGCCTAAAGTGGAGATCCT-3′ and 5′-GCGCTGTGGGAGTTGCAT-3′; Myf-6, 5′-ATGGTACCCTATCCCCTTGC-3′ and 5′-TAGCTGCTTTCCGACGATCT-3′.