Abstract: The BMP-2/BMP-7 heterodimer (BMP-2/-7) is a potent osteogenic inducer. It is well established that BMP-2/-7 activates mitogen activated protein kinases (MAPKs) in some cells. It is also widely accepted that MAPK signaling cascades play a crucial role in the immediate osteogenic response to BMPs. However, the precise mechanism and role of the MAPK signaling network in osteogenesis is still controversial. We investigated whether BMP-2/-7 regulates osteogenic gene expression in human periodontal ligament (HPDL) cells, and how MAPKs might be involved in this process, by treatment of HPDL cells with BMP-2/-7 with or without MAPK inhibitors. We found that the Jun-N-terminal kinase (JNK) is essential for the expression of almost all osteogenic marker genes. Inhibition of extracellular signal-regulated kinase (ERK) using inhibitor had no effect on the expression of early osteogenic marker genes, whereas it significantly down-regulated the expression of late osteogenic marker genes such as Bone sialoprotein and Osteocalcin. These results indicate that MAPKs play a crucial role in osteogenesis of HPDL cells.

Keywords: Bone morphogenetic protein-2/-7, Osteogenic differentiation, JNK pathway, ERK pathway.

Introduction

Bone Morphogenetic Protein (BMP) is a member of the transforming growth factor-β family, which induces osteogenic differentiation of mesenchymal stem cells in vivo and in vitro. The BMP-2/BMP-7 heterodimer (BMP-2/-7) is one of the most potent of the known osteogenic inducers. The dimer binding to pre-dimerized receptor (BMPR) complexes induces Smad1/5/8 phosphorylation and Smad4 activation. Smad1/5/8 forms a complex with Smad4 and this complex translocates to the nucleus where it promotes the transcription of marker proteins of osteogenic differentiation. BMPR are divided into two different types, type I and type II. Type I receptors are activin receptor-like kinases (ALK). Four different kinds of ALK (ALK1/2/3/6) are known to function as BMP receptors. There are three different kind of type II receptors; BMPR-II, activin type II receptor (ActR-II) and activin type II receptor B (ActR-IIB). The roles of these receptors are different in different cell types.
this mineralized tissue. Recent studies have reported that human periodontal ligament fibroblasts are a heterogeneous population of cells, among which precursor cells of various cell lineages exist. Furthermore, PDL cells are known to respond to bone inducers such as BMP\(^{18}\). PDL cells have been regarded as the source of cementoblasts and osteoblasts\(^{19}\). In addition, PDL fibroblasts, i.e. PDL cells, have been shown to have osteogenic potential\(^{20}\).

The purpose of this study was to investigate whether BMP regulates osteogenic genes in human periodontal ligament (HPDL) cells and how such regulation is mediated by MAPK signaling.

Materials and methods

Cell culture and osteogenic differentiation

Normal human periodontal ligament (HPDL) cells were purchased from Lonza (Basel, Switzerland) and cultured in Bullet Kit Stromal cell growth medium (SCGM, Lonza). Cells of passages 5 to 8 were seeded at a density of 1 x 10\(^5\) cells/cm\(^2\) in SCGM for each assay. Osteogenic differentiation was promoted by replacing the SCGM with osteogenic differentiation medium (OBM), composed of á-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 2 % FBS (Invitrogen), 50 ìg/ml, L-ascorbic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 10 mM á-glycerophosphate (Wako), with or without 100 ng/ml of the BMP-2/-7 heterodimer (R&D System, Abingdon, UK). The cells were also treated with or without 25 ìM SP600125 (JNK inhibitor; Calbiochem, Darmstadt, Germany) or 25 ìM FR180204 (ERK1/2 inhibitor; Calbiochem) in the presence of BMP-2/-7, following which the culture media were not changed for up to 96 h. The cells were maintained at 37 °C in 5 % CO\(_2\) for 48 or 96 h.

Assay of alkaline phosphatase (ALP) activity

The cells were fixed in 4 % paraformaldehyde for 5 min at room temperature, rinsed with phosphate-buffered saline (PBS) and then incubated with ALP substrate solution (Roche Diagnostics, Basel, Switzerland) for 1 h at room temperature. After three washes with distilled water, images were captured using the macroscopic appearance of the cells was visually assessed.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was used to examine the expression of osteogenic differentiation markers. Following incubation for 96 h in the differentiation medium, total RNA was extracted using the QIAzol reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s protocols. cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR analysis was performed using the Premix Ex Taq\(^{\text{TM}}\) reagent (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. The target genes were RUNX2, alkaline phosphatase (ALP), type I collagen (COL1A1), Osterix (OSX), Bone sialoprotein (BSP) and osteocalcin (OCN).

Relative expression of genes of interest was estimated using \(\Delta \Delta \text{Ct}\) method.

Statistical analysis

Each experiment was repeated three times. When ANOVA indicated differences between groups, multiple comparisons of each experimental group were performed using the Bonferroni test.

Results

Effect of JNK and ERK inhibitors on BMP-induced ALP activity in HPDL cells

To determine whether activation of JNK or ERK1/2 is required for osteogenic differentiation of HPDL cells, we assayed BMP-2/-7-induced ALP activity in HPDL cells in the presence or absence of specific JNK and ERK inhibitors. For these experiments, HPDL cells were cultured to confluence before the inhibitor was added. The treatment with BMP-2/-7 upregulated ALP activity in HPDL cells. Inhibition of JNK activation using the widely used JNK-specific inhibitor SP600125 markedly inhibited BMP-2/-7-induced ALP activity in HPDL cells. In contrast, BMP-2/-7-induced ALP activity in the presence of 25 ìM of the specific ERK1/2 inhibitor FR180204 was similar to that in cells treated without these inhibitors (Fig. 1).

Effect of inhibition of JNK and ERK1/2 pathways on the mRNA expression of early osteogenic differentiation marker genes

RUNX2 is an essential transcription factor for osteogenic differentiation at the early phase of osteogenesis. To further analyze
Figure 2. Effect of MAPK inhibitors on the expression of early osteogenic differentiation marker genes in HPDL cells. The mRNA expression level of the early osteogenic differentiation marker genes *RUNX2* (A), *ALP* (B) and *COL1A1* (C) in HPDL cells was measured using RT-PCR at 0 h, 48 h and 96 h after treatment with 100 ng/ml BMP-2/-7 (BMP), 100 ng/ml BMP-2/-7 +25 μM SP600125 (BMP+JNKi), 100 ng/ml BMP2/7 +25 μM FR180204 (BMP+ERKi) or control. All experiments were repeated three times. The data are presented as means ± SD. The Bonferroni correction for multiple comparisons was applied. *p<0.01; **p<0.001

the role of JNK and ERK in osteogenesis, we investigated the effect of JNK and ERK1/2 inhibitors on BMP-2/-7-induction of early osteogenic marker genes, using qRT-PCR. BMP-2/-7 treatment significantly increased *RUNX2* mRNA expression at 48 h and 96 h after its addition. JNK inhibitor treatment significantly decreased BMP-2/-7-induced *RUNX2* mRNA at these time points (Fig. 2A). ERK1/2 inhibitor treatment induced no significant change in *RUNX2* expression at 48 h after BMP-2/-7 treatment (Fig. 2A). The BMP-2/-7-induced mRNA expression of other early osteogenic differentiation marker genes, *ALP* and *COL1A1*, was also markedly decreased in JNK inhibitor-treated cells at 48 h and 96 h (Fig. 2B and 2C, respectively). However, their expression was unaffected by the presence of ERK1/2 inhibitor (Fig. 2B and 2C, respectively).

Effect of inhibition of JNK and ERK1/2 pathways on the mRNA expression of late osteogenic differentiation marker genes

Osterix (OSX) is a transcription factor that is required for the differentiation of pre-osteoblasts into mature osteoblasts. It is therefore involved in the latter phase of osteogenic differentiation.
Figure 3. Effect of MAPK inhibitors on the expression of late osteogenic differentiation marker genes in HPDL cells. The mRNA expression levels of the late osteogenic differentiation marker genes OSX (A), BSP (B) and OCN (C) in HPDL cells were measured using RT-PCR at 48 h and 96 h after treatment with 100 ng/ml BMP-2/-7 (BMP), 100 ng/ml BMP-2/-7 +25 μM SP600125 (BMP+JNKi), 100 ng/ml BMP2/7 +25 μM FR180204 (BMP+ERKi) or control. All experiments were repeated three times. The data are presented as means ± SD. The Bonferroni correction for multiple comparisons was applied. **p<0.001

BSP and OCN are also established late osteogenic differentiation marker genes. OSX mRNA expression was not detectable prior to BMP-2/-7 treatment but was strongly induced at 48 h and 96 h after its treatment (Fig. 3). BMP-2/-7 also induced the mRNA expression of BSP and OCN at 48 h and at 96 h. The JNK inhibitor markedly decreased the mRNA expression of all of these late osteogenic marker genes at both 48 h and 96 h after BMP-2/-7 treatment (Fig. 3A, B and C). The ERK1/2 inhibitor significantly decreased BMP-2/-7-induced OSX mRNA expression (Fig. 3C). This result was in contrast to the earlier experiments described above indicating that ERK1/2 inhibitor treatment did not alter the mRNA expression of RUNX2. The mRNA expression of BSP and OCN was also remarkably decreased by ERK1/2 inhibitor treatment at 48 h and 96 h after BMP-2/-7 addition (Fig. 3B and C, respectively)
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Discussion

There have been several reports of differences in the BMP mediated osteogenic response of human and rodent bone marrow-derived stem cells (MSC)\textsuperscript{21, 23}. BMP-2 treatment of rodent MSC culture enhanced the gene expression of ALP and other osteogenesis related genes\textsuperscript{24, 27}, whereas human MSCs displayed a very poor response to BMP-2 stimulation\textsuperscript{28}. A recent study showed that human MSCs markedly elevated ALP expression when the cultures were stimulated with BMP-2 in the presence of an ERK1/2 inhibitor\textsuperscript{21}. On the other hand, stimulation of mouse MSC and murine preosteoblasts MC3T3-E1 cells with BMP-2 also promoted both ERK expression and ERK activity\textsuperscript{12}. Reducing ERK activity decreased ALP activity, suggesting that BMP-2 induced ERK activation and that this ERK activation is required for ALP activation\textsuperscript{29,30}. These contradictory reports indicated that the effects of BMP-2 on osteogenic differentiation are regulated by MAPK signaling pathways but that their mechanism of regulation is very complicated and is still not well understood.

Although analysis of osteogenic regulation implied that MAPK pathways may modulate the transcription of genes expressed early in osteogenic differentiation, it is very difficult to predict whether the genes expressed during osteogenic differentiation are up-regulated or down-regulated by MAPK signaling. We therefore investigated whether two major MAPKs, JNK and ERK could regulate osteogenic marker gene expression in HPDL cells.

The role of ERK activation in osteogenic differentiation has been the subject of many studies, but no clear association of ERK and osteogenesis was presented. There are several reports of negative or negligible effects of ERK on the expression of early osteogenic genes in human MSCs\textsuperscript{31,32}. However, other reports indicated that both BMP and MAPK signaling pathways play a crucial role in the regulation of osteogenic-specific gene expression and differentiation\textsuperscript{11}. BMPs induce MSC condensation and differentiation into osteogenic lineages. BMPs are secreted by immature osteogenic cells and are necessary for induction of the differentiated phenotype in these cells\textsuperscript{33}. MC3T3-E1 cells, like primary osteogenic cultures, produce BMP-2, -4 and -7\textsuperscript{29}. These BMPs transduce their signals through the Smad pathway. Secretion of BMPs from MC3T3-E1 cells is required for their osteogenic differentiation, suggesting that BMPs and their cellular signaling are essential for osteogenic differentiation\textsuperscript{34}. The effect of BMPs on osteogenesis critically depends on the cell type and the cell differentiation stage. One of the reasons for the complicated effects of BMPs may be due to their complex signal transduction mechanism. In general, BMP-induced Smad and MAPK pathways converge on common signaling intermediates. RUNX2 is a candidate common intermediate. BMPs have been reported to up-regulate RUNX2 transcription and ERK activation is required for RUNX2-dependent transcription\textsuperscript{35}. But this general signaling cascade has many exceptions. The role of ERK pathways in BMP signaling is less clear and appears to vary with cell origin, cell type and the experimental system used. These variations may be

<table>
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<tr>
<th>Gene symbol</th>
<th>Gen Bank accession no.</th>
<th>Primer sequence: sense/antisense</th>
<th>Probe no.</th>
<th>Amplicon</th>
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related to variations in the BMP receptors expressed in different cell types and resulting differences in the subsequent signaling cascade. BMP signaling is transduced through Smad pathways as well as through non-Smad pathways including MAPK pathways. It has been reported that osteocalcin (OCN) expression was blocked by ERK inhibitor while Bone sialoprotein (BSP) expression was not inhibited by ERK inhibition suggesting that the BSP gene must be controlled by mechanisms different from those that regulate OCN expression in mouse MC3T3-E1 cells\(^{29}\).

We here show that human PDL cells also show many difference in the regulation of osteogenic differentiation marker proteins expression compared to mouse cells. Thus BMPs regulate or are regulated by many different kinds of factors which might be the reason for this complexity. Therefore we have to investigate each cell to elucidate BMP signaling cascade. The inhibition of JNK pathway blocked both early and late osteogenic differentiation gene expression, while the inhibition of ERK pathway had little effect, or even enhanced, early osteogenic marker gene expression in HPDL cells. Interestingly, the ERK1/2 inhibitor blocked late osteogenic mRNA expression. These results suggested that ERK activation is not required for the early stage of osteogenic differentiation but that it is an essential regulator for late stage of that in HPDL cells (Fig. 3). JNK activations play important roles in insulin-like growth factor-1 (IGF-1) signaling, which is a critical factor in BMP induction of osteogenic differentiation. BMP and IGF-1 stimulation are essential for osteogenic differentiation\(^{30}\). We have not yet determined whether BMPs can actually stimulate MEK and ERK activity in HPDL cells, although ERK inhibition clearly caused down-regulation of the expression of genes that are markers of the late stage of osteogenic differentiation that is promoted by BMP. Further studies will be necessary to determine whether MAPK pathways have a direct or a permissive role in the induction of gene expression by BMPs. In conclusion, ERK activity is essential for the expression of genes that are markers of the late stage of osteogenic differentiation that is induced by BMPs in HPDL.

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