Hepatocyte growth factor-induced BMP-2 expression is mediated by c-Met receptor, FAK, JNK, Runx2, and p300 pathway in human osteoblasts

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Abstract

Hepatocyte growth factor (HGF) has been demonstrated to stimulate osteoblast proliferation and participated bone remodeling. Bone morphogenetic protein-2 (BMP-2) is a crucial mediator in bone formation during fracture healing. However, the effects of HGF in BMP-2 expression in human osteoblasts are large unknown. Here we found that HGF induced BMP-2 expression in human osteoblasts dose-dependently. HGF-mediated BMP-2 production was attenuated by c-Met inhibitor or siRNA. Pretreatment with FAK inhibitor or JNK inhibitor (SP600125) also blocked the potentiating action of HGF. Stimulation of osteoblasts with HGF enhanced FAK phosphorylation, JNK phosphorylation, RunX2 translocation from cytosol to the nucleus. HGF-mediated Runx2 binding to BMP-2 promoter was inhibited by c-Met inhibitor, FAK inhibitor, and SP600125. The binding of Runx2 to the BMP-2 promoter, as well as the recruitment of p300 and the enhancement of histone H3 and H4 acetylation on the BMP-2 promoter was enhanced by HGF. Our results suggest that HGF increased BMP-2 production in human osteoblasts via the c-Met receptor/FAK/JNK/Runx2 and p300 signaling pathway.

Running head: HGF induces BMP-2 expression

Key Words: HGF; BMP-2; Osteoblasts; Runx2

Introduction

Bone is a complex tissue composed of several cell types which are continuously undergoing a process of renewal and repair termed “bone remodeling”. The two major cell types responsible for bone remodeling are osteoclasts, which resorb bone, and osteoblasts, which form new bone. Bone remodeling is regulated by several systemic hormones (e.g., parathyroid hormone, 1, 25-dihydroxybitamin D_3, sex hormones, and calcitonin), and local factors (e.g., nitric oxide, prostaglandins, growth factors, and cytokines) [1]. When resorption and formation of bone are not coordinated and bone breakdown overrides bone building, osteoporosis results [2]. Since new bone formation is primarily a function of the osteoblast, agents that act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts can enhance bone formation [3, 4].

Bone morphogenetic proteins (BMPs), with more than 20 members, belong to the TGF-β superfamily and were originally identified by their unique ability to induce ectopic cartilage and bone formation *in vivo* [5, 6]. BMPs play important roles in bone formation and bone cell differentiation by stimulating alkaline phosphatase activity and synthesis of proteoglycan, collagen, fibronectin, and osteocalcin [6, 7]. It has been shown that BMP-2 and BMP-4 are synthesized by osteoblasts [8]. Among BMP family members, BMP-2 has been extensively studied and demonstrated to play a crucial role in inducing osteoblast differentiation and bone formation during embryonic skeletal development and postnatal bone remodeling [9]. After specific receptors binding, BMP-related effects are mediated by different signaling pathways including the Ras/MAPK system, different Smad proteins, Ca^{2+}, cAMP, the Runx/Cbfa1 pathway, and the Wnt/β-catenin system [10, 11].

Hepatocyte growth factor (HGF) was identified in the early 1980s [12, 13] and was subsequently determined to be a heterodimeric molecule composed of an alpha and beta chain [14]. The importance of HGF in organ development is demonstrated by HGF null mutation mice, which exhibit embryonic lethality [15]. HGF exhibits strong angiogenic properties through its ability to induce expression of vascular endothelial

growth factor, another angiogenic factor, but also has angiogenic properties of its own [16]. Osteoblasts and osteoclasts express c-Met, the receptor for HGF and produce HGF [17]. HGF has been demonstrated to stimulate both osteoblast proliferation and osteoclast chemotactic migration [17]. In combination with vitamin D, HGF promotes osteoblast differentiation of vertebral bone marrow cells [18]. We hypothesized that HGF controls BMP-2 expression in osteoblasts. This study was designed to test this hypothesis and also determine the precise signaling pathway.

Previous studies have shown that HGF modulates osteoblastic bone formation [17, 18]. HGF-mediated bone formation may involve activation of c-Met receptor. However, the effect of HGF on BMP-2 (an osteoblastic formation gene) expression in human osteoblasts is mostly unknown. In this study, we found that HGF induced BMP-2 expression in human osteoblasts. In addition, c-Met receptor, focal adhesion kinase (FAK), JNK, Runx2, and p300 signaling pathways may be involved in the increase of BMP-2 expression by HGF.

Materials and Methods

Materials

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, p-JNK, JNK, p-FAK, FAK, Runx2, p300, acetylated-H3, acetylated-H4, and RNA polymerase II, and the small interfering RNAs (siRNAs) against c-Met, FAK, Runx2, and a control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that does not lead to specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies specific for BMP-2, c-Met, and BMP-2 enzyme immunoassay kit were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human HGF was purchased from PeproTech (Rocky Hill, NJ). All other chemicals were purchased from Sigma-Aldrich

(St. Louis, MO). The phosphorylation site mutant of FAK(Y397F) was provided by Dr. J. A. Girault (Institut du Fer a´ Moulin, Moulin, France). The JNK dominant negative mutant was provided by Dr. M Karin (University of California, San Diego, CA).

Cell culture

The MG-63 and hFOB cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD). The human osteoblast-like cell line MG-63 was cultured in MEM supplemented with 10% FBS and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin). The conditionally immortalized human fetal osteoblastic cell line, hFOB, was maintained in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (GIBCO-BRL; Gaithersburg, MD) containing 10% FBS supplemented with geneticin (300 μg/ml) and antibiotics at 33.5℃, the permissive temperature for the expression of the large T antigen. All experiments with hFOB cells were carried out at the permissive temperature of 33.5℃.

Measurement of BMP-2 production

Human osteoblasts were cultured in 24-well culture plates. After reaching confluency, cells were treated with HGF and then incubated in a humidified incubator at 37℃ for 24 h. To examine the downstream signaling pathways involved in HGF treatment, cells were pretreated with various inhibitors for 30 min before addition of HGF (30 ng/ml) administration. After incubation, the medium was removed and stored at -80℃ until the assay was performed. BMP-2 in the medium was assayed using BMP-2 enzyme immunoassay kits, according to the procedure described by the manufacturer.

Quantitative real time PCR

Total RNA was extracted from osteoblasts by using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Two μg of total RNA was reverse transcribed into cDNA by using

oligo(dT) primer [19, 20]. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA, USA). Two µl of total cDNA were added per 25-µl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, Foster City, CA, USA). qPCR assays were carried out in triplicate on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 ℃ followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

Western blot analysis

Cellular lysates were prepared as described [21, 22]. Proteins were resolved using SDS-PAGE and transferred to Immobilon polyvinyldifluoride membranes. The membranes were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit antibodies against human p-FAK, FAK, p-JNK, JNK, or actin (1:1000) for 1 h at room temperature. After three washes, the blots were incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Immunofluorescence staining

Cells were cultured in 12-mm coverslips. After treatment with HGF, cells were fixed with 4% paraformaldehyde at room temperature. Thirty minutes later, 4% nonfat milk in PBS containing 0.5% Triton X-100 was added to the cells. The cells were then incubated with rabbit anti-Runx2 (1:100) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology Inc., St Louis, MO, USA) for 1 h, respectively. The FITC was detected using a Zeiss

fluorescence microscope.

siRNA transfection

The siRNA against c-Met, FAK, Runx2, and control were purchased from Santa Cruz Biotechnology. Cells were transfected with siRNAs (100 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously [23]. DNA immunoprecipitated with an anti-Rux2 or p300 Ab (2 μg) were purified and extracted with phenol-chloroform. The purified DNA was quantified by quantitative real-time PCR and normalized with the input DNA, which was performed in triplicate with SYBR green mix using the StepOnePlus sequence detection system. The primers: 5'-GGGTTGGAACTCCAGACTGT-3' and 5'-GAAGAGTGAGTGGACCCCAG-3' were utilized to amplify across the BMP-2 promoter region (-169 to +16) [23].

Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's *t* test. The difference was significant if the p value was <0.05.

RESULTS

HGF induces BMP-2 production in human osteoblasts

HGF has been reported to stimulate proliferation and differentiation of osteoblasts [17, 18]. To examine the effects of HGF on BMP-2 expression, MG63 and hFOB cells were exposed to HGF, and the mRNA expression of BMP-2 was

determined. Treatment of osteoblasts with HGF (3–30 ng/ml) for 24 h induced BMP-2 mRNA expression (Fig. 1A). In addition, stimulation of cells with HGF also led to increased protein expression of BMP-2 by using ELISA and western blotting (Fig. 1B&C). It has been reported that HGF exerts its effects through interaction with a specific receptor c-Met [24]. Pretreatment of osteoblasts with c-Met inhibitor reduced HGF-increased BMP-2 expression (Fig. 1D-F). In addition, transfection of cells with c-Met siRNA also reduced HGF-increased BMP-2 expression (Fig. 1D&E). Therefore, an interaction between HGF and c-Met is very important for BMP-2 production in human osteoblasts.

The signaling pathways of FAK and JNK are involved in the potentiating action of HGF

It has been reported that FAK is involved in the c-Met-mediated cell functions [25, 26]. To determine whether FAK was involved in HGF triggered BMP-2 expression, osteoblasts were pretreated with FAK inhibitor for 30 min and then incubated with HGF for 24 h. As shown in Fig. 2A&B, pretreatment with FAK inhibitor reduced HGF-induced BMP-2 expression. Transfection of cells with FAK siRNA or mutant also reduced HGF-induced BMP-2 expression (Fig. 2A&B). We then directly measured FAK phosphorylation in response to HGF. Stimulation of MG-63 cells led to a significant increase in phosphorylation of FAK (Fig. 2C&D). In addition, pre-incubated with c-Met or FAK inhibitor reduced HGF-mediated FAK phosphorylation (Fig. 2C&D). Taken together, these results indicate that the FAK pathway is involved in HGF-induced BMP-2 production.

FAK-dependent JNK activation is involved in the regulation of gene expression [27, 28]. Therefore, we investigated the role of JNK in mediating HGF-induced BMP-2 expression with the specific JNK inhibitor SP600125. As shown in Figure 3A&B, HGF-induced BMP-2 expression was markedly attenuated by pretreatment of cells for 30 min with SP600125 or transfected of cells for 24 h with JNK mutant. To directly confirm the crucial role of JNK in BMP-2 expression, we measured the level

of JNK phosphorylation in response to HGF. As shown in Figure 3C, treatment of MG-63 cells with HGF resulted in a time-dependent phosphorylation of JNK. Pretreatment of cells with c-Met inhibitor, FAK inhibitor, or SP600125 markedly inhibited the HGF-induced JNK phosphorylation (Fig. 3C). Based on these results, HGF appears to act through a signaling pathway involving c-Met receptor, FAK, and JNK to enhance BMP-2 expression in human osteoblasts.

Involvement of Runx2 in HGF-induced BMP-2 expression

Runx2 has been involved in bone formation [29]. To examine the role of the Runx2 activation in HGF-mediated BMP-2 expression, the Runx2 siRNA was used. Transfection of cells with Runx2 siRNA reduced HGF-enhanced BMP-2 expression (Fig. 4A&B). Runx2 activation was further evaluated by analyzing the accumulation of Runx2 in nucleus as well as by a chromatin immunoprecipitation assay. Treatment of cells with HGF increased accumulation of Runx2 in nucleus (Fig. 4C). Pretreatment of cells with FAK inhibitor and SP600125 reduced HGF-increased Runx2 accumulation in nucleus (Fig. 4C). We next investigated whether Runx2 binds to BMP-2 promoter after HGF stimulation. The *in vivo* recruitment of Runx2 to the BMP-2 promoter (-169 to +16) was assessed via chromatin immunoprecipitation assay. *In vivo* binding of Runx2 to BMP-2 promoter occurred after HGF stimulation (Fig. 4D). The binding of Runx2 to BMP-2 promoter by HGF was attenuated by c-Met inhibitor, FAK inhibitor, and SP600125 (Fig. 4E). However, we did not find the Runx2 binding site [OSE2 element (ACCACA)] between BMP-2 promoter region (-169 to +16). Therefore, Runx2 may binds to OSE2-like element on BMP-2 promoter. Taken together, these data suggest that the activation of the c-Met, FAK, JNK, and Runx2 pathway is required for the HGF-induced increase in BMP-2 expression in human osteoblasts.

HGF increases the recruitment of p300 with Runx2 in osteoblasts

Runx2 has been reported to associate with histone acetyltransferase p300 and

then to increase the gene expression [30]. Chromatin was immunoprecipitated with anti-p300 Ab, which contained the essential binding sites for transcriptional activators and was amplified by qPCR. As shown in Fig. 5A, *in vivo* binding of p300 to the BMP-2 promoter was increased after treatment with HGF. In addition, pretreatment of cells with c-Met inhibitor, FAK inhibitor, and SP600125 also reduced HGF-induced recruitment of p300 to BMP-2 promoter (Fig. 5B). It has been shown that p300, after recruitment to target gene promoters, can acetylate lysine residues within the core histone tails to facilitate the binding of nuclear factors to chromatin by destabilizing the promoter-bound nucleosomes; it then complexes with RNA polymerase II holoenzyme to form enhanceosome, initiating gene transcription [31]. As shown in Fig. 5C&D, the acetylation of histones H3 and H4 and the assembly of RNA polymerase II on the BMP-2 promoter were increased in response to HGF, and these effects were attenuated by c-Met inhibitor, FAK inhibitor, and SP600125. These results imply that Runx2 modulates the promoter recruitment of p300, as well as its histone acetyltransferase activity, leading to the acetylation of core histones and association.

Discussion

In this study with osteoblasts, elevation of BMP-2 mRNA and protein levels followed recombinant HGF protein treatment through c-Met receptor induction of the FAK, JNK, Runx2, and p300 signaling pathways. Our findings provide the first evidence that HGF increased BMP-2 expression, providing a link and molecular mechanism between HGF family and BMP-2 in the physiology of bone.

HGF plays an essential role in the development and regeneration of the liver and also stimulates the growth, motility, and morphogenesis of a variety of cell types [32]. Although initially thought to be of mesodermal origin, HGF is expressed almost ubiquitously, including by osteoblasts [33]. It has been postulated to participate in

bone remodeling and formation [17]. The biological activity of HGF is mediated by binding to cell surface c-Met receptor. Here, we confirmed that c-Met receptor is required for HGF-induced BMP-2 expression. Pretreatment of cells with c-Met inhibitor reduced HGF-induced BMP-2 expression. This was further confirmed by the result that the c-Met siRNA inhibited the enhancement of BMP-2 production by HGF. Therefore, the interaction between HGF and c-Met is very important for BMP-2 production by human osteoblasts.

FAK, a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling [34]. It has been shown that FAK is involved in the c-Met-mediated gene expression [25, 26]. We demonstrated that HGF increased phosphorylation of tyrosine 397. Pretreatment of cells with FAK inhibitor reduced HGF-promoted BMP-2 expression. Furthermore, the FAK mutant and siRNA antagonized the HGF-mediated potentiation of BMP-2 expression, suggesting that tyrosine 397 of FAK activation is an obligatory event in HGF-induced BMP-2 production in human osteoblasts.

JNK plays a critical role in the induction of gene transcription [35]. Because JNK has been reported to be the downstream effector of FAK [27, 28]. We examined the potential role of JNK in the signaling pathway HGF-induced BMP-2 expression. Treatment of cells with SP600125 or transfection of cells with JNK mutant reduced HGF-mediated BMP-2 expression. In addition, we also found that treatment of osteoblasts with HGF induced increases in JNK phosphorylation. These effects were inhibited by c-Met inhibitor, FAK inhibitor, and SP600125, indicating the involvement of c-Met/FAK-dependent JNK activation in HGF-mediated BMP-2 induction.

During osteoblastic differentiation, BMP-2 mRNA is induced, and maintains the sustained phenotype of mature osteoblasts [36]. Previous studies have indicated that the BMP-2 gene regulation during limb morphogenesis and osteoblast differentiation may involve multiple mechanisms and signaling pathways, such as ER, prostaglandin E2, retinoic acid, Hoxa13, Gli2/3, interferon, and interleukins [37, 38]. The results of

our current study show that Runx2 activation contributes to HGF-induced BMP-2 expression in osteoblasts. Transfection of cells with Runx2 siRNA reduced HGF-increased BMP-2 expression. Therefore, the Runx2 binding site is likely to be the most important site for HGF-induced BMP-2 production. The interaction between Runx2 and p300 was critical for the induction of gene expression [30]. We revealed that HGF enhanced recruitment of p300 to the BMP-2 promoter. The acetylations of histone H3 and H4 on the BMP-2 promoter were increased as well. Therefore, p300-dependent acetylation of histones and recruitment of basal transcription machinery may be involved in the HGF-induced BMP-2 gene transcription. It has been reported that acetylation of histone H3 and H4 is a prerequisite for TNF-α production in monocytes and macrophages [39], and acetylation of histone H4-mediated oxidative stress-regulated proinflammatory gene, such as IL-8 expression in human pulmonary epithelial cells [40]. The recruitment of transcriptional coactivators accompanied with histone acetylation may be also necessary for the BMP-2 gene expression.

In conclusion, we explored the signaling pathway involved in HGF-induced BMP-2 expression in human osteoblasts. We found that HGF increased BMP-2 expression by binding to the c-Met receptor and activating FAK and JNK, which enhanced binding of Runx2 to the BMP-2 promoter. This coordinated with the recruitment of p300 and enhanced the acetylation of histone H3 and H4, resulting in the transactivation of BMP-2 production.

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mutant.

Conflict of Interest Statement

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence our work

FIGURE LEGENDS

Figure 1 HGF increases BMP-2 expression through c-Met receptor.

(A) Cells were incubated with HGF for 24 h, and BMP-2 mRNA was examined by qPCR. (B&C) MG63 cells were incubated with HGF for 24 h, and BMP-2 protein was examined by ELISA and western blotting. (D&E) Cells were pretreated for 30 min with c-Met inhibitor (3 μM) or transfected with c-Met siRNA for 24 h followed by stimulation with HGF for 24 h, and BMP-2 expression was examined by western blotting, qPCR and ELISA. (E; upper) MG63 cells were transfected with c-Met siRNA for 24 h, and c-Met expression was examined by western blotting. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with HGF-treated group.

Figure 2 FAK is involved in HGF-induced BMP-2 expression.

(A&B) Cells were pretreated for 30 min with FAK inhibitor (200 nM) or transfected with FAK mutant and siRNA followed by stimulation with HGF for 24 h. Media and total RNA were collected, and the expression of BMP-2 was analyzed with ELISA and qPCR. (A; upper) MG63 cells were transfected with FAK siRNA for 24 h, and FAK expression was examined by western blotting. (C&D) MG63 cells were pretreated for 30 min with c-Met inhibitor or FAK inhibitor for 30 min, followed by stimulation with HGF for different time intervals, and FAK phosphorylation was determined by western blotting. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with HGF-treated group.

Figure 3 JNK is involved HGF-induced BMP-2 expression.

(A&B) Cells were pretreated for 30 min with SP600125 (3 μM) or transfected with JNK mutant followed by stimulation with HGF for 24 h. Media and total RNA were collected, and the expression of BMP-2 was analyzed with ELISA and qPCR. (C&D) MG63 cells were pretreated for 30

min with c-Met inhibitor, FAK inhibitor, or SP600125 for 30 min, followed by stimulation with HGF for different time intervals, and JNK phosphorylation was determined by western blotting. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with HGF-treated group.

Figure 4 Runx2 is involved in HGF-mediated BMP-2 production in osteoblasts.

(A&B) Cells were transfected with Runx2 siRNA followed by stimulation with HGF for 24 h. Media and total RNA were collected, and the expression of BMP-2 was analyzed with ELISA and qPCR. (A; upper) MG63 cells were transfected with Runx2 siRNA for 24 h, and Runx2 expression was examined by western blotting. (C) MG63 cells were pretreated for 30 min with FAK inhibitor or SP600125 followed by stimulation with HGF for 2 h, and Runx2 immunofluorescence staining was examined. (D&E) MG63 cells were treated with HGF for different time intervals or pretreated with c-Met inhibitor, FAK inhibitor, and SP600125 followed by stimulation with HGF for 120 min. A chromatin immunoprecipitation assay was then performed. The chromatin was immunoprecipitated with anti-Runx2. One percent of the precipitated chromatin was assayed to verify equal loading (input). *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with HGF-treated group.

Figure 5 HGF increases the recruitment of p300 with Runx2 in human osteoblasts.

MG63 cells were treated with HGF for different time intervals or pretreated with c-Met inhibitor, FAK inhibitor, and SP600125 followed by stimulation with HGF for 120 min. A chromatin immunoprecipitation assay was then performed. The chromatin was immunoprecipitated with anti-p300, acetylated-H3, acetylated-H4, or RNA polymerase II. One percent of the precipitated chromatin was assayed to verify equal loading (input). *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with

HGF-treated group.

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