Bone Morphogenetic Protein-2 Suppresses Invasiveness of TSU-Pr1 Cells with the Inhibition of MMP-9 Secretion

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Abstract. *Bone morphogenetic protein (BMP), which is a member of the transforming growth factor β (TGF-β) superfamily, has powerful osteoinductive effects and various biological activities in a variety of cells. The effect of BMP-2 on the human carcinoma cell line TSU-Pr1 was examined. BMP-2 was found to inhibit the migration and invasiveness, but not the proliferation, of TSU-Pr1 cells. Gel zymography for matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) detection revealed that BMP-2 down-regulated the activity of MMP-9, but not of MMP-2 or uPA. BMP-2 also reduced the secretion of MMP-9 into the culture media, but did not affect the secretion of MMP-2, Timp-1, Timp-2 or Timp-3. These results suggest that BMP-2 inhibits migration and decreases MMP-9 secretion to suppress the invasiveness of TSU-Pr1 cells. This is the first report of a role for BMP signaling in reducing the invasiveness of cancer cells.*

Bone morphogenetic proteins (BMPs) were initially identified on the basis of their abilities to induce bone and cartilage formation when implanted subcutaneously or intramuscularly into animals (1, 2). BMPs are structurally similar to the transforming growth factor β (TGF-β) superfamily (1, 3). As with TGF-β, the effects of BMPs are mediated by specific type I and type II serine-threonine kinase receptors (BMPR) (4, 5). The binding of BMP-2 to the type II receptor induces oligomerization of the receptor complex, resulting in the phosphorylation of the type I receptor and the recruitment of the downstream signaling proteins, Smad1, Smad5 and Smad8 (6, 7). Type I BMPR-phosphorylated Smad1, -5 or -8 heterodimerized with Smad4 translocates to the nucleus to act as a transcription factor and induces the expression of genes that mediate the biological activities of BMPs (8).

Tumor invasion and metastasis are complex processes that require controlled degradation of the extracellular matrix (ECM). Several proteinases, including matrix metalloproteinases (MMPs), which are endopeptidases that can degrade most ECM components, regulate the tumor microenvironment and promote cancer progression, playing a major role in this process (9). MMPs constitute a multigene family of cell surface enzymes (10, 11). Components of the ECM, cytokines, growth factors and hormones modulate the transcription of MMP genes and the activity or clearance of the proteins (12, 13).

Under physiological conditions, MMP expression, secretion and activity are highly-regulated. Their expression and activation are elevated in most cancers compared with normal tissue. The role of MMPs in human carcinoma have been studied during the last decade (14-18). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) preferentially degrade denatured collagens (gelatin) and native collagen type IV, which is one of the main components of the ECM and basal membranes. The invasion and metastasis of tumor cells require disruption of the basement membrane and ECM, therefore, MMP-2 and MMP-9 have been implicated as playing critical roles in such processes.

BMP mRNAs and BMP receptors are expressed in a variety of normal and cancerous tissues (19-25) and the effects of BMPs on cancer cells have been examined in several studies. However, the function of BMP-2 in cancer cells, especially during metastasis, is not fully understood.

In the present study, the effect of BMP-2 on the human cancer cell line TSU-Pr1 was investigated. BMP-2 was found to modulate the invasiveness of these cells and to inhibit MMP-9 secretion in ECM. BMP-2 had no effect on the secretion of Timps or uPA. These findings suggest that BMP signals reduce the invasiveness of human carcinoma cells *via* the inhibition of MMP-9 activity.

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Key Words: Bone morphogenetic protein-2, TSU-Pr1 cells, invasion, MMP.

Materials and Methods

Cell culture. The human carcinoma cell line TSU-Pr1 was maintained in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS).

Proliferation assay. The cells ($1x10^3$ cells) were pre-cultured at 37˚C for 1 day in 96-well microplates and treated with 100 ng/ml BMP-2 for 3 days. The cells were washed with 0.9% NaCl and stained with crystal violet for 30 min. The stained cells were dissolved with 1% sodium lauryl sulfate and the relative viable cell number was measured at 595 nm with a spectrophotometer (Microplate Reader Model 550, Bio-Rad, Tokyo, Japan).

Soft-agar colony formation. The cells were incubated with 100 ng/ml BMP-2 for 7 days, harvested, washed and then replated in 0.35% soft agar at the same viable cell number per plate. After 14 days, the colonies were stained with *p*-iodo nitrotetrazolium violet solution and were counted.

In vitro migration and invasion assay. To examine the migratory ability, the cells were suspended in RPMI 1640 at a density of $2x10^4$ cells/ml and seeded into the upper chamber of a chamber with no coated filters (Becton Dickinson, Bedford, MA, USA) with various concentrations of BMP-2 (0-500 ng/ml). The lower wells were filled with RPMI 1640 with 10% FBS. Following an 8-h incubation, the cell penetration was quantified by Giemsa staining. The penetrated cells were counted under a microscope. To examine the invasion ability, chambers with Matrigel-coated filters (Becton Dickinson) were used. The cells were incubated for 16 h and the penetrated cells were counted in the same manner.

Preparation of conditioned medium and zymography. The cells were cultured in serum-free medium in both the absence and presence of 100 ng/ml BMP-2 for up to 24 h. The conditioned media were collected and clarified by centrifugation and then concentrated by centrifugation through a Microcon-YM10 tube (Millipore, Bedford, MA, USA). Gelatinolytic and fibrinolytic activities in the conditioned medium were analyzed by zymography, as described previously (26, 27). In brief, the conditioned medium was mixed with the SDS sample buffer without β-mercaptoethanol and heated for 30 min at 37˚C. The cell number-standardized conditioned medium was analyzed by electrophoresis on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 1 mg/ml of gelatin (for gelatin zymography) or 12 mg/ml of fibrinogen and 0.1 NIH unit/ml of plasminogen (for fibrin zymography). The gel was washed in 2.5% Triton X-100 to remove SDS, incubated overnight at 37˚C in 200 mM NaCl containing 40 mM Tris-HCl and 10 mM $CaCl_2$ (pH 7.5) and stained with Coomassie Brilliant Blue. The presence of gelatinolytic or fibrinolytic activities was identified as clear bands on a uniform blue background after destaining.

Western blot analysis. Cell number-standardized conditioned medium samples were used for the detection of MMPs and Timps. For the detection of Smads and Id1, the cells were lysed in lysis buffer and the protein content was standardized. The proteins were loaded and run on an SDS-PAGE gel, then transferred electrophoretically to nitrocellulose membranes (Bio-Rad). The membranes were washed with TBS-T (25 mM Tris-HCl, 137 mM NaCl, 2.86 mM KCl, 0.1% Tween 20) and blocked with 5% non-fat milk in TBS-T at room temperature for 1 h. The membrane was probed overnight at 4˚C with the appropriate primary antibody in TBS-T. After being washed, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody in TBS-T and incubated for 1 h and the immune complexes were detected by chemiluminescence with an ECL detection kit (Amersham Life Sciences, Piscataway, NJ, USA).

Reagents and antibodies. BMP-2 was generously provided by Astellas Pharma Inc. (Tokyo, Japan). The anti-MMP-2 and MMP-9 antibodies were purchased from Calbiochem (San Diego, CA, USA) and Daiichi Fine Chemical (Takaoka, Japan), respectively. The anti-Timp-1, Timp-2 and Timp-3 antibodies were purchased from Chemicon (Temecula, CA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Daiichi Fine Chemical, respectively. The anti-phospho-Smad1/5/8 and anti-Smad 1 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology. The anti-Id-1 antibody was obtained from Santa Cruz Biotechnology.

Results

Effect on the proliferation and malignancy of TSU-Pr1 cells. To investigate the ability of BMP-2 to alter the characteristics of TSU-Pr1 cells, the proliferation, soft-agar colony-formation, migration and invasion of these cells were studied (Figure 1). BMP-2, at a concentration of 100 ng/ml, did not show any effect on the proliferation or colony-forming ability of TSU-Pr1 cells (Figure 1A and 1B). The TSU-Pr1 cells were treated with several concentrations of BMP-2 in order to investigate the effects of BMP-2 on cell migration and invasive ability. The cells showed significantly lower migration (Figure 1C) and invasion (Figure 1D) than that shown by the untreated control cells. In the migration assay, BMP-2 inhibited the migration of the TSU-Pr1 cells through a non-coated filter in a dose-dependent manner. This effect was minimal at a concentration of 20 ng/ml BMP-2 (9% inhibition), sub-maximal at 100 ng/ml (38% inhibition) and highest at 500 ng/ml (42% inhibition). In the invasion assay, BMP-2 reduced the invasion of TSU-Pr1 cells through the Matrigel-coated filter. A minimal effect was observed at 20 ng/ml BMP-2 (52% inhibition). The greatest effect was observed at 100 ng/ml (84% inhibition) and a sub-maximal effect was observed at 500 ng/ml (61% inhibition).

BMP signals are transduced in TSU-Pr1 cells. To study the intracellular signaling of BMP-2 in TSU-Pr1 cells, the phosphorylation of Smad 1, Smad 5 and Smad 8 downstream of the BMP receptors was analyzed using an antibody that recognizes the phosphorylated forms of these proteins. Stimulation of the TSU-Pr1 cells for up to 60 min with 100 ng/ml of BMP-2 in the absence of FBS caused increased phosphorylation of Smad1, 5 and 8 proteins (Figure 2A).

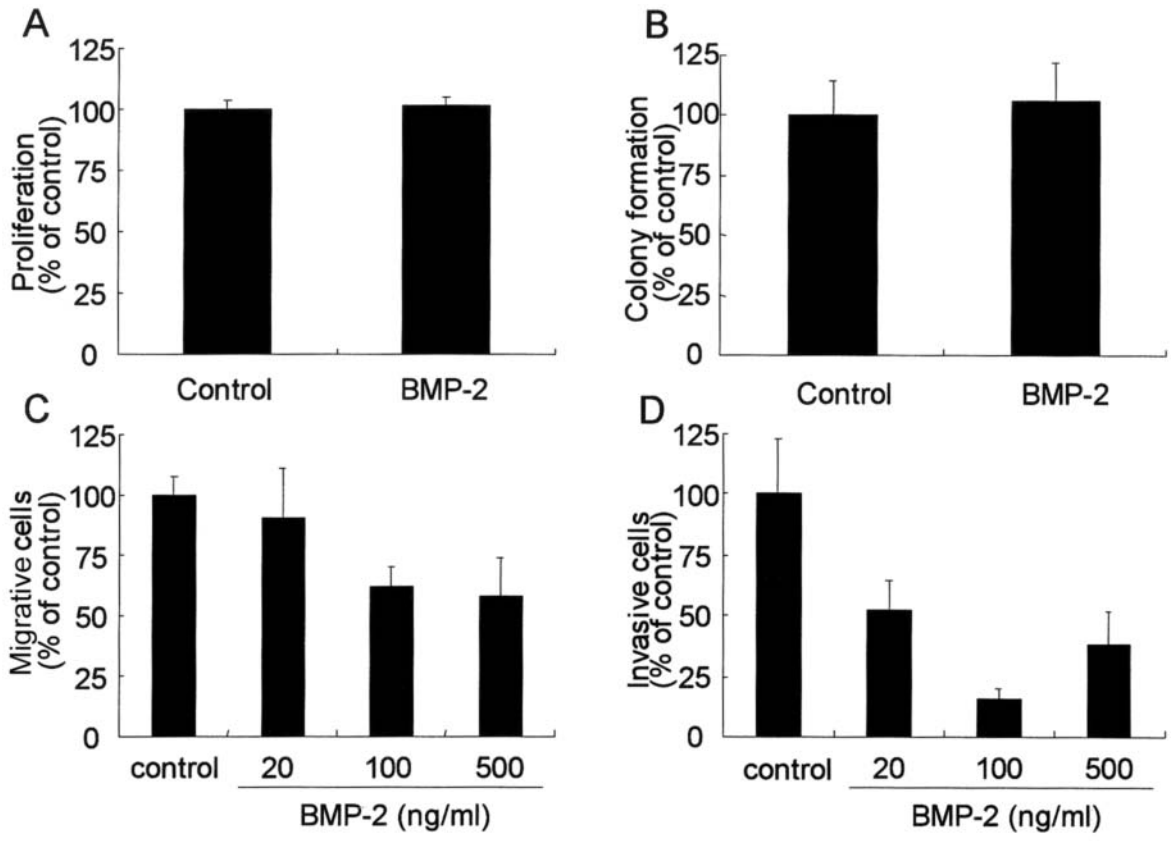

Figure 1. *Proliferation, soft-agar colony-formation, migration and invasion assays of TSU-Pr1 cells treated with BMP-2. For the proliferation assay (A), cells (1x10^3 cells) were treated with 100 ng/ml BMP-2 for 3 days in 96-well microplates and stained with crystal violet. The relative number of viable cells was measured at 595 nm with a spectrophotometer. For the soft-agar colony-forming assay (B), the cells were treated with 100 ng/ml BMP-2 for 7 days and replated in 0.35% soft agar at the same viable cell number per plate. After 14 days, the colonies were stained with p-iodo nitrotetrazolium violet solution and counted. For the migration assay (C), the cells (1x10^4/well) were seeded on a non-precoated filter into a chamber with various concentrations of BMP-2 (0-500 ng/ml) and incubated for 8 h. Those cells that passed through the pores in the filter were fixed, stained and counted with the aid of a microscope. For the invasion assay (D), the experimental conditions were the same as those for the migration assay except that a Matrigel-coated filter was used and the cells were incubated for 16 h. The values shown are the mean±SD of triplicate measurements. Each assay was repeated 3 times.*

To further confirm that BMP signals are transduced in TSU-Pr1 cells, the effect of BMP-2 on *Id-1* expression in the absence of FBS were examined. *Id-1* is a target gene of BMP-2. Western blot analysis was carried out on the TSU-Pr1 cells treated with BMP-2 for 24 h (Figure 2B). The *Id-1* expression was increased significantly by BMP-2, suggesting that BMP signals are transduced in TSU-Pr1 cells.

BMP-2 suppresses MMP-9 but not MMP-2 or uPA activity. To address how BMP-2 modulates TSU-Pr1 invasiveness, the proteolytic activity of the TUS-Pr1 cells was measured by gelatin (Figure 3A) and fibrin zymography (Figure 3B). Treatment of TSU-Pr1 cells with BMP-2 decreased both the pro- and active forms of MMP-9 activity over time. MMP-2 activity was very weak and did not show any change in response to treatment, while the uPA activity was not affected by BMP-2.

BMP-2 suppresses MMP-9 secretion by TSU-Pr1 cells. To examine the effect of BMP-2 on the MMP system, the expressions of MMP-2, MMP-9, Timp-1, Timp-2 and Timp-3 were analyzed by Western blotting (Figure 4). BMP-2 significantly decreased the expression of the pro- and active forms of MMP-9, but not those of MMP-2, Timp-1, Timp-2 or Timp-3 by the TSU-Pr1 cells.

Discussion

Members of the TGF-β superfamily are multifunctional growth factors and the nature of their effects depends on the cellular context (28). The effects of BMP-2, a member of the TGF-β superfamily, on the invasiveness of cancer cells remains unclear, although various biological activities of BMP-2 on cancer cells have been described (21, 29-31). The present study provides evidence for the role of BMP-2

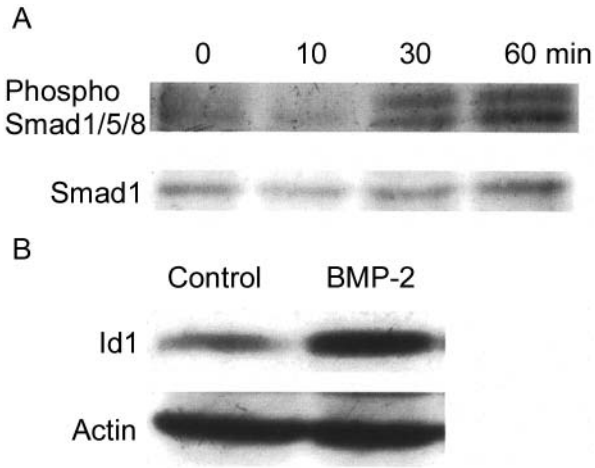

Figure 2. *Western blot analysis of phospho-Smad1/5/8, Smad1 and Id1 protein in TSU-Pr1 cells. BMP signals are transduced in TSU-Pr1 cells. BMP-2-induced the phosphorylation of Smad1/5/8 (A) and Id-1 expression (B). The TSU-Pr1 cells were treated for up to 60 min (for Smad1/5/8 phosphorylation) or 24 h (for Id-1 expression) and subjected to Western blot analysis with anti-phospho-Smad1/5/8 antibody (A, upper panel), anti-Smad1 antibody (A, lower panel), anti-Id-1 antibody (B, upper panel) or anti-actin antibody (B, lower panel).*

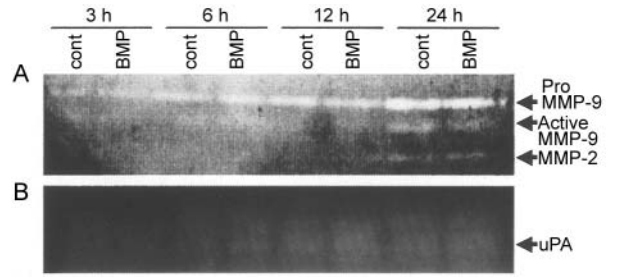

Figure 3. *Effect of BMP-2 on the gelatinolytic and fibrinolytic activities of TSU-Pr1 cells. The conditioned media from TSU-Pr1 cells were tested by gelatin (A) and fibrin zymography (B) as described in the "Materials and Methods" section. Treatment of TSU-Pr1 cells with BMP-2 decreased the pro- and active forms of MMP-9 but not the MMP-2 or uPA activity over time. The serum-free medium was analyzed on SDS-PAGE containing 0.1% gelatin for gelatin zymography (A) or 12 mg/ml of fibrinogen and 0.1 NIH unit/ml of plasminogen for fibrin zymography (B).*

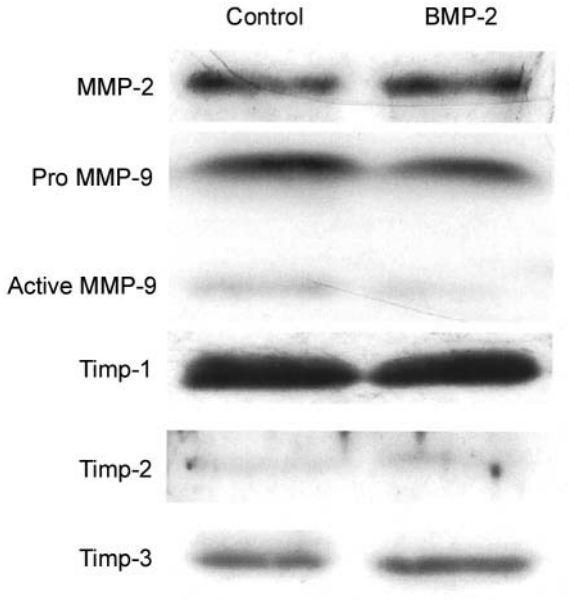

Figure 4. *Western blot analysis of MMP-2, MMP-9, Timp-1, Timp-2 and Timp-3 in TSU-Pr1 cells. The treatment of TSU-Pr1 cells with BMP-2 decreased the expressions of the pro- and active forms of MMP-9, but not those of MMP-2, Timp-1, Timp-2 or Timp-3. The TSU-Pr1 cells were treated for 24 h and then subjected to Western blot analysis.*

in a human carcinoma cell line, TSU-Pr1, especially with regard to malignancy and the MMP system.

Previous reports showed that the growth of TSU-Pr1 cells was not affected by BMPs (21, 32). We also found that BMP-2 failed to inhibit proliferation of TSU-Pr1 cells (Figure 1A). To examine whether BMP signals are activated by BMP-2 in TSU-Pr1 cells, we analyzed Smad1, 5, 8 and *Id-1* expressions. Smad1, 5 and 8 are BMP-specific R-Smads and *Id-1* is a BMP target gene. As shown in Figure 2, R-Smads are phosphorylated and *Id-1* expression is increased in response to BMP-2, indicating that BMP signals are transduced by BMP-2 in TSU-Pr1 cells.

BMP-2 was shown to significantly inhibit the migration and invasion of highly metastatic TSU-Pr1 cells and to decrease the expression of MMP-9 over time. The acquisition of migration and invasive abilities is an important aspect of tumor progression and a principal factor related to cancer morbidity and mortality. It has been reported that cell migration and the production of proteases, including MMPs and uPA, are essential components of the invasion process (33). The reduced invasiveness may be largely due to the decreased production of MMP-9, since this enzyme is important for the degradation of the ECM.

Although several studies have shown that BMPs stimulate cancerous cell invasion (34, 35), the effects on invasion may vary depending on the cell type. For instance, TGF-β inhibited the growth of normal epithelial cells (36-38) and, in early tumor stages, transformed epithelial cells which usually remain sensitive to TGF-β-mediated growth inhibition (39, 40). However, in the later stages of tumorigenesis, epithelial tumor cells frequently escape from TGF-β-induced growth control and, once this has occurred, TGF-β can promote tumor progression (41, 42). TGF-β inhibited the trophoblast cell invasion associated with a

decrease in proteases (43). In contrast, TGF-β promoted the highly invasive nature of carcinoma cells (44).

In conclusion, our data showed that BMP-2 inhibited the migration and invasion of TSU-Pr1 cells and that this inhibition of invasiveness may be associated with a reduced secretion of MMP-9. These findings suggest that BMP-2 may be a therapeutic target for highly metastatic cancers. Further studies will provide additional information with respect to the mechanisms involved in the BMP-2-induced effects.

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Received September 8, 2005
Revised November 29, 2005
Accepted December 1, 2005