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RESEARCH

Activation of the PI3K/Akt Pathway Mediates Bone Morphogenetic Protein 2-Induced Invasion of Pancreatic Cancer Cells Panc-1

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Abstract Bone morphogenetic proteins (BMPs) signaling has an emerging role in pancreatic cancer. However, because of the multiple effects of different BMPs, no final conclusions have been made as to the role of BMPs in pancreatic cancer. In our studies, we have focused on bone morphogenetic protein 2(BMP-2) because it induces an epithelial to mesenchymal transition (EMT) and accelerates invasion in the human pancreatic cancer cell line Panc-1. It has been reported that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway mediates invasion of gastric and colon cancer cells, which is unrevealed in pancreatic cancer cells. The objective of our study was to investigate whether BMP-2 mediated invasion might pass through the PI3K/Akt pathway. Our results show that expression of phosphorylation of Akt was increased by treatment with BMP-2, but not Noggin, a BMP-2 antagonist. Then pretreatment of Panc-1 cells with LY294002, an inhibitor of the PI3K/AKT pathway, significantly inhibited BMP-2-induced EMT and invasiveness. The data suggest that BMP-2 accelerates invasion of panc-1 cells via the PI3K/AKT pathway in panc-1 cells, which gives clues to searching new therapy targets in advanced pancreatic cancer.

Keywords PI3K/Akt · Bone morphogenetic protein-2 (BMP-2) · Invasion · Epithelial to mesenchymal transition (EMT)

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Introduction

Although the management and treatment of patients with pancreatic cancer have improved in the last few decades, the overall 5-year survival rate remains at less than 5%. Patients rarely exhibit symptoms before the cancer has become locally advanced or metastatic [1, 2]. BMPs have an emerging role in pancreatic cancer. BMPs belong to the transforming growth factor β (TGF-β) superfamily. BMPs exert their effects via two different types of serine/threonine kinase receptors, BMP type I (BMPR-IA and BMPR-IB) and type II (BMPR-II) receptors. BMP receptor type I is activated upon ligand binding and subsequently phosphorylates receptor -activated Smad proteins (Smad-1, Smad-5 and Smad-8). The phosphorylated Smads then bind to a common mediator Smad (Co-smad, Smad-4), translocate into the nucleus and activate transcription of target genes [3, 4]. BMPs have broad functions including the regulation of many types of normal tissue patterning [5–7] and epithelial-mesenchymal interaction in the developmental process [8]. More recently, it has been reported that BMPs induced EMT and invasion of Panc-1 cells, which was related to activation of Smad1, 5, 8 pathway [9].

Although Smads are critical for BMP family signaling, recent data have implicated multiple non-Smad pathways, including PI3K/Akt, NF-κB, or RAS/ERK pathways, in mediating BMP signaling [10]. It has been found that the PI3K/Akt pathway plays an important role in maintaining the neoplastic phenotype of pancreatic cancer cells, Inhibition of the pathway significantly improved the

survival of nude mice bearing pancreatic tumor xenografts [11, 12]. Increased expression of the PI3K catalytic subunit genes and an increase in Akt activity has been observed in pancreatic cancer cells [13]. Interestingly, which may lead to activation of Akt, was shown to be correlated with metastatic pancreatic cancer [11, 14]. Furthermore, activation of Akt has been suggested to be associated with chemoresistance of aggressive pancreatic cancer [15, 16].

It has been reported that BMP-2 causes EMT and accelerates invasion of Panc-1 cells, and more recently, two studies observed that BMP-2 accelerates the motility and invasiveness of gastric and colon cancer cells via activation of the PI3K/Akt pathway [17, 18], but the role of the PI3K/Akt pathway in invasiveness of pancreatic cancer cells induced by BMP-2 has not been fully investigated. In the present study, we explored the role of the PI3K/Akt pathway in BMP-2-induced EMT and cellular invasiveness. Our results suggest that the BMP-2 signaling pathway induces metastatic functions of pancreatic cancer through the recruitment of the PI3K/Akt pathway.

Materials and Methods

Cell Culture

Human pancreatic cancer cell line Panc-1 was purchased from the Institute of Cell Biology, Shanghai, China, and cultured in Dulbecco's modified Eagle's medium. The medium was supplemented with 4.5 g/L glucose and L-glutamine, 100 IU penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum.

Reagents and Antibodies

Recombinant human BMP-2 and noggin were purchased from R&D Systems (Minneapolis, MN). LY294002 were purchased from Calbiochem (San Diego, CA). Antibodies specific for Akt and phospho-Akt, were obtained from Cell Signaling Technology (Beverly, MA). Antibodies specific for E-cadherin, Slug and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Western Blotting

Cells were lysed by the addition of lysis buffer containing150 mM NaCl,50 mM Tris-HCl, 1% Nonidet P40, and 0.5% sodium deoxycholate. Cell lysates were cleared by centrifugation at 16,000 rpm, 4°C for 15 min. Cleared lysates were boiled for 5 min at 100 C after the addition of 5× sample loading buffer containing 1 M Tris-HCl (pH 6.8), sodium dodesylsulphate, glycerol, and bromphenolblue. The samples were electrophoresed at 200 V on 12.5% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked with 5% non-fat dry milk, and incubated with the primary antibody. The primary antibodies used in this study were as follows: The primary antibodies were as follows: anti-Akt (Cell Signaling Technology), anti-phospho-Akt (Ser 473; Cell Signaling Technology), anti-E-cadherin (Santa Cruz Biotechnology, Inc.), anti-Slug (Santa Cruz Biotechnology, Inc.), anti-β-actin (Santa Cruz Biotechnology, Inc.).

Matrigel Invasion Assay

In vitro invasion assays were performed using 24-well Matrigel-coated transwells (BD Biosciences). 1×10^5 cells/well were seeded in the upper chamber, serum-free medium containing BMP-2 or control vehicle was added to the lower chamber. After 24 h of incubation, non-migrating cells were removed from the upper chamber with a cotton swab and migrating cells on the underside were fixed and stained with crystal violet. The invasing cells were then counted by microscopy. All experiments were repeated three times.

Statistical Analysis

Statistical comparisons were performed by two-tailed Student's t test. Data are given as the mean ± SEM. Significance was established when $P<0.05$.

Results

BMP-2 Up-Regulates Protein Level of the PI3K/Akt Pathway in Panc-1 Cells

To determine whether the PI3K/Akt pathway was involved in the BMP-2-mediated cellular response in panc-1 cells, we first compared levels of phosphorylated and non-phosphorylated Akt in Panc-1 cells treated with BMP-2 or control vehicle by using Western blotting. Stimulation with BMP-2 led to a significant increase in phosphorylation of ser473 in Akt (Fig. 1a). However, there was no change observed in the expression of total Akt, irrespective of the presence of BMP-2. To further confirm the effect of BMP-2 on the expression or kinase activation of Akt, we inhibited the BMP-2 pathway by treating cells with Noggin and then performed Western blot analysis for phosphorylated Akt. As expected, phosphorylation of Akt was markedly reduced in cells that were treated with Noggin when compared with cells treated with control vehicle (Fig. 1a), suggesting that BMP-2 signaling plays a critical role in Akt activation. LY294002,an inhibitor of the PI3K/Akt pathway, attenuated the effect of BMP-2 on the PI3K/Akt pathway. In spite of

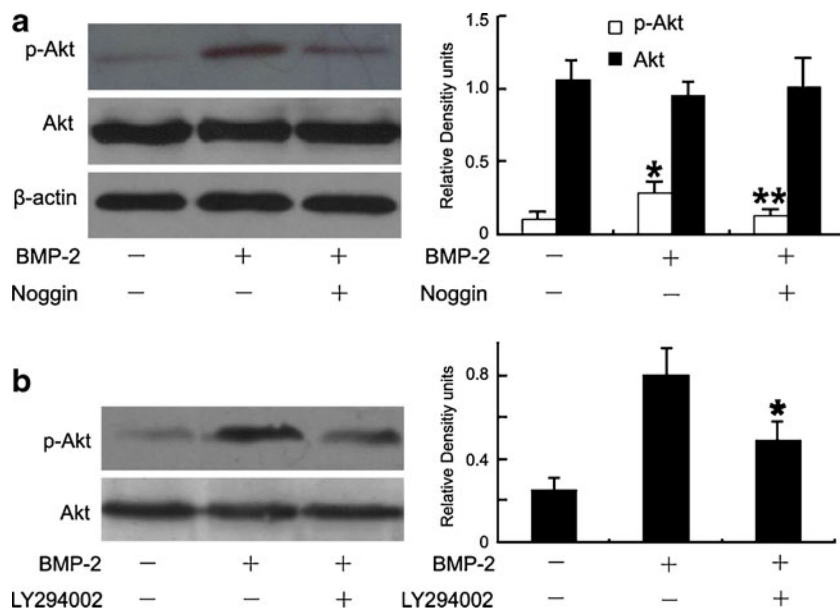

Fig. 1 BMP-2 up-regulates protein level of the PI3K/Akt pathway in Panc-1 cells. (**a**) Panc-1 cells were pretreated with Noggin (5 μg/ml) for 60 min, and then stimulated with BMP-2(100 ng/ml) for 60 min. The total protein was harvested for Western blot analysis using specific antibodies against p-Akt, Akt, and β-actin. $^{*}P<0.01$, compared with cells that were treated with control vehicle. $^{**}P<0.01$, compared with cells that were treated with BMP-2. (**b**) Cells were pretreated with 20 μM LY294002 + DMSO for 30 min followed by stimulation with BMP-2 for 60 min. Protein extracts were prepared for Western blot analysis using specific antibodies against p-Akt and Akt. $^{*}P<0.01$, compared with cells that were treated with BMP-2 + DMSO

the presence of added BMP-2, treatment with LY294002 significantly decreased phosphorylation of Akt (Fig. 1b).

Inhibition of the PI3K/Akt Pathway Decreases BMP-2-Induced Invasion in Panc-1 Cells

BMP-2 has recently been demonstrated to increase the invasiveness of pancreatic cancer cells, BMP-2 treatment of Panc-1 cells dramatically increased the invasion compared with untreated cells through Matrigel [9].To determine the roles of the PI3K/Akt pathway during BMP-2-induced invasion in Panc-1 cells, we examined the effects of blocking the PI3K/Akt pathway on BMP-2-induced invasion. Treatment with LY294002 of Panc-1cells before BMP-2 stimulation significantly decreased invasion than that compared with cells treated with BMP-2 alone. Taken together, these findings show that the BMP-2 signaling pathway modulates the invasion of Panc-1 cells through PI3K/Akt signals. In addition, invasive activity in response to LY294002 by Panc-1 cells treated with BMP-2 was blocked (Fig. 2).

Inhibition of the PI3K/Akt Pathway Attenuates EMT Induced by BMP-2 in Panc-1 Cells

Recent studies have reported that BMP-2 induces EMT, a crucial step for cancer invasion and metastasis, in pancreatic cancer cells. To determine the roles of the PI3K/Akt

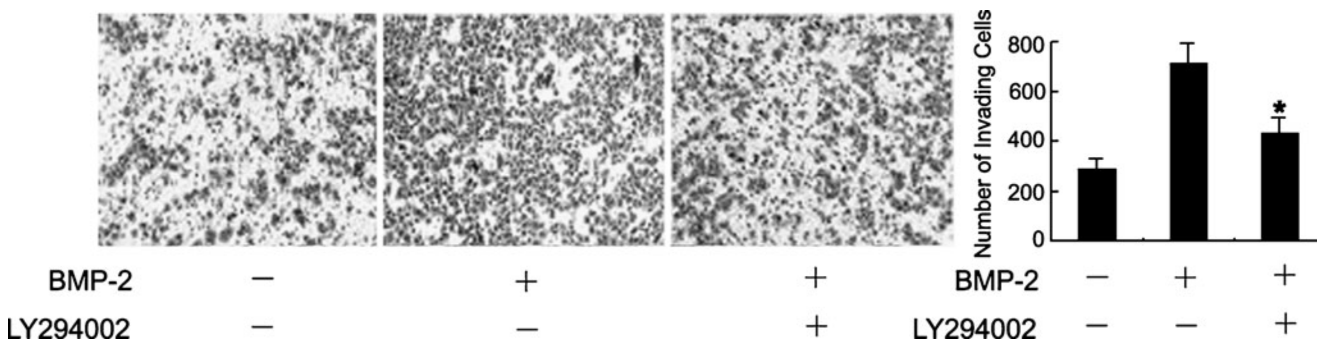

Fig. 2 Invasive activity in response to LY294002 by Panc-1 cells treated with BMP-2 was blocked. Panc-1 cells treated with LY294002 (20 μM) in the presence of BMP-2 (100 ng/ml) were then evaluated in a performance invasion assay.$^{*}P<0.01$, compared with cells that were treated with BMP-2 plus DMSO

pathway during BMP-2-induced EMT in Panc-1 cells, we examined the effects of blocking the PI3K/Akt pathway on BMP-2-induced EMT. Panc-1 cells were pretreated with LY294002 before BMP-2 stimulation and Western blotting for E-cadherin and Slug was performed. We found that decreasing levels of expression of E-cadherin protein by BMP- 4 was completely reverted in Panc-1 cells when the PI3K/Akt pathway was blocked by LY294002 (Fig. 3). Similarly, pretreatment with LY294002 also blocked Slug expression that results from BMP-2 stimulation (Fig. 3). Collectively, these findings strongly suggest that the PI3K/Akt pathway is involved in the EMT response to BMP-2.

Discussion

Recently, several studies have suggested that BMP signaling plays a role in the control of the invasiveness of pancreatic cancer cells. For example, Gordon et al. [9] reported that BMP-2 and BMP-4 is highly overexpressed in human pancreatic cancer and stimulates tumor growth and motility in Panc-1 cells. Hamada et al. [19] also demonstrated that the BMP signaling pathway modulates EMT, resulting in enhancement of invasion in Panc-1 cells. Although these reports demonstrated the role that Smad pathway plays in invasion of pancreatic cancer cells, little is known about that PI3K/Akt pathway regulation plays on BMP-2 induced metastatic behavior of pancreatic cancer cells. The PI3K/Akt pathway is a major cascade stimulating cell migration and invasion in various human cancers [20–22]. Moreover, the PI3K/Akt pathway has been shown to be activated by BMP-2 [23]. Several groups have found that the PI3K/Akt pathway is correlated with the acquisition of migratory and invasive capabilities by BMP-2 [17, 18]. To better understand the molecular mechanism by which BMP-2 promotes invasion of Panc-1 cells. Firstly, we examined that in this study using recombinant human BMP-2 to activate the PI3K/Akt pathway in panc-1 cells. Our results showed that Stimulation with BMP-2 led to a significant increase in phosphorylation of ser473 in Akt, which was blocked by LY294002. Then, using matrigel invasion assay, we observed that increasing invasive capability of Panc-1 induced by BMP-2 was recovered. These findings suggesting that the PI3K/Akt pathway is involved in the invasion response to BMP-2.

EMT, a complex process that leads to loss of epithelial morphology and gain of an invasive fibroblast-like mesenchymal phenotype, is a crucial step for cancer invasion and metastasis in various cancer cell [24–27].EMT is often associated with a decrease or loss of epithelial markers, E-cadherin, and a gain of mesenchymal markers, Slug, which is known to repress expression of the E-cadherin gene. Recent studies have reported that BMPs induces EMT in pancreatic cancer cells and this contributes to increased invasiveness [19].To further confirm the role of the PI3K/Akt pathway in BMP-2 induced invasion of Panc-1 cells, we demonstrated that blockage of the PI3K/Akt pathway by the PI3K inhibitor, LY294002, attenuates Panc-1cells responsive to BMP-2 mediated EMT, indicating that the PI3K/Akt pathway modulates BMP-2 signaling in pancreatic cancer invasion. Interestingly, It has been founded that the activation of PI3K signaling pathways was not detected in BMP-4 treated panc-1 cells. Therefore, to understand this difference of BMP-2 and BMP-4 in pancreatic cancer, further studies will be needed. More recently, Kang MH et al. [17, 18] demonstrated that BMP2 promotes the invasiveness of gastric and colon cancer cells via activation of the PI3K/Akt pathway, which is highly in accordance with our findings.

In summary, BMPs signaling has an important role in pancreatic cancer. Based on our data, we suggest that BMP-2-induced EMT and invasiveness of Panc-1 cells is accomplished by activation of the PI3K/Akt pathway and the precise mechanism is yet to be further defined, which is relevant to our investigation of therapeutic molecular targets to Pancreatic cancer.

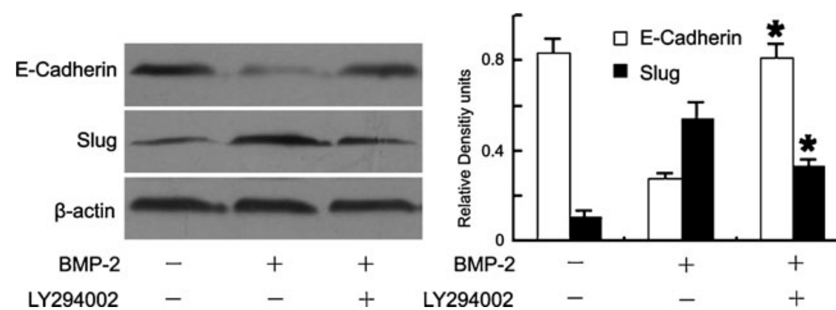

Fig. 3 Inhibition of the PI3K/Akt pathway attenuates EMT induced by BMP-2 in Panc-1 cells. Panc-1 cells were pretreated for 30 min with LY294002 (20 μM) followed by stimulation with BMP-2 (100 ng/ml). Western blotting with anti-E-cadherin, anti-Slug, or anti-β-actin was carried out. *$P<0.01$, compared with cells that were treated with BMP-2 plus DMSO

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