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Growth/differentiation Factor-5 Induces Osteogenic Differentiation of Human Ligamentum Flavum Cells through Activation of ERK1/2 and p38 MAPK

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Key Words

Growth/differentiation factor-5 • Ligamentum flavum • Ossification • Osteogenic differentiation • Mitogen-activated protein kinases

Abstract

Ossification of ligamentum flavum (OLF) is a pathological ectopic ossification in the spinal ligament, leading to spinal canal stenosis, but little was known about its pathogenesis. A previous study has found growth/differentiation factor (GDF)-5 expression at ossified sites of the ligaments from OLF patients. This study aimed to investigate the osteogenic effects of GDF-5 on cultured human ligamentum flavum cells (LFCs). LFCs were isolated from human spinal ligamentum flavum, and treated with or without recombinant human (rh) GDF-5. Alkaline phosphatase (ALP) activity was measured. Expression of osteocalcin was assessed by reverse transcriptase-PCR, Western blotting and immuno-fluorescence. Matrix mineralization was assessed by alizarin red staining. Activation of mitogen-activated protein kinases (MAPK) ERK1/2, p38 and JNK were detected by Western blotting. We found that rhGDF-5 treatment increased ALP activity and osteocalcin expression in a time- and dose-dependent manner, and induced mineralized nodule form. In addition, rhGDF-5 challenge mediated the ERK1/2 and p38 activation but not JNK. Inhibiting this activation pharmacologically, using U0126, a ERK1/2 inhibitor, or SB203580, a p38 inhibitor, resulted in significantly lower ALP activity and osteocalcin protein expression. The present study shows that rhGDF-5 induces osteogenic differentiation of human LFCs through activation of ERK1/2 and p38 MAPK. These findings give some new insight into the pathogenesis of OLF.

Introduction

Ossification of ligamentum flavum (OLF) is characterized by an ectopic bone formation in the spinal ligamentum flavum which is normally composed of elastic and collagen fibers [1]. OLF is a well-recognized cause of spinal canal stenosis resulting in myeloradiculopathy, particularly in Asian population [2]. Previous studies have suggested that some factors, such as genetic background

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[3], diabetes mellitus [4], fluorosis [5], ankylosing spondylitis [6], mechanical stress [7] and regional cytokines or growth factors [8, 9], were involved in the onset and progression of OLF, but the underlying pathogenesis of this disease is still largely unknown.

Growth/differentiation factor (GDF)-5 also called as bone morphogenetic protein (BMP)-14 and cartilage-derived morphogenetic protein (CDMP)-1 is a number of the transforming growth factor beta superfamily [10, 11]. The osteogenic potential of recombinant human (rh) GDF5 has been intensively examined *in vitro* and *in vivo* for a decade. The *in vitro* response to rhGDF-5 resulted in osteogenic differentiation in various cell types, such as pluripotent mesenchymal precursor cell C2C12 [12], adipose-derived stromal cells [13], bone marrow mesenchymal stem cells [14] and periosteum-derived cells [15]. Matrices loaded with rhGDF-5 induced ectopic bone formation when implanted in rat subcutaneous or intramuscular sites [16, 17]. Application of rhGDF-5 combined with collagen matrix enhanced spinal fusion in rabbit or sheep models [18, 19]. Recently, GDF-5 expression has been only detected in ossified spinal ligaments from OLF patients, which indicated GDF-5 as an important contributor to the pathogenesis of OLF [20]. However, the molecular mechanism of the osteogenic effects of GDF-5 on OLF has not been explored.

Mitogen-activated protein kinases (MAPK), a family of serine/threonine kinases, play an important role in signal transduction and regulation of gene transcription in response to changes of the cellular environment [21]. Three major subfamilies of MAPK, extracellular signal-regulated kinase (ERK), p38 kinase (p38) and c-Jun N-terminal kinase (JNK) were associated with human pathological conditions [22]. Moreover, MAPK regulated key cellular functions such as proliferation, differentiation, migration and apoptosis [21]. Previous study found that the MAPK signaling cascade was activated along with the expression of various osteomarkers during the differentiation of osteogenic cells, which indicated that MAPK play an important role in cell osteogenic differentiation [23].

We hypothesized that GDF-5 can induce osteogenic differentiation of human ligamentum flavum cells (LFCs). This study aimed to test this hypothesis by investigating: (1) the osteogenic effects of rhGDF-5 on cultured LFCs by detecting the change of alkaline phosphatase (ALP) activity, osteocalcin expression and mineralized nodule formation, (2) the activation of MAPK signalings, and (3) involvement of activated MAPK in rhGDF-5-induced osteogenic differentiation of LFCs.

Materials and Methods

Ligamentum Flavum Specimens

The ligamentum flavum specimens were harvested from eight patients who underwent surgical decompression for the fracture of thoracic spine or thoracolumbar junction spine. Surrounding tissues were carefully removed under a dissecting microscope. To avoid any possible contamination by osteogenic cells, the ligaments were carefully extirpated at a distance from ligament-bone insertions. Any ossification case was excluded with images of x-ray, computed tomography and magnetic resonance imaging. Informed consent was obtained from each patient, and the project was approved by the Ethics Committee of Southern Medical University.

LFCs Cultures and Treatment

LFCs cultures were performed as previously described [24]. Briefly, The collected ligaments were washed with phosphate-buffered saline (PBS) several times, minced into about 0.5 mm^2 pieces, and then digested at 37 °C for 60 min by 0.2% collagenase type I (Sigma-Aldrich. St. Louis, MO, USA). Collagenase-treated ligament pieces were washed with DMEM (Gibco BRL, Melbourne, Victoria, Australia) and then placed in 6-well plates (Corning-Costar, Oneonta, NY, USA) in DMEM supplemented with 10% fetal bovine serum (Gibco BRL.), 100 U/ml penicillin and 100 pg/ml streptomycin, and incubated in 95% air/5% CO_2 atmosphere at 37 °C, LFCs were harvested with 0.2% trypsin (Sigma-Aldrich)/0.02% ethylenediamine tetraacetic acid once they migrated from the explants and became confluent. The third passage LFCs were used in the following studies [25].

In the differentiation experiments, LFCs were treated with different concentrations of rhGDF-5 (ProSpec-Tany TechnoGene, Rehovot, Israel) ranging from 0 to 500 ng/ml for 10 days, or with 100 ng/ml rhGDF-5 for 0 to 14 days, and then subjected to analysis of alkaline phosphatase (ALP) activity, mRNA and protein expression of osteocalcin. In addition, LFCs were treated with or without 100 ng/ml rhGDF-5 for 4 weeks to detect matrix mineralization. In order to detect MAPK activation, LFCs were exposed to 100 ng/ml rhGDF-5 at 0, 5, 15, 30, 60, 120 and 240 minutes. Inhibition experiments were subsequently carried out by pretreatment for 60 minutes with or without different MAPK inhibitors.

Alkaline phosphatase (ALP) activity assay

After LFCs were collected, cell extract was prepared with RIPA lysis buffer (Beyotime Bio, Haimen, China). ALP activity in the cell lysates was assayed at the end of the incubation with ALP substrate buffer (Jiancheng Bio, Nanjing, China) containing the soluble substrate p-nitrophenyl phosphate according to the manufacturer's instruction. The enzyme activity was normalized against cellular protein concentration and was expressed as U/g protein. Protein concentration was determined using the Bradford method [26].

Reverse transcriptase (RT)-PCR

For semiquantitative measurement of osteocalcin mRNA expression, total RNA was extracted from LFCs using the TRI

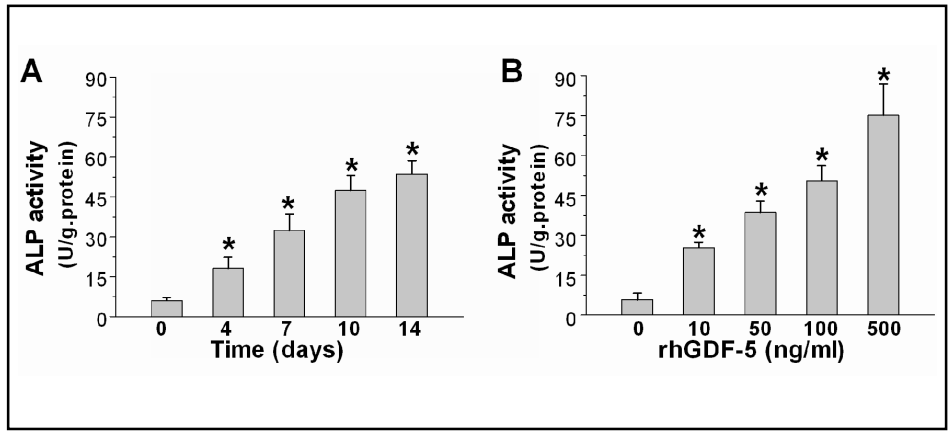


Fig. 1. Effect of rhGDF-5 on alkaline phosphatase (ALP) activity in LFCs. LFCs were incubated with 100 ng/ml of rhGDF-5 for the indicated time, or with different concentration of rhGDF-5 for 10 days. The total proteins of cells were extracted and subjected to ALP activity assay. rhGDF-5 treatment increased the ALP activity in LFCs in time- (A) and dose- (B) dependent manner. Date from three independent experiments are presented as mean ± SE, ANOVA $P<0.05$ in A and B. *$P<0.05$ vs control.

Reagent ® (Molecular Research Center, Cincinnati, OH, USA). 0.5 μg of each RNA sample was converted to cDNA using PrimeScript™ RT reagent kit (Takara, Dalian, China). Equal amount of cDNA was subsequently amplified by PCR using GoTaq® Green Master Mix (Promega, Madison, WI, USA). Specific oligonucleotide primers were designed on the basis of human sequences in GenBank as following: osteocalcin: Sense 5'-ATG AGA GCC CTC ACA CTC CTC-3', and antisense 5'-GCC GTA GAA GCG CCG ATA GGC-3'; β-actin: sense 5'-GAC TAC CTC ATG AAG ATC CT-3', and antisense 5'-CCA CAT CTG CTG GAA GGT GG-3'. PCR conditions consisted of 35 cycles of denaturing at 94 °C for 30s, annealing at 60 °C for osteocalcin, 55°C for β-actin for 30s, and extension at 72 C for 30s. PCR products (osteocalcin: 294 bp; β-actin: 510 bp) were electrophoresed on 1.5% agarose gel, stained with ethidium bromide. Bands were visualized and analyzed using Eel Documentation and Analysis System (Uvitec, Cambridge, UK). Relative expression of osteocalcin was estimated by using β-actin mRNA as an internal control.

Western blotting

Western blotting was carried out as described previously [27]. Briefly, LFCs were washed with cold PBS and lysed with RIPA lysis buffer. Cell lysates were separated by tricine-SDS-PAGE for osteocalcin analysis or 10% SDS-PAGE for other protein analyses and electrotransferred subsequently onto polyvinylidene difluoride membranes. The membrane blots were probed with a primary antibody overnight at 4 °C, and incubated with appropriate horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature, and visualized by enhanced chemiluminescent kit (Pierce, Rockford, IL, USA). The primary antibodies were goat anti-osteocalcin, rabbit anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-ERK1/2, rabbit anti-phosphor-ERK1/2, rabbit anti-p38, rabbit anti-phosphor-p38, rabbit anti-JNK, rabbit anti-phosphor-JNK (Cell Signaling Technology, Danvers, MA, USA).

Immunofluorescence

LFCs were analyzed for osteocalcin expression by immunofluorescence staining as described previously [24]. Briefly, the cell monolayers on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100. After washing and blocking, the cells were incubated with a goat anti-osteocalcin antibody (1:25, Santa Cruz Biotechnology) overnight at 4 °C. After washing, the cells were incubated for 60 minutes with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG (1:100, ZSGB-BIO Inc, Beijing, China) at 37 °C. The nuclear was subsequently stained for 10 minutes with propidium iodide (PI) (Sigma-Aldrich.) at 37 °C. Immunostained cells were observed under LSM-510 confocal scanning microscopy (Carl Zeiss, Göttingen, Germany).

Alizarin red staining

We adopted the Alizarin-red staining method [28] to determine mineralization of extracellular matrix. Cells were washed with PBS, and fixed with ice-cold 70% ethanol for 60 minutes. After washed, cells were stained with 40 mM Alizarin red (Sigma-Aldrich.), pH 4.2, for 30 minutes at room temperature. Following the staining, cells were rinsed five times with distilled water and incubated in PBS for 15 min with gentle agitation to remove nonspecific Alizarin-red stain. Stained cultures were photographed using microscopy (IX71, Olympus, Tokyo, Japan).

Statistical Analysis

All experiments were repeated at least three times. Data are presented as mean ± standard error (SE). Differences between groups were assessed using Repeated Measures ANOVA. The statistical software package SPSS 13.0 was used for statistical analyses in this study. Two-tailed $P < 0.05$ was considered statistically significant.

Results

rhGDF-5 induced osteogenic differentiation of LFCs

ALP activity, osteocalcin expression and matrix mineralization are the main markers of osteogenesis [29]. We examined these markers in LFCs treated with or

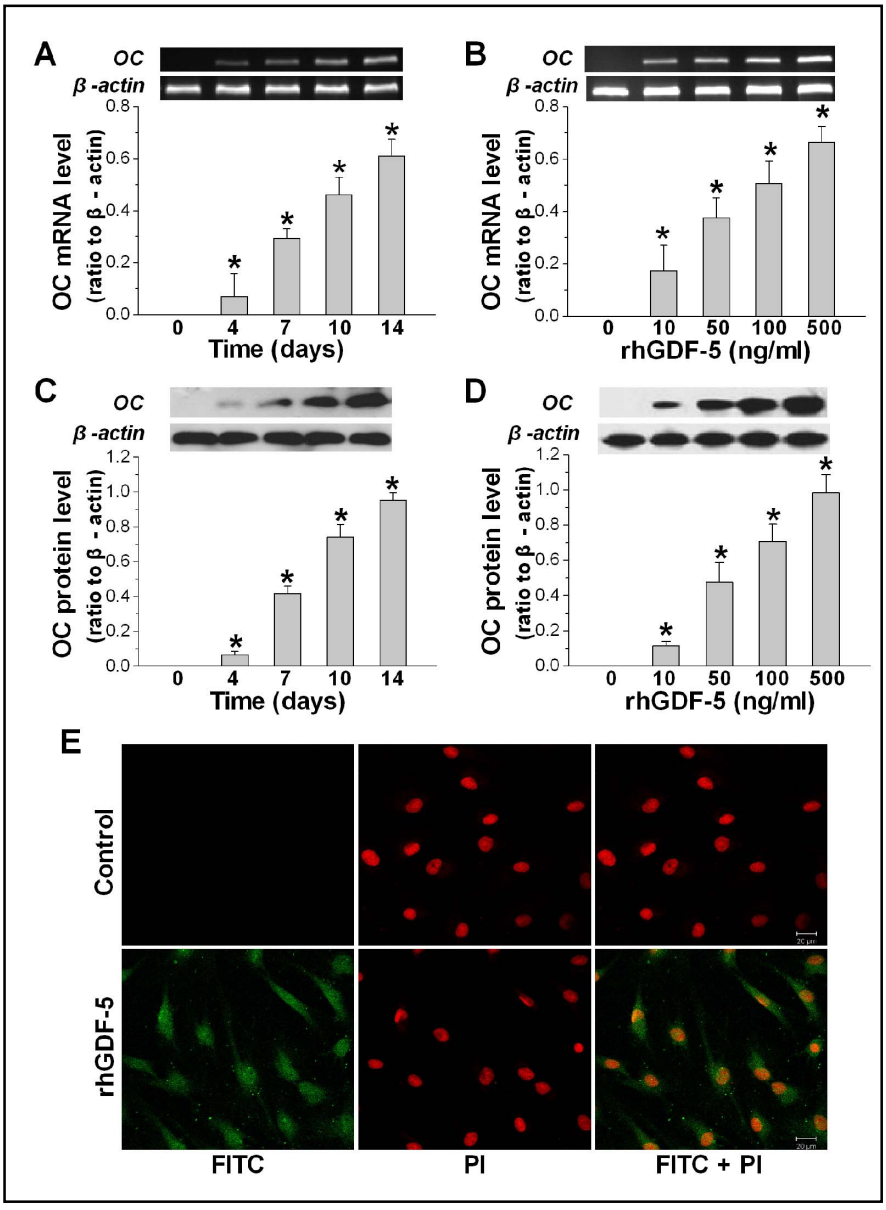


Fig. 2. Effect of rhGDF-5 on the expression of osteocalcin (OC) in LFCs. LFCs were incubated with 100 ng/ml of rhGDF-5 for the indicated time, or different concentration of rhGDF-5 for 10 days. The total RNA was extracted and OC mRNA expression was detected by semiquantitative RT-PCR analysis (A & B). The total proteins were extracted and OC protein expression was detected by immunoblotting (C & D). In other experiments, human LFCs were incubated with or without 100 ng/ml rhGDF-5 for 10 days, OC protein expression was examined by immunofluorescent staining (E), OC were stained with fluorescein isothiocyanate (FITC, green) and nuclei were stained with propidium iodide (PI, red) (bar =20 μm). Data and pictures shown are representative of three independent experiments, all values are presented as mean ± SE, ANOVA $P<0.05$ in A, B, C and D. *$P<0.05$ vs control.

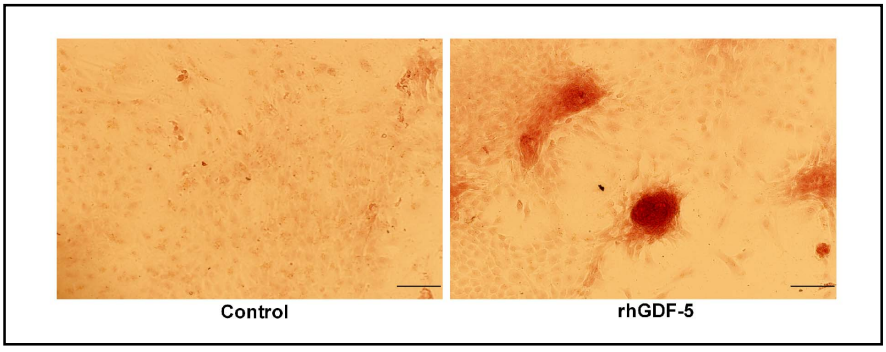


Fig. 3. Effect of rhGDF-5 on mineralized nodule formation in LFCs. LFCs on confluence were incubated with or without 100 ng/ml rhGDF-5 for 4 weeks. The mineralized nodule formation was determined by Alizarin red staining (bar =50 μm). Pictures shown are representative of three independent experiments.

without rhGDF-5. As shown in Fig. 1, rhGDF-5 treatment increased significantly ALP activity in time- and dose-dependent manners. In addition, rhGDF-5 treatment enhanced the expression of osteocalcin at both mRNA (Fig. 2A and B) and protein level (Fig. 2C and D) in time- and dose-dependent manners. Furthermore, the immunofluorescence studies demonstrated that osteocalcin expression was predominantly located at the

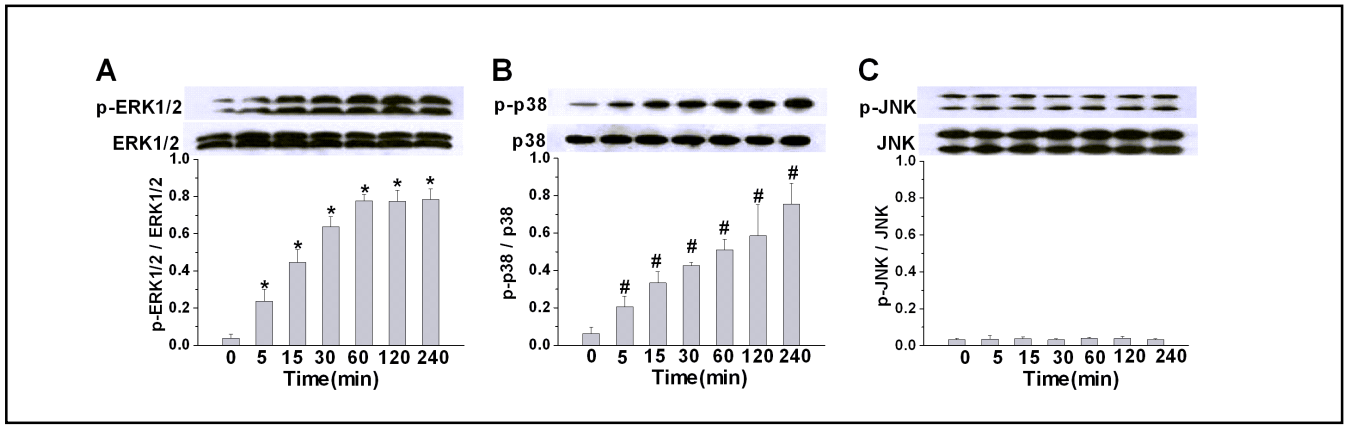
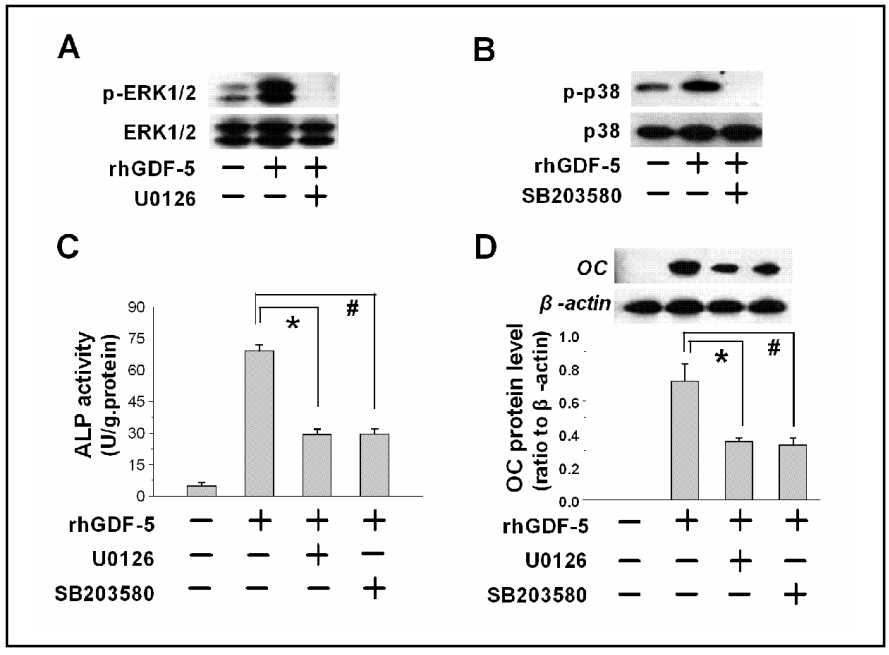


Fig. 4. Effect of rhGDF on MAPK activation in LFCs. Human LFCs were treated with 100 ng/ml of rhGDF-5 for the indicated time. Phospho-ERK 1/2 (p-ERK 1/2) and ERK 1/2 (A), phospho-p38 (p-p38) and p38 (B), and phospho-JNK (p-JNK) and JNK (C) were detected by immunoblotting. Representative blot of three independent experiments are shown, all values are presented as mean ± SD, ANOVA, $P<0.05$ in A, B, and $P=0.68$ in C; * # $P<0.05$ vs control.

Fig. 5. Effects of U0126 (ERK inhibitor) and SB203580 (p38 inhibitor) on rhGDF-5-induced osteogenic differentiation of LFCs. A and B: LFCs were cultured for 60 min in the presence or absence of rhGDF-5 (100 ng/ml) combined with or without U0126 or SB203580 at 20 μM. Phosphorylation of ERK 1/2 and p38 were detected by immunoblotting. C and D: LFCs were cultured for 10 days in the presence or absence of rhGDF-5 (100 ng/ml) combined with or without U0126 or SB203580 at 20 μM. ALP activity and the expression of OC protein were measured. Data and pictures shown are representative of three independent experiments, all values are presented as mean ± SE, * # $P<0.05$ vs rhGDF-5 group.



cytoplasm of the rhGDF-5-challenged cells, but not in the control cells (Fig. 2E). More analysis of osteogenic differentiation by Alizarin red staining revealed that there was the presence of mineralized nodules in the rhGDF-5 group, but not in the control cultures (Fig. 3).

rhGDF-5 activated the ERK1/2, p38 but not JNK in LFCs

To gain insight into the molecular mechanism underlying the osteogenic effects of rhGDF-5 on LFCs, we investigated the activity of MAPK in LFCs treated with rhGDF-5. As shown in Fig. 4A and B, rhGDF-5 treatment had not significant effect on the expression levels of ERK1/2, p38 and JNK, but induced the phosphorylation of ERK1/2 (Fig. 4A) and p38 (Fig. 4B) in a time-dependent manner, while the phosphorylation of JNK (Fig. 4C) was not observed. The phosphorylated ERK1/2 and p38 MAPK were increased approximately 15-fold and 12-fold, respectively, in rhGDF-5 group compared with the control cells.

rhGDF-5-induced osteogenic differentiation of LFCs was mediated by ERK 1/2 and p38 MAPK

To further investigate whether the activation of ERK1/2 and p38 was linked to the osteogenic effect of rhGDF-5 in LFCs, we used specific inhibitors to block

the phosphorylation of ERK1/2, or p38, respectively, and the markers of cell osteogenic differentiation were then measured. In these experiments, LFCs were stimulated with rhGDF-5 in the presence or absence of ERK 1/2 specific inhibitor U0126 or p38 specific inhibitor SB203580 at the concentration of 20 μM. As shown in Fig. 5, ERK1/2 and p38 phosphorylation were completely inhibited by their specific inhibitors, respectively (Fig. 5A and B). Moreover, U0126 and SB203580 significantly inhibited rhGDF-5-induced the increases of ALP activity and osteocalcin protein expression (Fig. 5C and D). These results suggested that rhGDF-5-induced osteogenic differentiation of LFCs was dependent on the activation of ERK 1/2 and p38 MAPK.

Discussion

OLF is definitely a pathological lesion of ectopic ossification originating from the ligamentum flavum tissue. Histologic study revealed that the developmental mode of OLF was mainly endochondral ossification [1]. *In vitro* and *in vivo* studies have indicated that the pathogenesis of OLF was associated with osteogenic differentiation of the spinal ligament cells [30-32]. Increased recognition of the nature of OLF has highlighted the importance of determining the molecular mechanisms of osteogenic differentiation of LFCs. The present study demonstrated that rhGDF-5 induced osteogenic differentiation of LFCs through the activation of ERK1/2 and p38 MAPK. To the best of our knowledge, this is the first study to report the osteogenic effect of GDF-5 on LFCs.

An important finding of this study was that rhGDF-5 treatment induced osteogenic differentiation of cultured LFCs. An *in vitro* study has shown that BMP-2, an importance osteogenic growth factor, induced high ALP activity in normal LFCs from patients without OLF [32]. Previous studies have also reported that BMPs and BMP receptors expressed in ossified ligament tissue from OLF patients and BMP induced OLF in a mice model [8, 31]. We have recently demonstrated that the cultured LFCs from OLF patients manifest phenotypic characteristics of osteoblasts as revealed by expression of osteocalcin, a specific maker of osteoblasts [24]. Therefore, all these studies suggested that LFCs had a great potential to differentiate into osteogenic cells, and this could be the key events in the onset and development of OLF.

Several studies revealed that GDF-5 plays a pivotal role in a variety of musculoskeletal processes including endochondral ossification [33, 34], joint formation [11, 35], tendon and ligament maintenance and repair [36, 37]. In addition, GDF-5 has been demonstrated to play a role in osteogenesis and ectopic bone formation [12-17]. Here, we found that rhGDF-5 induced osteogenic differentiation of LFCs as revealed by the increase of ALP activity, expression of osteocalcin and mineralized nodule formation. The present results supported the evidence that GDF-5 had the potential for osteogenic differentiation. A previous study has shown GDF-5 expressed in spindle-shaped cells and chondrocyte-like cells at ossified sites of the ligaments from OLF patients [20]. Taken together, these findings indicated that endogenous GDF-5 may regulate osteogenic differentiation of the spinal ligamant cells in an autocrine and/or paracrine manner.

GDF-5 can bind to the type I receptor BMPR-IB and the type II receptors BMPRII and ActRII [38]. Through these BMP receptors, GDF-5 initiates intracellular signaling through the activation of downstream substrates, including MAPK signaling molecules. Here, we provided two lines of evidence supporting rhGDF5-induced activation of MAPK in LFCs. First, the presence of rhGDF-5 induced specifically the phosphorylation of ERK1/2 and p38, but had not effect on JNK. Second, the pretreatment of U0126, an ERK1/2 specific inhibitor, and SB203580, a p38 specific inhibitor, blocked rhGDF-5-induced the phosphorylation of ERK1/2 and p38, respectively. These findings suggested that rhGDF-5 induced activation of ERK1/2 and p38 MAPK in LFCs. Consistent with our result, previous studies have shown that GDF-5 induced the phosphorylation of ERK1/2 and p38 in ATDC5 cell line [39] and activated ERK1/2 in human umbilical vein smooth muscle cells [40].

Evidence has accumulated to indicate that MAPK play an important role in the regulation of cell osteogenic differentiation [23, 41, 42]. Recent studies have demonstrated that the activation of MAPK signaling cascade contributes to osteogenic differentiation of cultured LFCs induced by mechanical stress and leptin, which were considered as the causes of OLF [25, 43]. However, whether the activation of ERK1/2 and p38 MAPK is involved in rhGDF-5 induced LFCs differentiation need to be further studied. In our study, the ERK1/2 inhibitor (U0126) or p38 inhibitor (SB203580) inhibited significantly rhGDF-5-induced increase in both ALP activity and osteocalcin expression. These results suggest that rhGDF-5-induced osteogenic

differentiation of LFCs is mediated by the activated ERK 1/2 and p38 MAPK.

In conclusion, this study demonstrates that rhGDF-5 induces osteogenic differentiation of cultured LFCs through the activation of ERK1/2 and p38 MAPK. These findings give some new insight into the pathogenesis of OLF, and may set the basis for pharmacological intervention of OLF patients.

Acknowledgements

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References

1 Okada K, Oka S, Tohge K, Ono K, Yonenobu K, Hosoya T: Thoracic myelopathy caused by ossification of the ligamentum flavum. Clinicopathologic study and surgical treatment. Spine 1991;16:280-287.

2 Aizawa T, Sato T, Sasaki H, Kusakabe T, Morozumi N, Kokubun S: Thoracic myelopathy caused by ossification of the ligamentum flavum: clinical features and surgical results in the Japanese population. J Neurosurg Spine 2006;5:514-519.

3 Mobbs RJ, Dvorak M: Ossification of the ligamentum flavum: Diet and genetics. J Clin Neurosci 2007;14:703-705.

4 Yonenobu K, Ebara S, Fujiwara K, Yamashita K, Ono K, Yamamoto T, Harada N. Ogino H, Ojima S: Thoracic myelopathy secondary to ossification of the spinal ligament. J Neurosurg 1987;66:511-518.

5 Wang W, Kong L, Zhao H, Dong R, Li J, Jia Z, Ji N, Deng S, Sun Z, Zhou J: Thoracic ossification of ligamentum flavum caused by skeletal fluorosis. Eur Spine J 2007;16:1119-1128.

6 Avrahami E, Wigler I, Stern D, Caspi D, Yaron M: Computed tomographic demonstration of calcification of the ligamenta flava of the lumbosacral spine in ankylosing spondylitis. Ann Rheum Dis 1988;47:62-64.

7 Maigne JY, Ayral X, Guerin-Surville H: Frequency and size of ossifications in the caudal attachments of the ligamentum flavum of the thoracic spine. Role of rotatory strains in their development. An anatomic study of 121 spines. Surg Radiol Anat 1992;14:119-124.

8 Hayashi K, Ishidou Y, Yonemori K, Nagamine T, Origuchi N, Maeda S, Imamura T, Kato M, Yoshida H, Sampath TK, ten Dijke P, Sakou T: Expression and localization of bone morphogenetic proteins (BMPs) and BMP receptors in ossification of the ligamentum flavum. Bone 1997;21:23-30.

9 Yayama T, Uchida K, Kobayashi S, Kokubo Y, Sato R, Nakajima H, Takamura T, Bangirana A. Itoh H, Baba H: Thoracic ossification of the human ligamentum flavum: histopathological and immunohistochemical findings around the ossified lesion. J Neurosurg Spine 2007;7:184-193.

10 Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJ, Kozak CA, Reddi AH, Moos M Jr: Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem 1994;269:28227-28234.

11 Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ: Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature 1994;368:639-643.

12 Yeh LC, Tsai AD, Lee JC: Cartilage-derived morphogenetic proteins induce osteogenic gene expression in the C2C12 mesenchymal cell line. J Cell Biochem 2005;95:173-188.

13 Shen FH, Zeng Q, Lv Q, Choi L, Balian G, Li X, Laurencin CT: Osteogenic differentiation of adipose-derived stromal cells treated with GDF-5 cultured on a novel three-dimensional sintered microsphere matrix. Spine J 2006;6:615-623.

14 Shimaoka H, Dohi Y, Ohgushi H, Ikeuchi M, Okamoto M, Kudo A, Kirita T, Yonemasu K: Recombinant growth/differentiation factor-5 (GDF-5) stimulates osteogenic differentiation of marrow mesenchymal stem cells in porous hydroxyapatite ceramic. J Biomed Mater Res A 2004;68:168-176.

15 Gruber R, Mayer C, Bobacz K, Krauth MT, Graninger W, Luyten FP, Erlacher L: Effects of cartilage-derived morphogenetic proteins and osteogenic protein-1 on osteochondrogenic differentiation of periosteum-derived cells. Endocrinology 2001;142:2087-2094.

16 Spiro RC, Liu L, Heidaran MA, Thompson AY, Ng CK, Pohl J, Poser JW: Inductive activity of recombinant human growth and differentiation factor-5. Biochem Soc Trans 2000;28:362-368.

17 Kakudo N, Wang YB, Miyake S, Kushida S, Kusumoto K: Analysis of osteochondro-induction using growth and differentiation factor-5 in rat muscle. Life Sci 2007;81:137-143.

18 Spiro RC, Thompson AY, Poser JW: Spinal fusion with recombinant human growth and differentiation factor-5 combined with a mineralized collagen matrix. Anat Rec 2001;263:388-395.

19 Jahng TA, Fu TS, Cunningham BW, Dmitriev AE, Kim DH: Endoscopic instrumented posterolateral lumbar fusion with Healos and recombinant human growth/differentiation factor-5. Neurosurgery 2004;54:171-180

20 Nakase T, Ariga K, Yonenobu K, Tsumaki N, Luyten FP, Mukai Y, Sato I, Yoshikawa H: Activation and localization of cartilage-derived morphogenetic protein-1 at the site of ossification of the ligamentum flavum. Eur Spine J 2001;10:289-294.

21 Turjanski AG, Vaque JP, Gutkind JS: MAP kinases and the control of nuclear events. Oncogene 2007;26:3240-3253.
22 Qi M, Elion EA: MAP kinase pathways. J Cell Sci 2005;118:3569-3572.
23 Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT: Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. J Bone Miner Res 2002;17:101-110.
24 Zhong ZM, Chen JT: Phenotypic characterization of ligamentum flavum cells from patients with ossification of ligamentum flavum. Yonsei Med J 2009;50:375-379.
25 Fan D, Chen Z, Chen Y, Shang Y: Mechanistic roles of leptin in osteogenic stimulation in thoracic ligament flavum cells. J Biol Chem 2007;282:29958-29966.
26 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.
27 Zhong ZM, Bai L, Chen JT: Advanced oxidation protein products inhibit proliferation and differentiation of rat osteoblast-like cells via NF-kappaB pathway. Cell Physiol Biochem 2009;24:105-114.
28 Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S: BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res 2003;18:1842-1853.
29 Aubin JE: Advances in the osteoblast lineage. Biochem Cell Biol 1998;76:899-910.
30 Murata H, Tanaka H, Taguchi T, Shiigi E, Mizokami H, Sugiyama T, Kawai S: Dexamethasone induces human spinal ligament derived cells toward osteogenic differentiation. J Cell Biochem 2004;92:715-722.
31 Miyamoto S, Takaoka K, Yonenobu K, Ono K: Ossification of the ligamentum flavum induced by bone morphogenetic protein. An experimental study in mice. J Bone Joint Surg Br 1992;74:279-283.
32 Tanaka H, Nagai E, Murata H, Tsubone T, Shirakura Y, Sugiyama T, Taguchi T, Kawai S: Involvement of bone morphogenic protein-2 (BMP-2) in the pathological ossification process of the spinal ligament. Rheumatology (Oxford) 2001;40:1163-1168.
33 Tsumaki N, Tanaka K, Arikawa-Hirasawa E, Nakase T, Kimura T, Thomas JT, Ochi T, Luyten FP, Yamada Y: Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. J Cell Biol 1999;144:161-173.
34 Kadomatsu H, Matsuyama T, Yoshimoto T, Negishi Y, Sekiya H, Yamamoto M, Izumi Y: Injectable growth/differentiation factor-5-recombinant human collagen composite induces endochondral ossification via Sry-related HMG box 9 (Sox9)expression and angiogenesis in murine calvariae. J Periodontal Res 2008;43:483-489.
35 Francis-West PH, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, MacPherson S, Luyten FP, Archer CW: Mechanisms of GDF-5 action during skeletal development. Development 1999;126:1305-1315.
36 Clark RT, Johnson TL, Schalet BJ, Davis L, Gaschen V, Hunziker EB, Oldberg A, Mikic B: GDF-5 deficiency in mice leads to disruption of tail tendon form and function. Connect Tissue Res 2001;42:175-186.
37 Nakase T, Sugamoto K, Miyamoto T, Tsumaki N, Luyten FP, Inui H, Myoui A, Tomita T, Yoshikawa H: Activation of cartilage-derived morphogenetic protein-1 in torn rotator cuff. Clin Orthop Relat Res 2002;399:140-145.
38 Nishitoh H, Ichijo H, Kimura M, Matsumoto T, Makishima F, Yamaguchi A, Yamashita H, Enomoto S, Miyazono K: Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. J Biol Chem 1996;271:21345-21352.
39 Nakamura K, Shirai T, Morishita S, Uchida S, Saeki-Miura K, Makishima F: p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. Exp Cell Res 1999;250:351-363.
40 Chen X, Zankl A, Niroomand F, Liu Z, Katus HA, Jahn L, Tiefenbacher C: Upregulation of ID protein by growth and differentiation factor 5 (GDF5) through a smad-dependent and MAPK-independent pathway in HUVSMC. J Mol Cell Cardiol 2006;41:26-33.
41 Gallea S, Lallemand F, Atfi A, Rawadi G, Ramez V, Spinella-Jaegle S, Kawai S, Faucheu C, Huet L, Baron R, Roman-Roman S: Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. Bone 2001;28:491-498.
42 Fu L, Tang T, Miao Y, Zhang S, Qu Z, Dai K: Stimulation of osteogenic differentiation and inhibition of adipogenic differentiation in bone marrow stromal cells by alendronate via ERK and JNK activation. Bone 2008;43:40-47.
43 Fan D, Chen Z, Wang D, Guo Z, Qiang Q, Shang Y: Osterix is a key target for mechanical signals in human thoracic ligament flavum cells. J Cell Physiol 2007;211:577-584.