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Blocking c-Met signaling enhances bone morphogenetic protein-2-induced osteoblast differentiation

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ABSTRACT

We previously demonstrated that blocking hepatocyte growth factor (HGF) receptor/c-Met signaling inhibited arthritis and articular bone destruction in mouse models of rheumatoid arthritis (RA). In the present study, we investigated the role of c-Met signaling in osteoblast differentiation using the C2C12 myoblast cell line derived from murine satellite cells and the MC3T3-E1 murine pre-osteoblast cell line. Osteoblast differentiation was induced by treatment with bone morphogenetic protein (BMP)-2 or osteoblast-inducer reagent in the presence or absence of either HGF antagonist (NK4) or c-Met inhibitor (SU11274). Osteoblast differentiation was confirmed by Runx2 expression, and alkaline phosphatase (ALP) and osteocalcin production by the cells. Production of ALP, osteocalcin and HGF was verified by enzyme-linked immunosorbent assay. Runx2 expression was confirmed by reverse transcription-PCR analysis. The phosphorylation status of ERK1/2, AKT, and Smads was determined by Western blot analysis. Both NK4 and SU11274 enhanced Runx2 expression, and ALP and osteocalcin production but suppressed HGF production in BMP-2-stimulated C2C12 cells. SU11274 also enhanced ALP and osteocalcin production in osteoblast-inducer reagent-stimulated MC3T3-E1 cells. SU11274 inhibited ERK1/2 and AKT phosphorylation in HGF-stimulated C2C12 cells. This result suggested that ERK and AKT were functional downstream of the c-Met signaling pathway. However, both mitogen-activated protein kinase/ERK kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) inhibitor suppressed osteocalcin and HGF production in BMP-2-stimulated C2C12 cells. Furthermore, SU11274, MEK, and PI3K inhibitor suppressed Smad phosphorylation in BMP-2-stimulated C2C12 cells. These results indicate that although the c-Met-MEK-ERK-Smad and c-Met-PI3K-AKT-Smad signaling pathways positively regulate osteoblast differentiation, c-Met signaling negatively regulates osteoblast differentiation, independent of the MEK-ERK-Smad and PI3K-AKT-Smad pathways. Therefore, blocking c-Met signaling might serve as a therapeutic strategy for the repair of destructed bone in patients with RA.

1. Introduction

Patients with rheumatoid arthritis (RA) often have severe systemic bone loss and increased risk of fracture due to increased bone resorption, and decreased bone formation [1]. Receptor activator of nuclear factor kappa-B ligand (RANKL), secreted by synovial tissues, plays a critical role in osteoclastogenesis [2]. Synovial fibroblasts from patients with RA express RANKL [3]. RANKL is also expressed by T cells in the synovial tissues of RA patients [4]. Bone formation requires coordination between osteoblasts and osteoclasts. This coordination is mediated by multiple growth factors and cytokines [5]. The bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily, and they play a central role in bone formation [6]. BMPs are expressed preferentially in mesenchymal tissues prefiguring the future skeleton, developing bones, and differentiated chondrocytes and osteoblasts [7]. Tumor necrosis factor (TNF)-α

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; ERK, extracellular signal-regulated kinase; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol-3-kinase; RA, rheumatoid arthritis; RT-PCR, reverse transcription-polymerase chain reaction

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is highly expressed in patients with RA, and it inhibits bone formation by affecting major osteoblast regulatory pathways [8,9].

Angiogenic growth factors such as fibroblast growth factor (FGF)-2 and FGF-4 [10,11], and vascular endothelial growth factor (VEGF) [12] act synergistically with BMP-2 to promote osteoblast differentiation. Conversely, hepatocyte growth factor (HGF), which is an angiogenic growth factor, has an inhibitory effect on osteoblast differentiation [13,14]. HGF enhances angiogenesis, and HGF receptor (c-Met)-mediated signaling events appear to induce synovial cell proliferations in RA. NK4 is a fragment of HGF that was constructed by proteolytic digestion, and it consists of 447 residues with a molecular weight of approximately 55–69 kDa. NK4 comprises the N-terminal hairpin and subsequent four-kringle domains of HGF, but lacks the 16 amino acids at the C-terminus of the α-chain and the whole β-chain. NK4 functions as an HGF antagonist by competitively binding to c-Met [15,16]. We previously demonstrated that the HGF antagonist, NK4, inhibits arthritis by suppressing angiogenesis and inflammatory cytokine production by $CD4^+$ T cells in SKG mice, an animal model of RA. We also demonstrated that articular bone destruction is inhibited by NK4 treatment [17]. In the present study, we investigated the role of c-Met signaling in osteoblast differentiation using C2C12 myoblasts, a cell line derived from murine satellite cells and the MC3T3-E1 murine pre-osteoblast cell line [18,19].

2. Materials and methods

2.1. Cell cultures

The C2C12 murine myoblast cell line and the MC3T3-E1 murine pre-osteoblast cell line were purchased from the American Type Culture Collection (Manassas, VA, USA) [18,19]. C2C12 or MC3T3-E1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C under a humid atmosphere of 95% air/5% CO_2.

2.2. Alkaline phosphatase (ALP) and osteocalcin assays

The ALP and osteocalcin assays were performed as described previously [20]. Briefly, C2C12 or MC3T3-E1 cells were seeded in 24-well tissue culture plates at a density of 1×10^5/mL/well. C2C12 cells were cultured with BMP-2 (300 ng/mL; R&D systems, Minneapolis, MN, USA) and MC3T3-E1 cells were cultured with the osteoblast-inducer reagent (2% β-glycerophosphate, 0.2% hydrocortisone, and 1% ascorbic acid-2-phosphate; TaKaRa, Shiga, Japan) and ALP or osteocalcin activities in the culture supernatants were determined after 7 or 10 days of culture, respectively. C2C12 cells were preincubated for 72 h in the presence of either mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (MEK) 1/2 inhibitor (PD98059) or phosphatidylinositol 3 kinase (PI3 K) inhibitor (Ly294002; Promega, Madison, WI, USA). After rigorous washing, the cells were stimulated with BMP-2 (300 ng/mL) for an additional 10 days, and the osteocalcin activities in the culture supernatants were determined. The concentrations of ALP or osteocalcin in the culture supernatants were determined using a mouse ALP (Bio Vision Research Products Mountain View, CA, USA) or osteocalcin (Biomedical Technologies, Inc., Stoughton, MA, USA) enzyme-linked immunosorbent assay (ELISA) kit, respectively.

2.3. HGF ELISA

The concentrations of HGF in the culture media were assayed using a mouse HGF ELISA kit (RayBio, Norcross, GA, USA).

2.4. Western blot analysis

Western blot analysis was performed as described previously [21]. Briefly, C2C12 cells were seeded in 12-well tissue culture plates at an initial density of 2×10^6 cells/mL/well, and then stimulated with BMP-2 (300 ng/mL). After 10 min of stimulation, Western blot analysis was performed. Briefly, the cells were lysed in radio -immunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology, CA, USA), and the protein content was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA), with bovine serum albumin as the standard. Each sample (20 μg) was resolved on a 10 % polyacrylamide gel under denaturing conditions and then transferred to a 0.45-μm nitrocellulose membrane. After blocking overnight at 4 °C with 5% nonfat milk in Tris-buffered saline containing 0.01% Tween® 20 (Santa Cruz Biotechnology), the membranes were incubated overnight at 4 °C with anti-phospho-ERK 1/2 antibody (1:1000 dilution in phosphate-buffered saline [PBS]; Santa Cruz Biotechnology), anti-phospho-AKT antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology), anti-phospho-smad1/5/8 antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology), or mouse anti-β-actin antibody (Cell Signaling Technology, Beverly, MA, USA). After washing the membranes with Tris-buffered saline containing 0.05% Tween® 20 (washing buffer), horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology) was added, followed by incubation for 45 min. After further washing, color was developed using luminol reagent (Santa Cruz Biotechnology), and the HRP activity of the blots was analyzed using a LAS1000 imager (Fuji film, Tokyo, Japan).

2.5. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Runx2 mRNA expression was determined by using quantitative RT-PCR. C2C12 cells were seeded in 24-well tissue culture plates at a density of 1×10^6 cells/mL/well. The cells were stimulated with BMP-2 (300 ng/mL). After 24 h of culture, RNA was extracted and quantitative RT-PCR was performed using a TaKaRa PCR kit (Takara). Primers used for Runx2 and β-actin RT-PCR assay were purchased from Applied Biosystems (Tokyo, Japan). Data represent the relative expression levels of Runx2 mRNA to control β-actin mRNA.

2.6. Statistical analysis

The results are expressed as the mean ± standard error (SE). The significance of the differences between the experimental results and the control values was determined by Student's *t*-test. *p* values less than 0.05 were considered significant.

3. Results

3.1. HGF antagonist (NK4) and c-Met inhibitor (SU11274) enhance osteoblast differentiation by C2C12 cells

Using C2C12 myoblasts, we examined the effect of NK4 on osteoblast differentiation. First, we examined the effect of NK4 on the ALP activity of BMP-2-stimulated C2C12 cells. NK4 treatment enhanced ALP production in BMP-2-stimulated C2C12 cells after 7 days of culture (Fig. 1A). Osteocalcin is a late osteoblast differentiation marker [22]. We examined the effect of NK4 treatment on osteocalcin production by the cells. NK4 treatment enhanced osteocalcin production in BMP-2-stimulated C2C12 cells after 10 days of culture (Fig. 1C). To confirm the effects of c-Met signaling on osteoblast differentiation, we next examined the