Biochemical and Biophysical Research Communications 463 (2015) 241–247

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

MEK5 suppresses osteoblastic differentiation

Shoichi Kaneshiro [a, b], Dai Otsuki [b], Kiyoshi Yoshida [b], Hideki Yoshikawa [b], Chikahisa Higuchi [b, *]

[a] Department of Orthopaedic Surgery, Japan Community Health Care Organization Osaka Hospital, 4-2-78 Fukushima, Fukushima Ward, Osaka City, Osaka 553-0003, Japan
[b] Department of Orthopaedic Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 30 April 2015
Available online 19 May 2015

Keywords:
MEK5
Osteoblastic differentiation
MEK5 inhibition
MEK5 inhibitor

ABSTRACT

Extracellular signal-regulated kinase 5 (ERK5) is a member of the mitogen-activated protein kinase (MAPK) family and is activated by its upstream kinase, MAPK kinase 5 (MEK5), which is a member of the MEK family. Although the role of MEK5 has been investigated in several fields, little is known about its role in osteoblastic differentiation. In this study, we have demonstrated the role of MEK5 in osteoblastic differentiation in mouse preosteoblastic MC3T3-E1 cells and bone marrow stromal ST2 cells.

We found that treatment with BIX02189, an inhibitor of MEK5, increased alkaline phosphatase (ALP) activity and the gene expression of ALP, osteocalcin (OCN) and osterix, as well as it enhanced the calcification of the extracellular matrix. Moreover, osteoblastic cell proliferation decreased at a concentration of greater than 0.5 μM. In addition, knockdown of MEK5 using siRNA induced an increase in ALP activity and in the gene expression of ALP, OCN, and osterix. In contrast, overexpression of wild-type MEK5 decreased ALP activity and attenuated osteoblastic differentiation markers including ALP, OCN and osterix, but promoted cell proliferation. In summary, our results indicated that MEK5 suppressed the osteoblastic differentiation, but promoted osteoblastic cell proliferation. These results implied that MEK5 may play a pivotal role in cell signaling to modulate the differentiation and proliferation of osteoblasts.

Thus, inhibition of MEK5 signaling in osteoblasts may be of potential use in the treatment of osteoporosis.

1. Introduction

Extracellular signal-regulated kinase 5 (ERK5) is a member of the mitogen-activated protein kinase (MAPK) family and is activated by MAPK kinase 5 (MEK5), which is a member of the MEK family [1]. The principal downstream target of MEK5 is ERK5, which is also known as MAPK7 or big MAP kinase 1(BMK1) [2]. Because ERK5 is the only known substrate of MEK5, all effects of MEK5 have been attributed to its ability to activate ERK5 [3].

The MEK5-ERK5 pathway is one of the lesser studied members among the MAPK family. This pathway has been implicated in cell survival, antiapoptotic signaling, angiogenesis, cell motility, differentiation and cell proliferation [4–6]. However, still much remains to be learnt regarding the potential activators of this pathway and the several intermediary molecules involved in signaling both the upstream and downstream activation of MEK5-ERK5.

MEK5-ERK5 is over-expressed or constitutively active in a number of cancers, such as prostate cancer and breast cancer, in comparison with the expression of the pathway in healthy cells [7,8]. On the other hand, the MEK5-ERK5 signaling pathway has been implicated in neurotrophin-mediated protective effects on apoptosis by serum withdrawal in neurons [9,10]. It has also been reported that MEK5-ERK5 is involved in cardiac hypertrophy [11] and cell fusion during skeletal muscle differentiation [12].

Our previous work demonstrated the functions of two other members of the MEK family, MEK1 and MEK2 in osteoblast differentiation. MEK1 could act as a positive modulator for the osteoblast differentiation, whereas MEK2 could act as a negative modulator [13,14].

These findings suggested a potential role of MEK5 in the osteoblastic differentiation. Thus far, this role has not been well elucidated, and no report has explained functions of MEK5 in osteoblasts.

* Corresponding author. Fax: +81 6 6879 3559.
E-mail address: c-higuchi@umin.ac.jp (C. Higuchi).

http://dx.doi.org/10.1016/j.bbrc.2015.05.035
0006-291X/

In this study, we examined the functions of MEK5 on osteoblastic differentiation and proliferation in mesenchymal cell lines.

2. Materials and methods

2.1. Cell culture

Mouse preosteoblastic MC3T3-E1 cells and bone marrow stromal ST2 cells were obtained from Riken Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Road Logan, UT, USA) as the growth medium at 37 °C under a humidified 5% CO_2 atmosphere. ST2 cells were cultured for cell growth in RPMI 1640 (Invitrogen) containing 10% FBS. For each assay, the growth medium was replaced and supplemented with 0.2 mM ascorbic acid (Sigma–Aldrich, St. Louis, MO, USA) and 4 mM β-glycerophosphate (Sigma–Aldrich), which was the differentiation medium. For all assays using ST2 cells, the differentiation medium was added with 50 ng/ml recombinant human BMP-2 (rhBMP-2; osteopharma, Osaka, Japan). The medium was renewed every 3 days.

2.2. Proliferation assay

MC3T3-E1 cells and ST2 cells were cultured in 96-well plates at 2.0×10^4 cells/cm^2 in the differentiation medium. Cell proliferation was assessed using the Premix WST-1 cell proliferation assay system (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. This assay was performed every 24 h for 3 days.

2.3. Alkaline phosphatase (ALP) staining and activity

Cells were seeded in 24-well plates at 2.0×10^4 cells/cm^2. After a 24 h incubation in the growth medium, cells were treated with BIX02189, MEK5 inhibitor (Selleck, Houston, TX, USA), in the differentiation medium for 3 days.

For ALP staining, cells were washed with phosphate-buffered saline (PBS) (Sigma–Aldrich) and fixed for 15 min with 10% formalin at room temperature. After fixation, the cells were incubated with the ProtoBlot II AP System with Stabilized Substrate (Promega, Madison, WI, USA) for 1 h at room temperature.

For measurement of ALP activity, cells were washed twice with PBS and lysed in mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL, USA) according to the manufacturer's protocol. ALP activity was assayed using *p*-nitrophenylphosphate as a substrate in an Alkaline Phosphatase Test Wako (Wako Pure Chemicals Industries, Ltd., Osaka, Japan). The protein content was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce).

2.4. Alizarin Red S staining

MC3T3-E1 cells were cultured for 28 days on 24-well plates in the differentiation medium. The cells were subsequently washed twice with PBS, fixed in 10% formalin for 10 min, and stained with Alizarin Red S (Sigma–Aldrich) at pH 6.3 for 1 h. Following staining, the Alizarin Red S solution was discarded and cells washed three times with distilled water. Bound Alizarin Red was dissolved in 200 μl of 100 mM hexadecylpyridium chloride (Sigma–Aldrich). The absorbance of the supernatant was measured at 570 nm.

2.5. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). First-strand cDNA was synthesized by reverse transcription PCR using SuperScript II RNase H-reverse transcriptase (Life Technologies Japan, Tokyo, Japan). Each cDNA was measured using quantitative real-time PCR of a Light Cycler system (Roche Applied Science, Basel, Switzerland). The SYBR Green assay using a Quantitect SYBR Green PCR Kit (Qiagen), in which each cDNA sample was evaluated in triplicate 20-μl reactions, was used for all target transcripts. Expression values were normalized to GAPDH. The following primers were used;

ALP (forward primer 5′-AATCGGAACAACCTGACTGACC-3′; reverse primer 5′-TCCTTCCAGCAAGAAGAA-3′),
Osteocalcin (forward primer 5′-CTCACTCTGCTGGCCCTG-3′; reverse primer 5′-CCGTAGATGCGTTTGTAGGC-3′),
Osterix (forward primer 5′-AGGCACAAAGAAGCCATAC-3′; reverse primer 5′-AATGAGTGAGGGAAGGGT-3′),
Runx2 (forward primer 5′-GCTTGATGACTCTAAACCTA-3′; reverse primer 5′-AAAAAGGGCCCAGTTCTGAA-3′), and
GAPDH (forward primer 5′-TGAACGGGAAGCTCACTGG-3′; reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′).

2.6. Western blotting

Cells were rapidly lysed on ice using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) containing protease and phosphatase inhibitors. The lysates were centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatants were used for the electrophoretic separations, following protein quantitation using BCA protein assay. Western blotting was performed using antibodies against ERK5 (Sigma–Aldrich), phospho-p42/44 MAPK (Thr202/Tyr204) (Cell Signaling Technology), p42/44 MAPK (Cell Signaling Technology), MEK1 (Cell Signaling Technology), MEK2 (Cell Signaling Technology), and MEK5 (Santa Cruz Biotechnology, Inc., Austin, TX, USA). To control protein loading, blots were also treated with β actin antibody (Cell Signaling Technology).

2.7. MEK5 knockdown by RNA interference

MC3T3-E1 cells and ST2 cells were transfected with small interfering RNA (siRNA) using Lipofectamine RNAiMAX (Invitrogen) according to the reverse transfection method in the manufacturer's protocol.

Two different sets of MEK5 siRNA oligos were purchased from Qiagen for MEK5 knockdown.

Cells transfected with siRNA were seeded in 24-well plates at a 1.0×10^4 cells/cm^2. They were incubated for 48 h and expression of MEK1, MEK2, and MEK5 was determined using western blotting. For assays of ALP staining, ALP activity, and quantitative real-time PCR of osteoblastic marker genes, the medium was subsequently replaced with the differentiation medium, and the cells were incubated for 3 days before use in experiments.

2.8. MEK5 overexpression by infection with adenovirus vectors

Adenovirus expressing MEK5 and β-galactosidase were purchased from Cell Biolabs, Inc. (San Diego, CA, USA). Each recombinant adenovirus was plaque purified, expanded, and tittered in 293 cells (Riken Cell Bank). MC3T3-E1 cells were infected and 2 days later, the medium was replaced with differentiation medium for each assay.

2.9. Statistical analysis

All data are expressed as means ± standard deviation (SD). A minimum of 3 independent experiments were performed for each assay. Statistical analysis was performed using a two-sided