RESEARCH COMMUNICATION

Effect of Insulin-like Growth Factor-1 on Bone Morphogenetic Protein-2 Expression in Hepatic Carcinoma SMMC7721 Cells through the p38 MAPK Signaling Pathway

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Abstract

Objective: To observe the effect of insulin-like growth factor-1 (IGF-1) on bone morphogenetic protein (BMP)-2 expression in hepatocellular carcinoma SMMC7721 cells. Methods: Cells were divided into blank control, IGF-1, IGF-1 + SB203580, and SB203580 groups. SB203580 was used to block the p38 MAPK signaling pathway. Changes in the expression of BMP-2, p38 MAPK, and phosphorylated p38, MERK, ERK and JNK were determined using reverse transcription polymerase chain reactions (RT-PCR) and Western blot analysis. Results: Protein expression of phosphorylated BMP-2, MERK, ERK, and JNK was significantly up-regulated by IGF-1 compared with the control group (1.138 ± 0.065 vs. 0.606 ± 0.013, 0.292 ± 0.005 vs. 0.150 ± 0.081, 0.378 ± 0.006 vs. 0.606 ± 0.013, and 0.299 ± 0.015 vs. 0.196 ± 0.017, respectively; P < 0.05). Levels of BMP-2 and phosphorylated MERK and JNK were significantly reduced after blocking of the p38MAPK signaling pathway (0.494 ± 0.052 vs. 0.165 ± 0.017, 0.073 ± 0.07 vs. 0.150 ± 0.081, and 0.018 ± 0.008 vs. 0.196 ± 0.017, respectively; P < 0.05), but such a significant difference was not observed for phosphorylated ERK protein expression (0.173 ± 0.07 vs. 0.150 ± 0.081, P > 0.05). Conclusion: IGF-1 can up-regulate BMP-2 expression, and p38 MAPK signaling pathway blockage can noticeably reduce the up-regulated expression. We can conclude that the up-regulatory effect of IGF-1 on BMP-2 expression is realized through the p38 MAPK signaling pathway.

Keywords: Insulin-like growth factor-1 - BMP-2 - p38 MAPK - hepatic carcinoma

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Introduction

Insulin-like growth factor-1 (IGF-1) is closely correlated with histiocyte proliferation, differentiation, and apoptosis as well as with tumor development. It promotes cell proliferation, differentiation, and maturation and inhibits cell apoptosis. Bone morphogenetic proteins (BMPs) are a group of functional proteins which participate in the biological processes of proliferation, differentiation, and apoptosis of many cells, and regulate cell growth, proliferation, and migration. Our previous study shows that BMP-2, BMP-6, and BMPR II are expressed in different degrees in hepatic carcinoma cells; BMP-2 can noticeably promote hepatic carcinoma cell proliferation and invasion, which might be realized by up-regulating the expression of BMP-2 and matrix metalloproteinase (MMP)-9 and down-regulating E-cadherin expression through the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway; BMP-2-targeting RNA intervention can significantly reduce the capacities of hepatic carcinoma cell migration and trans-membrane in vitro (Wu et al., 2011). These results are consistent to those reported (Xu et al., 2006; Xia et al., 2008). However, to the best of our knowledge, the mechanism leading to BMP-2 high expression in hepatic carcinoma cells has not been reported to date. Many physiological functions of BMP-2 are dependent on the activation of the MARP and P13K pathways; IGF-1 can promote BMP-2 bioactivity and up-regulate its expression through the P13K/Akt signaling pathway to increase cell structure and matrix syntheses; the other way round, the cell differentiation-promoting function of BMP-2 is dependent on the signaling pathway activated by IGF-1 (Noda et al., 2005; Osyczka and Leboy, 2005; Mukherjee et al., 2010). Therefore, the tumor-related bioactivity of BMP-2 is presumably correlated with IGF-1, and both BMP-2 and IGF-1 might become new targets of hepatic carcinoma molecule targeted therapy.
In the current study, the p38 MAPK signaling pathway was blocked using the p38 MAPK specific blocker SB203580. Changes in the associated protein expression were then detected. Whether IGF-1 up-regulates BMP-2 through the p38 MAPK signaling pathway was then investigated.

Materials and Methods

Cell culture

SMMC7721 cell lines were cultured with 100 mL/L

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FBS-containing DMEM medium in a 100 mL/L CO_2 incubator. The medium was changed every two days. When the cells overgrew the bottle, they were digested with 0.25% trypsin for generation at a 1:2 or 1:4 ratio. Cells in logarithmic growth phase were taken.

Grouping

The cells were divided into the blank control, IGF-1, IGF-1 + SB203580 (50 μM), and SB203580 (50 μM) groups. In the early preliminary experiment, the authors of the current study found that the 48-h 50 ng/mL IGF-1 action brought about a significant difference in SMMC7721 proliferation compared with other action methods (Figure 1, $P < 0.05$) based on the experimental grouping method used by Furundzija et al. (2010). This action method was then applied in the current study.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRNzol reagent (Beijing Tiangeng, China). The A260/280 ratio was measured by an ultraviolet spectrophotometer for RNA purity identification. After quantification, samples were stored at -80 °C. RT-PCR procedures were performed according to the instructions indicated in the kit (Dalian TaKaRa, China). The primer sequences of BMP-2 were 5'-AAC CTG CAA CAG CCA ACT-3' (upstream) and 5'-GGA GCC ACA ATC CAG TCA T-3' (downstream). Those of p38 were 5'-CGG AGT GGC ATG AAG CTG TAG-3' (upstream) and 5'-CCC TAG GAA ACC AAC ACA GCA-3' (downstream). Those of β-actin (the internal standard) were 5'-CGG GAA ATC GTG CGT GAC-3' (upstream) and 5'-TGG AAG GTG GAC AGC GAG G-3' (downstream). The amplification conditions consisted of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 57 °C for 45 s and 72 °C for 45 s, and a final extension step at 72 °C for 8 min. PCR products were analyzed in a 10 g/L electrophoresis gel. Then, images were analyzed by the Gel-Pro analyzer software.

Western blot analysis

The protein expression of BMP-2, uPA, and phosphorylated p38, MERK, ERK and JNK were determined using Western blot analysis. Cells were washed twice with PBS, lysed, and then incubated at 4 °C for 30 min. After centrifugation, the supernatant was collected. Protein samples were analyzed and quantified using the Brandford method. The samples (60 μg/well) were fractionated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (4 °C and 50 V). The membrane was treated with confining solution containing 5% skimmed milk at room temperature for 2 h and incubated with primary antibody against BMP-2 (1:200 dilutions; Santa Cruz, USA) at 4 °C overnight. After washing, it was then incubated with secondary antibodies for 2 h. After washing, protein on the membrane was visualized using the ECL detection kit (Santa Cruz, USA). Protein expression was measured by the Gel-Pro analyzer software as the ratio between the gray value of target band and that of GAPDH. The experiment was repeated three times.

Statistical analysis

Data were presented as and analyzed by the SPSS 17.0 software. T-test was performed for comparison between groups based on one-factor ANOVA. $P < 0.05$ was considered significant.

Results

BMP-2 gene and protein expression

RT-PCR did not show any significant difference in BMP-2 gene expression among the groups after 48-h IGF-1 action (50 ng/mL). Western blot analysis showed that BMP-2 protein expression in the IGF-1 group was significantly higher than that in the control group (1.138 ± 0.065 vs. 0.606 ± 0.013, $P < 0.05$), and that in the IGF-1 + SB203580 was also significantly higher than that in the SB203580 group (0.494 ± 0.052 vs. 0.165 ± 0.017, $P > 0.05$).

P38 and phosphorylated p38 protein expression

Western blot analysis showed that the IGF-1 and IGF + SB203580 groups did not display significant differences in p38 mRNA expression compared with the blank control group (0.099 ± 0.015, 0.222 ± 0.029, and 0.061 ± 0.012, respectively; $P > 0.05$), but the SB203580 group did display a significant difference (1.059 ± 0.082 vs. 0.061 ± 0.012, $P < 0.05$). The SB203580 and IGF-1 + SB203580 groups showed a significant difference in phosphorylated p38 protein expression (1.063 ± 0.088 vs. 0.424 ± 0.012; $P < 0.05$), but the IGF-1 and the control groups did not show a significant difference (0.621 ± 0.043 vs. 0.507 ± 0.059; $P > 0.05$). The results are shown in Figures 2 and 3.

Phosphorylated MERK, ERK, and JNK protein expression

Western blot analysis showed that IGF-1 significantly up-regulated the expression of phosphorylated MERK, ERK, and JNK (0.150 ± 0.081 vs. 0.292 ± 0.005, 0.606 ± 0.013 vs. 0.378 ± 0.006, and 0.196 ± 0.017 vs. 0.299 ± 0.015; $P < 0.05$). The blocking of the p38 MAPK signaling pathway significantly inhibited the expression of phosphorylated MERK and JNK (0.073 ± 0.07 vs. 0.150 ±

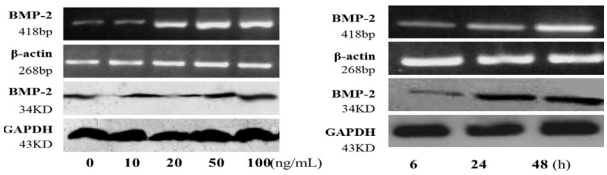

Figure 1. Effects of IGF-1 at Different Concentrations in Different Phases on BMP-2 mRNA and Protein Expression

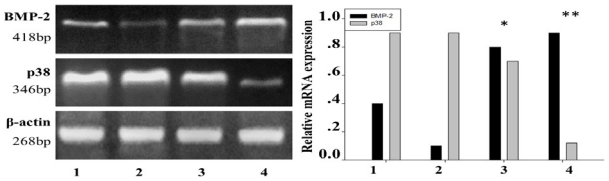

Figure 2. The mRNA Expression of BMP-2 and p38 in Different Groups. 1: the blank control group, 2: the IGF-1 + SB203580 group, 3: the IGF-1 group, and 4: the SB203580 group; *$P < 0.05$ compared with the blank control group, and **$P > 0.05$ compared with the IGF-1 group; n = 3, χ±s

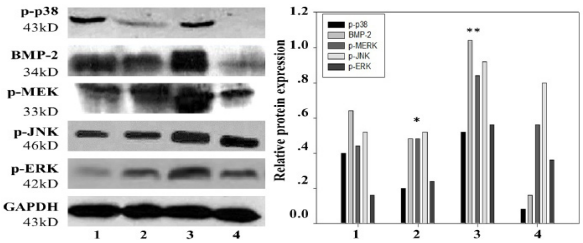

Figure 3. The Protein Expression of Phosphorylated p38, BMP-2, MERK, ERK, and JNK in Different Groups. 1: the blank control group, 2: the IGF-1 + SB203580 group, 3: the IGF-1 group, and 4: the SB203580 group; *P < 0.05 compared with the blank control group, **P < 0.05 in the protein expression of phosphorylated MERK and JNK compared with the control group, and P > 0.05 in phosphorylated ERK protein expression compared with the control group; n = 4, χ±s

0.081 and 0.018 ± 0.008 vs. 0.196 ± 0.017; P < 0.05) but rather than the phosphorylated ERK expression (0.147 ± 0.009 vs. 0.148 ± 0.010; P > 0.05). The results are shown in Figure 3.

Discussion

IGFs play important roles in normal physiological activities. They are closely correlated with embryonic tissue differentiation, ontogeny, and substance metabolism, and are present in many human tissues such as mammary glands, colon, and the prostate. They, along with IGF-1 in circulations, regulate histiocyte proliferation and differentiation through autocrine and paracine secretions, and meanwhile perform an anti-apoptosis effect. IGFs primarily consist of the growth factors IGF-1 and IGF-2 and their corresponding receptors IGF1R and IGF2R, as well as IGF binding proteins (Jones and Clemmons, 1995). IGF-1 plays its role by acting on IGF1R and IGF2R through the MAPK pathway and the phosphatidylinositol 3-kinase (P13K/Akt) pathways (Salehi et al., 2009; Santen et al., 2009). BMPs are subject to the transforming growth factor-β superfamily. They participate in the biological processes of proliferation, differentiation and apoptosis of many cells (Wozney, 1998). BMPs are mainly synthesized and secreted by osteoblasts, and have more than 20 subtypes among which BMP-2 is a critical bone formation regulatory factor (Date et al., 2004). IGF-1 can promote the BMP-2-activated process of osteoblast differentiation, maturation and growth through the P13K/Akt signaling pathway to regulate the BMP-2-induced expression of alkaline phosphatase and osteopontin in bone marrow stromal cells (Gutierrez et al., 1995). In addition, IGF-1 can up-regulate BMP-2 expression in bone marrow stromal cells to increase cell structure and matrix syntheses. On the other side, the activation effect of BMP-2 on osteoblast differentiation seems to depend on the IGF-1-activated pathway signals. The interaction between BMP-2 and the PI3K/Akt pathway activated by IGF-1 performs an important role in osteoblast differentiation and maturation and long bone development; BMP-2 and IGF-1 exert a synergistic effect during the process of cartilage development, and BMP-2 noticeably increases its promotive effect on cartilage cell proliferation in a time-dependent manner after combined with IGF-1; although IGF-1 itself cannot induce cell differentiation, it presumably promotes bone remodeling and fracture repair by activating the related signaling pathways (Chen et al., 2009; Mukherjee and Rotwein, 2009; Steinert et al., 2009; Wang et al., 2009).

There are at least three MAPK signal transduction pathways present in mammalian cells: The ERK1/2 pathway is mainly responsible for cell growth and differentiation regulation while the JNK and p38 pathways participate in stress responses such as inflammation and apoptosis. Many physiological functions of BMP-2 are realized after the MAPK and P13K pathways are activated (Vinals et al., 2002; Guicheux et al., 2003). BMP-2 binds to BMPR-I and BMPR-II to form receptor compounds; these compounds promote the phosphorylation of Smad-1/5/8 to transmit cellular signals, thus, to regulate the target gene (Nishimura et al., 1998). P38 blockers can inhibit the processes of Smad-1 phosphorylation and nuclear transfer induced by BMP-2, suggesting that the Smad-1 signaling pathway is closely correlated with PI3K/Akt, p38, and its downstream nuclear factor NF-κB (Noth et al., 2003). BMP-2/BMP receptor in prostate carcinoma cells might activate the Smad signaling pathway through signaling molecules, such as PI3K/Akt, p38 MAPK, and NF-κB (Graham et al., 2009).

The present study shows that IGF-1 can noticeably up-regulate the gene and protein expression of MBP-2, p38, and phosphorylated p38, MERK, ERK and JNK; the blocking of p38 MAPK can inhibit the protein expression of BMP-2 and phosphorylated ERK in different degrees (but rather than that of phosphorylated MERK and JNK in SMMC7721 cells). These results suggest that the up-regulatory effect of IGF-1 on BMP-2 expression is realized through the MAPK/ERK signaling pathway. The MAPK signaling pathways have been know that they extensively participate in cell proliferation, differentiation, and apoptosis, perform important roles in the transduction pathways of varied signals, and participate in vascular endothelial hyperplasy and invasion and promote neoangiogenisis. BMP-2 can increase tumor vascularization and promote tumorigenesis, which might be realized through the p38 MAPK pathway (Raida et al., 2005). The BMPs/MAPK signal transduction pathway performs its role by interacting with the classic BMPs/BMPR/Smads signaling pathway (Martin et al., 2007). However, whether there exist other stimulating factors in the upstream of BMP-2 and what the action mechanisms between these factors and other downstream signaling pathways of BMP-2 are like still remain to be explored.

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