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BMP-2 modulates the proliferation and differentiation of normal and cancerous gastric cells

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Abstract

Bone morphogenetic protein 2 (BMP-2), a member of the transforming growth factor β super-family, has been shown to act as an antiproliferative agent for a variety of cell lines by activating signaling cascades that cause cell cycle arrest. However, the biological effect and mechanism of action of BMP-2 on gastric cells remain unknown. In the present study, we showed that recombinant human BMP-2 dose-dependently inhibited the growth of OUMS37 rat gastric cells and MKN74 human gastric cancer cells. The antiproliferation seems to be due to cell cycle arrest in the G_1-phase, which was revealed by flow cytometric assays. BMP-2 increased the level of p21/WAF1/CIP1, suggesting that BMP-2-mediated inhibition of cell proliferation may be induced through p21/WAF1/CIP1. In addition, BMP-2 increased the expression of pepsinogen II, a differentiation marker of the stomach, in MKN74 cells. These results indicate that BMP-2 plays important roles in modulating the proliferation and differentiation of gastric epithelial cells.

Keywords: BMP-2; Proliferation; p21/WAF1/CIP1; Differentiation; Pepsinogen

Bone morphogenetic proteins (BMPs) were initially identified based on their ability 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) super-family [3,4]. BMP-2, a member of this large family of proteins, stimulates the growth and differentiation of osteogenic and chondrogenic cells during bone remodeling and also plays important roles in embryogenesis [2,3,5,6]. Similar to TGF-β, BMPs exert their effect via specific type I and type II serine–threonine kinase receptors (BMPR). Binding of BMP-2 to the type II receptor induces oligomerization of the receptor complex, resulting in phosphorylation of the type I receptor and recruitment of downstream signaling protein, Smad1, Smad5, and Smad8 [7,8]. Among the latter, Smad1 has been extensively studied as the target of BMPR signaling. Type I BMPR-phosphorylated Smad1 heterodimerizes with Smad4, translocates to the nucleus to act as a transcription factor, and then induces genes that mediate the biological activity of BMP-2 [9].

Epithelial–mesenchymal interactions are necessary for the normal development of various digestive organs [10,11]. When the proventricular epithelium begins to form glands, only BMP-2 of the BMP family is expressed in the chicken proventricular mesenchyme at a high level. Virus-mediated BMP-2 overexpression results in an increase in the number of glands formed and expression of embryonic chicken pepsinogen (ECPg), one of the differentiation markers of glandular epithelial cells. On the other hand, the gland formation and expression of ECPg are inhibited by ectopic expression of noggin [12], which can block the action of BMP by binding directly to BMP-2 or BMP-4 with high affinity. Two type I receptors for BMP are expressed in the mouse embryonic stomach [13], and the earliest discernible defect in some Smad5-deficient mouse embryos is a vestigial foregut [14]. Thus, the BMP pathway may be involved in gastric differentiation.

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Juvenile polyposis (JP) is an autosomal dominant gastrointestinal hamartomatous polyposis syndrome, in which patients are at risk for developing gastrointestinal cancers [15,16]. Thirteen of 54 JP patients had germline *BMPRIA* mutations, and patients with *BMPRIA* mutations had a more prominent JP phenotype than those without [17], suggesting high tumor-suppressive activity of BMPRIA. It has been reported that *BMP-2* and *BMP-4* mRNA expression was found in some human gastric cancer cell lines [18]. This accumulated evidence indicates that the BMP pathway might be involved in gastric cell growth control.

BMP-2 inhibits the proliferation of smooth muscle cells, primary mesangial cells, prostate cancer cells, and breast cancer cells [19–22]. Up to now, there have been a number of studies on TGF-β–Smad signaling molecules in gastric carcinogenesis [23,24]. However, although BMPs and their receptors are closely related to the respective TGF-β ligands and receptors, their potential roles in gastric carcinogenesis are still unknown. Here, we examined the effects of BMP-2 on normal and cancerous gastric cells.

Materials and methods

Cell lines and cell culture. The cell lines used in this study comprised a rat gastric epithelial cell line, OUMS37 [25] (kind gift from Dr. Masayoshi Namba, Okayama University Medical School, Japan), and a human gastric cancer cell line, MKN74 (obtained from Riken Cell Bank, Tsukuba, Japan). For routine culture, OUMS37 cells were grown in Dulbecco's modified Eagle's medium and MKN74 cells in RPMI 1640. Both cell lines were cultured at 37 °C under a 5% CO_2 and 95% air atmosphere in the respective medium supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin.

cDNA synthesis and PCR analysis. Total RNA was isolated from the cell lines using a Quickprep RNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, England). Isolated RNA (2 μg) was pre-incubated with 0.4 μg of 12–18-mer oligo(dT) at 70 °C for 10 min and then with 10 mM dNTP, 0.1 M dithiothreitol, and 1 μl Superscript II (RNaseH (−) reverse transcriptase; Life Technologies, Gaithersburg, MD) at 42 °C for 1 h, according to the protocols recommended by the manufacturer. The synthesized cDNA was then amplified by the PCR method. Each PCR cycle consisted of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 1 min, followed by final extension at 72 °C for 10 min. The PCRs for *BMPRIA* and *BMPRII* comprised 24 cycles in a 25 μl mixture containing 1 μl of the synthesized cDNA. The product sizes and primer sequences used were as follows: *hBMPRIA* (GenBank Accession No. NM_004329), 316 bp, 5′-ATCATGG CTGACATCTACAGC-3′ and 5′-ACATCTTGGGATTCAACCAT C-3′; and *hBMPRII* (GenBank Accession No. XM_028038), 587 bp, 5′-AGCAGCAGAACCCTTCCCAAG-3′ and 5′-CCAGAGAATTA GGCCTCTGT-3′. These primers were designed from the sequences with high homology between the human and rat, and the PCR products were confirmed by sequencing. As an internal control for RT-PCR, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcripts were amplified from the same cDNA samples as described previously [26].

For RT-PCR analysis of *pepsinogen C* (GenBank Accession No. NM_002630), which encodes protein pepsinogen II, total RNA (2 μg) from MKN74 cells treated with 100 ng/ml of recombinant human BMP-2 (rhBMP-2) (obtained from Yamanouchi Pharmaceutical Company, Japan) or the same volume of vehicle (LF6: 5 mM glutamic acid, 5 mM sodium chloride, 2.5% glycine, 0.5% galactose, and 0.01% Tween 80) for 48 h was used for cDNA synthesis. The synthesized cDNA was amplified by the semi-quantitative RT-PCR method, as follows. We used a forward primer (5′-GACACAGGCACCTCTCT GCTC-3′) and a reverse primer (5′-GACCTGAGGAAGACATCCC C-3′). PCR for *pepsinogen C* was performed for 30 cycles and 35 cycles in a 25 μl mixture consisting of 1 μl cDNA, 2.5 μl of 10× buffer, 2 μl of 2.5 mM dNTP, 10 pmol of each primer, and 1 U *Taq* DNA polymerase (Biotech International, Bentley, Australia), and each PCR cycle consisted of 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 1 min, with final extension at 72 °C for 10 min. The PCR products were confirmed by sequencing.

Cell proliferation assays. Cell proliferation assays with rhBMP-2 were conducted as follows. OUMS37 cells and MKN74 cells were plated at 2×10^4 or 5×10^4 cells/well, respectively, in a 24-well plate in the respective medium. After culturing for 4–5 h, rhBMP-2 was added to give different concentrations (10, 50, and 100 ng/ml). Cell proliferation was evaluated on day 3 (OUMS37) or 6 (MKN74) by determining the number of cells with a Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) according to the protocol recommended by the manufacturer. As a control, the same volume of vehicle was added to each well.

Flow cytometric analysis. For cell cycle analysis, near 50% confluent cells were synchronized in the G_0/G_1 phase by overnight incubation in serum-free medium. Cells were then incubated in the complete medium containing 100 ng/ml rhBMP-2. After 48 h (OUMS37) or 72 h (MKN74) incubation, the cells were trypsinized and washed with PBS, and then fixed in 70% ethanol for 30 min at −20 °C. The cells were then centrifuged at 1500*g* for 4 min. For nuclear staining, the cells were treated with 1 ml of 100 ng/ml RNaseA at 37 °C for 20 min, centrifuged and re-suspended in 69 μM propidium iodide, and then incubated at room temperature for 20 min. The cells were then analyzed by flow cytometry on FACStar Plus (Becton–Dickinson Immunocytometry System, San Jose, CA). Data were analyzed for 20,000 viable cells as determined by forward and right angle light scatter, stored as frequency histograms, and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

Luciferase assay. The human *p21/WAF1/CIP1* promoter luciferase reporter (*p21*-Luc) construct was described previously [27]. This reporter construct contains the *p21* promoter from −2699 to +45 bp in the pGL2-basic vector. MKN74 cells were plated at 5×10^4 cells/well (24-well plate) 1 day before transfection. The cells were transfected with 300 ng of *p21*-Luc construct and 10 ng pRL-SV40 vector using Trans IT-LT1 transfection reagent (Mirus, Madison, WI) according to the protocol recommended by the manufacturer. Beginning on the next day, the cells were incubated for another 24 h with or without 100 ng/ml rhBMP-2. The cells were then harvested and a dual luciferase test was performed using a Dual Luciferase Assay Kit (Promega, Madison, WI) as described by the manufacturer with a Lumicounter 700 (Microtech Niti-on, Chiba, Japan). Each assay was performed in duplicate and experiments were repeated three times. The results were expressed as fold activation, that is, the ratio of normalized luciferase activity on treatment with rhBMP-2 to that with the vehicle.

Western blot analysis. Approximately 30–50% confluent cultures of OUMS37 and MKN74 cells were incubated with 100 ng/ml rhBMP-2 for 24 and 48 h, respectively. The cells were lysed with NP-40 lysis buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris, pH 8.0, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 50 μg/ml PMSF). Protein concentrations were measured with a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). The total cell lysates (80 μg per lane) were separated on 12% SDS–polyacrylamide gels and the proteins were electroblotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA). The blots were probed separately with antibodies against p21/WAF1/CIP1 diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), and α-tubulin diluted 1:2000 (Santa Cruz). Secondary goat anti-rabbit and anti-mouse antibodies were purchased from Santa Cruz Biotechnology.

Determination of the secreted pepsinogen II protein was performed as follows. About 20% confluent culture of MKN74 cells was incubated with 100 ng/ml rhBMP-2 for 4 days. The medium was then changed and rhBMP-2 (100 ng/ml) was added for further 2 days, then 40 μl aliquots of the medium of cultured cells were separated on 10% SDS–polyacrylamide gels and the products were blotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA). The blots were probed with antibodies against pepsinogen II diluted 1:1000 (The Binding Site Limited, Birmingham, UK). Secondary sheep anti-human pepsinogen II (IgG fraction) antibodies were purchased from The Binding Site Limited.

Results

BMP receptors are expressed in OUMS37 and MKN74 cells

To determine if the BMP-2 signaling pathway is intact, we investigated the mRNA expression of *BMPRIA* and *BMPRII* in OUMS37 and MKN74 cells. RT-PCR analysis revealed that *BMPRIA* and *BMPRII* were abundantly expressed in both cell lines (Fig. 1).

BMP-2 inhibits the proliferation of OUMS37 and MKN74 cells

We analyzed the effects of BMP-2 on the proliferation of OUMS37 and MKN74 cells with the Cell Counting kit 8. Growing OUMS37 and MKN74 cells were treated with increasing concentration of rhBMP-2. As shown in Fig. 2, BMP-2 inhibited cell growth in a dose-dependent manner in both cell types. Approximately 60% and 50% growth inhibition of OUMS37 and MKN74 cells was observed with 100 ng/ml BMP-2. We also directly calculated the number of cells with a hemocytometer, and obtained similar results (data not shown).

The responsiveness of OUMS37 and MKN74 cells to rhBMP-2 was also evidenced by alterations in cell shape.

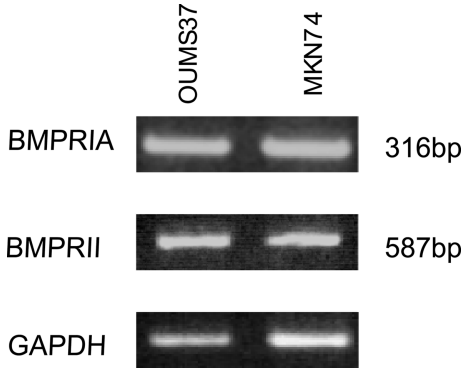

Fig. 1. *BMPRIA* and *BMPRII* are expressed in OUMS37 and MKN74 cell lines. Total RNA (2 μg) isolated from OUMS37 and MKN74 cell lines was used for cDNA synthesis, and then PCRs were performed as described under Materials and methods. The upper panel shows type *IA BMP receptor*, and the middle one shows type *II BMP receptor*. *GAPDH* was used as a control.

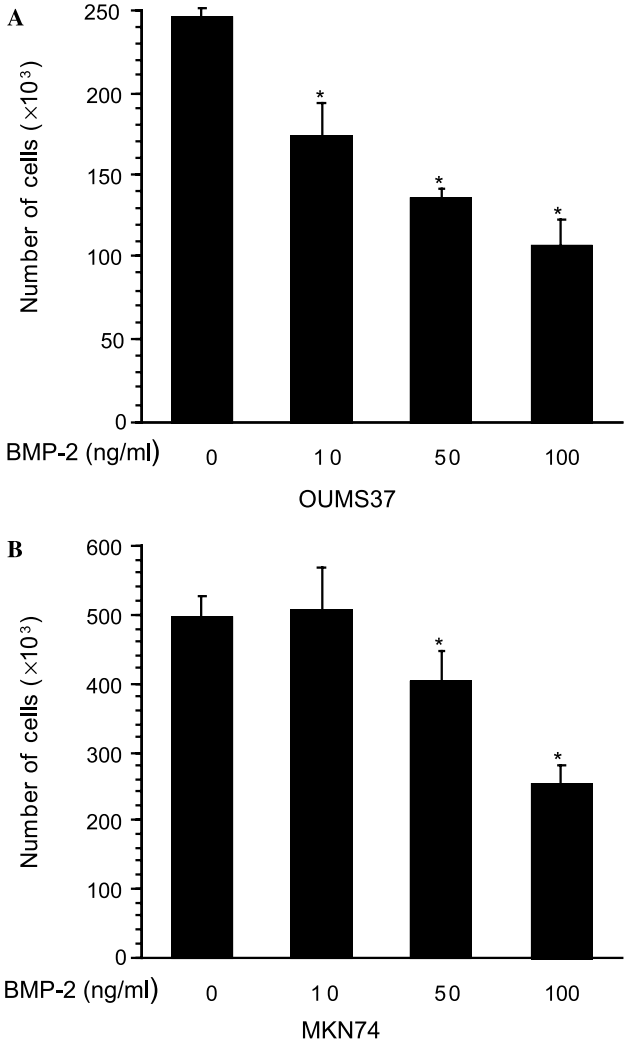

Fig. 2. Effects of BMP-2 on OUMS37 (A) and MKN74 (B) cell proliferation. OUMS37 and MKN74 cells were incubated with different concentrations of rhBMP-2 as described under Materials and methods. The numbers of cells were determined for each treatment. Means ± SD of three independent experiments. $^{*}P < 0.05$ vs untreated cells.

The majority of OUMS37 and MKN74 cells treated with rhBMP-2 adopted a more enlarged shape with close intercellular contacts and a decreased proportion of nucleus to cytoplasm compared with untreated cells (Fig. 3). These data indicated that BMP-2 dose-dependently inhibited the proliferation of OUMS37 and MKN74 cells, and changed the cell morphology.

BMP-2 causes cell cycle arrest in the G_1-phase in OUMS37 and MKN74 cells

A possible explanation for the observed growth inhibition of OUMS37 and MKN74 cells in response to BMP-2 is either apoptosis or inhibition of new DNA synthesis. We examined the effects of BMP-2 on OUMS37 and MKN74 cell cycle progression by means of FACS analysis. OUMS37 and MKN74 cells

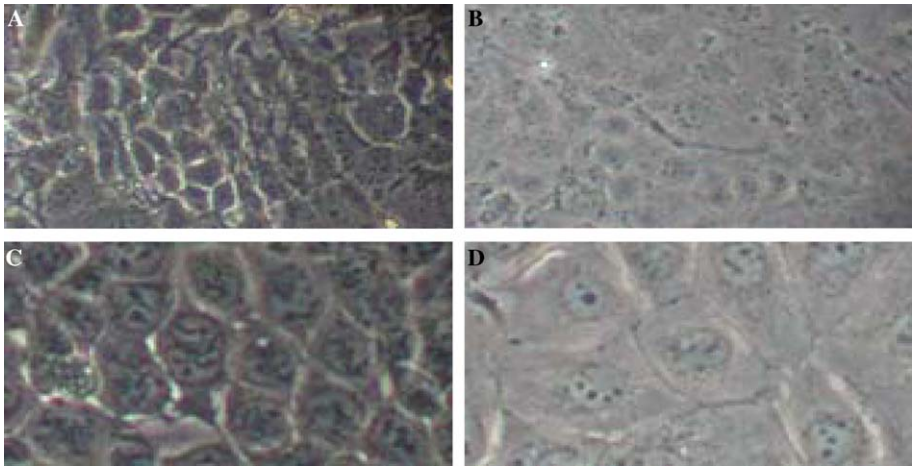

Fig. 3. Morphological changes of OUMS37 and MKN74 cells. The upper panels (A and B) show OUMS37 cells and the lower ones (C and D) MKN74 cells. OUMS37 and MKN74 cells were incubated with 100 ng/ml concentration of rhBMP-2 for 4 or 5 days, respectively. Cells treated with BMP-2 (B and D) adopted a more enlarged shape with close intercellular contacts and a decreased proportion of nucleus as to cytoplasm compared with untreated cells (A and C). Magnification 200×.

Table 1
Flow cytometric analysis of OUMS37 and MKN74 cells for G_1- and S-phase

Name of cell	BMP-2	% of cells in G_1-phase	% of cells in S-phase
OUMS37	–	69.0 ± 1.2	24.1 ± 0.8
	+	78.0 ± 0.4	15.5 ± 0.2
MKN74	–	54.3 ± 2.3	40.3 ± 1.4
	+	65.8 ± 1.8	28.2 ± 0.5

incubated with 100 ng/ml rhBMP-2 showed approximately 36% and 30% decreases in the S-phase, respectively (Table 1). The decrease in the S-phase population was accompanied by an increase in the cell number at the G_1-phase of the cell cycle. These data suggest that rhBMP-2 inhibits OUMS37 and MKN74 cell proliferation by arresting them at the G_1-phase of the cell cycle.

BMP-2 up-regulates the expression of CDK inhibitor p21/WAF1/CIP1 in OUMS37 and MKN74 cells

Cyclin-dependent kinase inhibitors (CDKIs) are involved in the cell cycle arrest induced by many growth inhibitors including TGF-β [28]. p21/WAF1/CIP1, one of the CDKI, was up-regulated by rhBMP-2 in some cell systems [19,22]. We therefore examined whether or not rhBMP-2 activates the *p21/WAF1/CIP1* promoter in MKN74 cells. rhBMP-2 stimulation resulted in about 2-fold transcriptional activation of the *p21/WAF1/CIP1* promoter construct compared with the same volume of vehicle (Fig. 4A).

To confirm the up-regulation of the endogenous p21/WAF1/CIP1 protein by BMP-2 in OUMS37 and MKN74 cells, we analyzed the protein level of p21/WAF1/CIP1 by Western blot assay. As shown in Fig. 4B, rhBMP-2 increased the p21 protein levels in

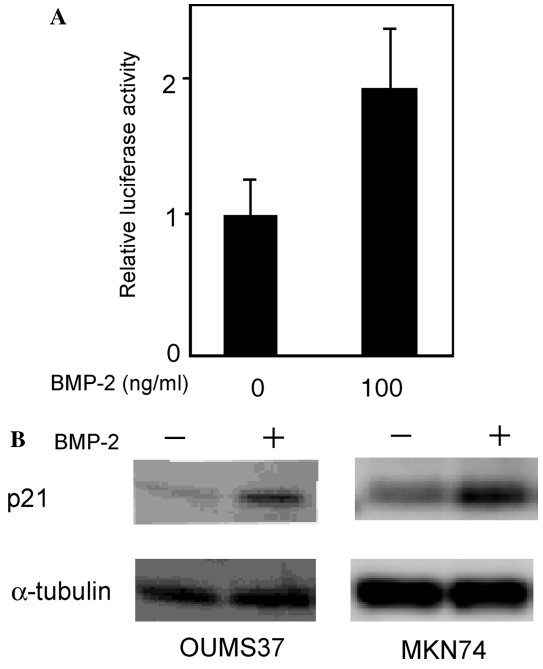

Fig. 4. (A) BMP-2 activates the *p21* promoter in MKN74 human gastric cancer cells. MKN74 cells were transfected with the *p21* promoter luciferase-reporter plasmid. Beginning on the next day, the cells were incubated for 24 h with or without 100 ng/ml rhBMP-2. The results were expressed as fold activation as described under Materials and methods. These results are representative of three independent experiments carried out in duplicate. (B) BMP-2 up-regulated the p21 protein level in OUMS37 and MKN74 cells. OUMS37 and MKN74 cells were incubated with 100 ng/ml BMP-2 for 24 and 48 h, respectively. Equal amounts of cell lysates were analyzed by Western blotting. α-Tubulin was used as a loading control.

OUM37 and MKN74 cells. These data constitute evidence that the growth inhibition of OUMS37 and MKN74 cells in response to BMP-2 may be caused by an increased level of p21/WAF1/CIP1.

BMP-2 up-regulates pepsinogen II expression

To determine whether or not BMP-2 up-regulates the transcription of endogenous *pepsinogen C*, a differentiation marker of glandular epithelial cells of the stomach, MKN74 cells treated with rhBMP-2 for 48 h were assayed for the *pepsinogen C* mRNA level by RT-PCR. MKN74 cells treated with the same volume of vehicle were used as a control. As shown in Fig. 5A, the internal standard *GAPDH* mRNA level remained constant in cells treated with rhBMP-2 or the same volume of vehicle (refer to the *GAPDH* panel). MKN74 cells treated with rhBMP-2 expressed the *pepsinogen C* transcript, whereas the control only showed a faint level of expression (refer to the *pepsinogen C* panel, 30 cycles), indicating that rhBMP-2 had up-regulated the transcription of the endogenous *pepsinogen C* gene in MKN74 cells.

To ascertain whether or not BMP-2 up-regulates the pepsinogen II protein, a translation product of *pepsinogen C*, we conducted Western blot analysis to determine the secreted pepsinogen II protein. The medium from MKN74 cells treated with rhBMP-2 contained pepsinogen II (a molecular mass of approximately 40 kDa), but the control exhibited only a very slight level of the protein (Fig. 5B), demonstrating that rhBMP-2 had also up-regulated the protein level of pepsinogen II in MKN74 cells.

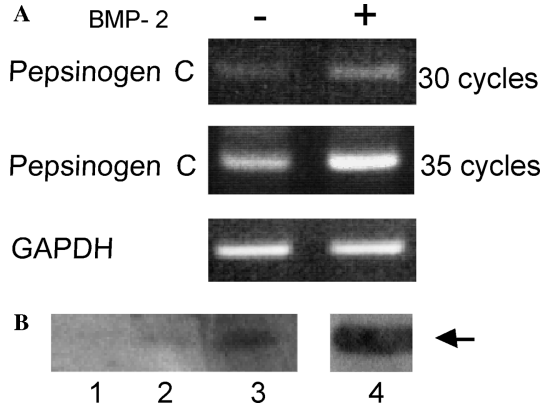

Fig. 5. Up-regulation of the pepsinogen II mRNA and protein in MKN74 cells. MKN74 cells were cultured with rhBMP-2 for 2 days. mRNA and proteins were analyzed as described under Materials and methods. (A) RT-PCR analysis of *pepsinogen C* encoding the pepsinogen II protein. *GAPDH* was used as a control. (B) Western blot analysis of pepsinogen II. Lane 1, fresh medium with 10% FBS; lane 2, medium of untreated MKN74 cells; lane 3, medium of MKN74 cells treated with rhBMP-2; and lane 4, human normal stomach mucosa as a positive control.

Discussion

Our data constituted the first evidence that BMP-2 inhibits the proliferation of OUMS37 rat gastric cells and MKN74 human gastric cancer cells. The inhibition may be due to an increased level of p21/WAF1/CIP1, a cyclin-dependent kinase inhibitor, in response to BMP-2 in both cell types. BMP-2 also up-regulated pepsinogen II, a differentiation marker of glandular epithelial cells of the stomach, in MKN74 cells.

BMPs are members of the TGF-β super-family of cytokines and, like TGF-β, employ similar signal transduction pathways. It has been reported that the TGF-β signal suppresses the growth of gastric epithelial cells [23] and is involved in gastric carcinogenesis [29–32]. Many gastric cancer cells including MKN74 exhibit functional impairment in the TGF-β pathway [33]. Because MKN74 cells express the wild type TGF-βRII and Smad4 proteins [33], the exact mechanism of resistance to TGF-β has not been clarified for MKN74.

BMP-2 is highly expressed in the chicken proventricular mesenchyme [12], and the receptors for BMP are expressed in the mouse embryonic stomach [13]. In addition, BMPs and their receptors are closely related to the respective TGF-β ligands and receptors. It is, therefore, possible that BMPs may play important roles in the control of gastric cell behavior. In the present study, we observed an antiproliferative effect of BMP-2 on OUMS37 and MKN74 cells, and both cell types showed the expression of receptors for BMP. It has been reported that Smad4 was expressed in MKN74 cells [33], thereby demonstrating that BMP receptors and the protein that mediates the BMP-2 signal to the nucleus were expressed in MKN74 cells. The susceptibility of OUMS37 and MKN74 cells to BMP-2 was supported by alterations in cell shape. Taken together, BMP signaling may participate in the regulation of gastric cell growth, which may be independent of the TGF-β pathway.

In other cell types, BMP-associated growth inhibitory responses were found to be transmitted, at least in part, by the CDK inhibitor p21/WAF1/CIP1. Inhibition of the cell growth of breast cancer cells and aortic smooth muscle cells by BMP-2 is mediated by p21/WAF1/CIP1 [19,22]. p21/WAF1/CIP1 binds to G_1 cyclin–CDK complexes, resulting in inhibition of CDK activity, and decreases phosphorylation of the retinoblastoma (RB) protein and then induces subsequent cell cycle arrest in the G_1-phase. In our study, BMP-2 up-regulated *p21/WAF1/CIP1* promoter activity and expression of the p21/WAF1/CIP1 protein in OUMS37 and MKN74 cells. Moreover, the BMP-2-treated cells exhibited cell cycle arrest in the G_1-phase. These data indicated that the growth inhibition by BMP-2 may be mediated by p21/WAF1/CIP1 in OUMS37 and MKN74 cells.

It was shown previously that p21/WAF1/CIP1 stimulates withdrawal from the cell cycle coupled to terminal differentiation [34]. In addition, epithelial–mesenchymal interactions are essential for normal development of the chicken proventriculus corresponding to the mammalian stomach [10]. *BMP-2*, one of the important genes expressed in the mesenchyme, contributes to proventricular gland formation and ECPg expression [12]. In the present study, rhBMP-2 increased the expression of pepsinogen II, a differentiation marker of stomach glandular cells, in MKN74 cells. Thus, BMP-2 may lead to differentiation of MKN74 cells. The inductive activation of cell differentiation by BMP-2 has been reported for several cell types including astrocytes, cardiomyocytes, and myoblast cells [5,35,36].

In conclusion, we revealed that BMP-2 caused cell cycle arrest in the G_1-phase in OUMS37 and MKN74 cells, and that this growth inhibitory action may be mediated by p21/WAF1/CIP1. We also showed that BMP-2 up-regulated pepsinogen II in MKN74 cells. Our findings indicate that the BMP pathway may play a pivotal role in modulating gastric epithelial cell behavior including that of gastric cancer cells. Further analyses of the effects of BMP-2 on gastric cells are necessary to better understand the roles of BMP-2 in gastric epithelial cell growth and differentiation.

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