Effect of BMP4 on the Growth and Clonogenicity of Human Leukemia and Lymphoma Cells

YUSUKE TAKAHASHI, TOMOHIRO ISHIGAKI, YUKI OKUHASHI, AYA ONO, MAI ITOH, NOBUO NARA and SHUJI TOHDA

Department of Laboratory Medicine, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

Abstract. *Background: Bone morphogenetic protein 4 (BMP4) signaling is involved in the maintenance of hematopoietic stem cells. However, the effects of BMP4 on leukemia and lymphoma cells are unknown. Materials and Methods: The effects of recombinant BMP4 on the in vitro growth of 12 leukemia and lymphoma cell lines were examined. Results: BMP4 treatment promoted the short-term growth of three cell lines and suppressed the growth of one. Induction of differentiation was not observed. BMP4 treatment suppressed the clonogenicity of four out of the six examined cell lines. BMP4 treatment promoted the growth of Jurkat cells but suppressed their ability to form colonies. BMP4 treatment up-regulated the phosphorylation of SMAD1/5/8 complex, indicating that BMP4 mediated signal transduction in the cells. Conclusion: BMP4 suppressed the clonogenicity of selected leukemia and lymphoma cell lines. The regulation of BMP4 signaling may be a useful therapeutic approach for leukemia and lymphoma, if appropriate cases are selected.*

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, are secreted ligands. To date, approximately 20 BMPs have been identified in mammals. BMP4 regulates the cell fate, proliferation, and differentiation of various cell types, including hematopoietic cells. BMP4 is known to maintain hematopoietic stem cells (1-3), whereas a low concentration of BMP4 induces proliferation and differentiation of hematopoietic progenitors, particularly erythroid progenitors (4, 5). The binding of BMP4 to type I and II receptors mediates the phosphorylation of the SMAD1/5/8 complex. The phosphorylated complex then translocates to the nucleus, in combination with SMAD4 and regulates the transcription of target genes.

Correspondence to: Shuji Tohda, MD, Ph.D., Department of Laboratory Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-Ku, Tokyo 113-8519, Japan. Tel: +81 358035334, Fax: +81 358035629, e-mail: tohda.mlab@tmd.ac.jp

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BMP4 is also involved in the pathophysiological mechanisms of a variety of cancer types (6). It was reported that exposure to BMP4 inhibits the tumorigenic potential of human brain tumor-initiating cells (7). In hematological malignancies, BMPs and their receptors are expressed in acute promyelocytic leukemia (8) and myeloma cells (9). It has also been reported that treatment with BMP4 inhibits the *in vitro* growth of myeloma cells (10).

In this study, we examined the effects of BMP4 on the growth of various leukemia and lymphoma cell lines in culture. We also examined the expression and activation of BMP4-related molecules in these cell lines. To our knowledge, this is the first report showing the effects of BMP4 on cells from various hematological malignancies.

Materials and Methods

Cells and reagents. Twelve human leukemia and lymphoma cell lines derived from acute T-lymphoblastic leukemia (T-ALL), B-cell malignant lymphoma (B-ML), chronic lymphocytic leukemia (B-CLL), acute myeloblastic leukemia (AML), and chronic myelogenous leukemia (CML) were used (Table I). T-ALL cell lines were provided as a gift from Dr. A. Harashima and Dr. K. Orita (Fujisaki Cell Center, Okayama, Japan). The TMD cell lines were established in our laboratory. NB4 (11) was kindly provided by Dr. M. Lanotte (Hôpital Saint-Louis, Paris, France). The OCI/AML cell lines were established at the Ontario Cancer Institute (Toronto, Canada). AA (12) was established by Dr. A. Arai (Tokyo Medical and Dental University, Japan) from acute pure erythroid leukemia cells. The other cell lines were supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Recombinant human BMP4 was purchased from R&D Systems (Minneapolis, MN, USA).

Short-term growth assay. The effects of BMP4 on short-term growth were examined using a colorimetric assay (WST-1 assay). The cells ($0.2\text{-}1\times10^4$ cells/well) were cultured in 0.1 ml of RPMI-1640 medium (GIBCO, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum, with or without BMP4. After 5 days of culture, a solution of WST-1 and 1-methoxy-5-methylphenazinium methylsulfate (Dojindo Laboratories, Kumamoto, Japan) was added to the cells. The optical density was measured using an enzyme-linked immunosorbent assay reader, to provide an indication of the

Table I. *Reverse transcription-polymerase chain reaction (RT-PCR) analysis of bone morphogenetic protein 4 (BMP4)-related gene expressions.*

	Cell lines	Qualitative				Quantitative (% of control)	
		BMP4	*BMPR1A*	*BMPR1B*	*BMPR2*	*ID1*	*ID2*
T-ALL	Jurkat	+	+	+	+	379	269
	DND-41	–	+	+	+	104	93
	KOPT-K1	+	+	-	+	231	77
B-ML	Daudi	–	+	+	+	484	193
	TMD8	–	+	–	+	158	93
CLL-AP	TMD2	–	+	–	+	132	120
AML	OCI/AML1	+	+	+	+	128	84
	OCI/AML4	w	+	w	+	55	49
	NB4	–	+	–	+	902	281
	THP-1	–	+	–	+	209	164
	AA	+	+	+	+	249	238
CML-BC	K562	–	+	w	+	326	311

T-ALL, acute T-lymphoblastic leukemia; B-ML, B-Cell malignant lymphoma; CLL-AP, chronic lymphocytic leukemia in acute phase; AML, acute myeloblastic leukemia; CML-BC, chronic myelogenous leukemia in blastic crisis; *BMPR*, bone morphogenetic protein receptor; *ID*, inhibitor of DNA binding; w, weakly positive. The expression levels of *ID1* and *ID2* genes examined by quantitative RT-PCR were normalized to those of *β-actin* mRNA. The values are shown as a percentage of the levels in control cells cultured without BMP4. More than 200% increase suggests significant up-regulation of the expression.

cell number. The Student's *t*-test was used to determine the statistical significance of the differences between the controls and the BMP4-treated cells.

Colony assay. The effects of BMP4 on colony formation were examined. Six cell lines that form distinct colonies were used. The cells were plated at 0.2-1×10^3 cells/well in 96-well culture plates, in 0.1 ml of RPMI-1640 medium containing 0.8% methylcellulose, with or without BMP4. After 7 days of culture, colonies containing more than 20 cells were counted under an inverted microscope.

Cell morphological analysis. To examine the effects of BMP4 on differentiation, cells cultured with or without BMP4 were observed under an inverted microscope. Cytospin preparations were created from the harvested cells, stained with Wright, and observed under a microscope.

Reverse transcription-polymerase chain reaction (RT-PCR). The expressions of the genes for BMP4 and its receptors were examined using qualitative RT-PCR. Total RNA was extracted, and first-strand cDNA was synthesized. PCR was performed using the primers shown in Table II. The products were electrophorised on an agarose gel and stained with ethidium bromide. The effects of BMP4 stimulation on gene expression were examined with quantitative RT-PCR using a FastStart DNA Master SYBR Green I kit and a LightCycler (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocols. We used QuantiTect primers (QIAGEN, Hilden, Germany) for inhibitor of DNA binding 1 (*ID1*) and *ID2* genes, which are down-stream target genes of BMP signaling (9), and LightCycler primer sets for the *β-actin* gene, used as a control, and for the Notch-related genes.

Immunoblot analysis. In order to confirm that BMP4 treatment mediated signaling transduction in these cell lines, we performed immunoblot analysis to examine the phosphorylation of the SMAD1/5/8 complex. Cells cultured for 24 hours, with or without 50 ng/ml BMP4, were harvested and lysed. The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were immunoblotted with antibodies against SMAD1, phosphorylated SMAD1/5/8 (Cell Signaling Technology, Danvers, MA, USA), and α-tubulin (Abcam, Cambridge, MA, USA), used as a loading control.

Table II. *Primers used for reverse transcription-polymerase chain reaction (RT-PCR).*

BMP4	Forward 5'-cttcagtctggggaggag-3'
	Reverse 5'-gatgaggtgcccaggcac-3'
BMPR1A	Forward 5'-caggttcctggactcagctc-3'
	Reverse 5'-ctttccttgggtgccataaa-3'
BMPR1B	Forward 5'-aaaggtcgctatggggaagt-3'
	Reverse 5'-gcagcaatgaaacccaaaat-3'
BMPR2	Forward 5'-gctaaaatttggcagcaagc-3'
	Reverse 5'-cttgggccctatgtgtcact-3'

The primer sequences were originally described by Piccirillo and colleagues (7).

Results

Effects of BMP4 on short-term growth. The effects of BMP4 on the short-term growth of 12 human leukemia and lymphoma cell lines are shown in Figure 1. BMP4 stimulation slightly promoted the growth of Jurkat, KOPT-K1, and TMD8 cells, whereas it suppressed the growth of the Daudi cells. The growth of the remaining eight cell lines was not significantly affected by BMP4. Each assay was repeated

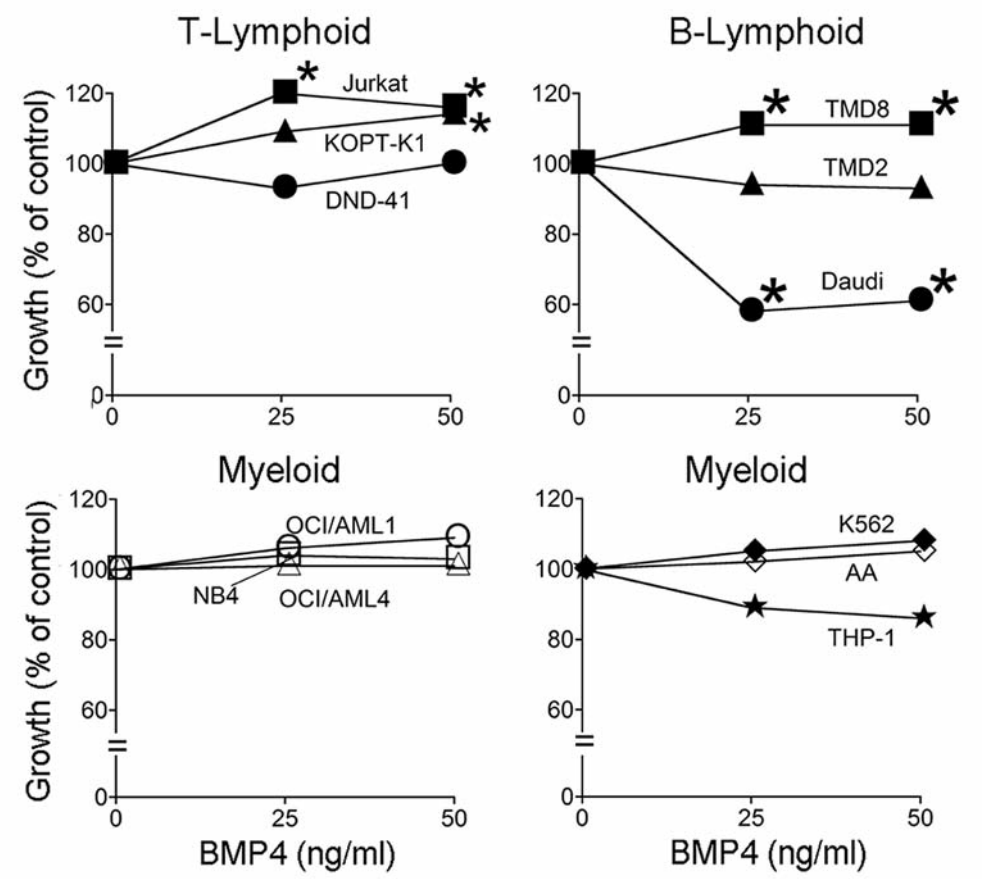

Figure 1. *Effects of bone morphogenetic protein 4 (BMP4) on the short-term growth of the leukemia and lymphoma cell lines examined by the WST-1 assay. The cells were cultured with or without BMP4 for 5 days, following which the optical density was measured. Growth is shown as a percentage of the mean O.D. value of the control cells cultured without BMP4. *p<0.05, significant difference from the control.*

more than three times independently, in order to verify reproducibility. No obvious morphological differences between the BMP4-treated cells and control cells were detected in the cytospin preparations of the 12 cell lines (data not shown).

Effects of BMP4 on colony formation. The effects of BMP4 on colony formation are shown in Figure 2. BMP4 significantly suppressed colony formation in four out of the six examined cell lines. There was no significant effect in the other two cell lines.

Gene expression. The results of the qualitative RT-PCR analysis are summarized in Table I. All the cell lines expressed genes for the BMP receptors, *BMPR1A* and *BMPR2*, whereas expression of the *BMPR1B* gene was not detected in five of the cell lines. The five cell lines did express *BMP4* mRNA. Next, we performed quantitative RT-PCR to examine the expression of *ID1* and *ID2* genes, the target genes of BMP signaling. As shown in Table I, BMP4 stimulation up-regulated the expression of *ID1*/*ID2* by more than 200% in seven cell lines. We also examined the expression of Notch-related genes, such as *NOTCH1*, *JAGGED1*, and hairy and enhancer of split 1 (*HES1*), because Notch signaling is closely related to BMP4 signaling (13). However, the expressions of these genes were not significantly affected by the BMP4 treatment (data not shown).

Effects of BMP4 on signaling proteins. Figure 3 shows the results from the immunoblot analysis of the three representative cell lines. The BMP4 treatment promoted the phosphorylation of the SMAD1/5/8 complex without affecting the amount of SMAD1 protein in these cell lines. This result indicates that the BMP4 treatment mediated signal transduction in these cells. We also examined the expression of the β-catenin protein, which was reported to play a role in homotypic cell aggregation and clonogenicity of Jurkat cells (14). We thought that an increase of β-catenin expression

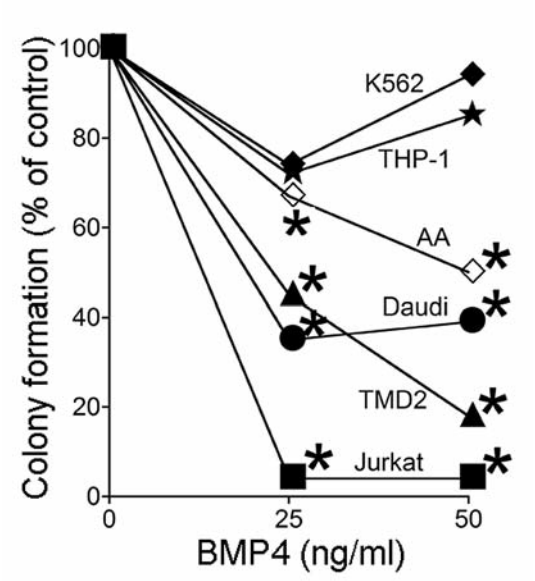

Figure 2. *Effects of bone morphogenetic protein 4 (BMP4) on colony formation in the leukemia and lymphoma cell lines. The cells were cultured in methylcellulose with or without BMP4 for 7 days, following which the colonies containing more than 20 cells were counted. The data are shown as the percentages of the mean colony numbers in the control cells cultured without BMP4. *p<0.05, significant difference from the control.*

would lead to suppression of colony formation in BMP4-treated cells. However, the amount of β-catenin protein was not affected by BMP4 stimulation (data not shown).

Discussion

This study demonstrated that BMP4 stimulation had diverse effects on the short-term growth of 12 human leukemia/lymphoma cell lines. We observed that BMP4 promoted the growth of three cell lines, suppressed the growth of one cell line, and did not affect the growth of eight cell lines. In contrast, it suppressed colony formation in four out of the six examined cell lines. It is interesting that BMP4 slightly promoted the short-term growth of the Jurkat cells but suppressed their ability to form colonies. These results suggest that BMP4 promotes the proliferation of leukemia cells in general but suppresses its function in leukemia stem/progenitor cells. Due to the fact that the suppression of clonogenicity by BMP4 was reported in brain tumor cells (6), this effect may indicate a common function of BMP4 in various malignant cells.

It has been reported that BMP4 signaling is involved in erythroid differentiation (5). In the cell lines examined here, despite AA and K562 cell lines which possess properties of the erythroid lineage, BMP4 treatment did not induce erythroid differentiation of these cell lines in our study.

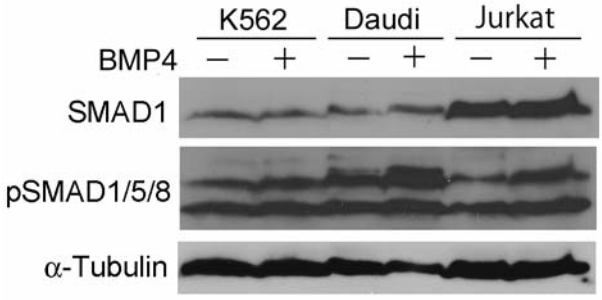

Figure 3. *Effects of bone morphogenetic protein 4 (BMP4) on the expression of SMAD1 and phosphorylation of the SMAD1/5/8 complex. The cells were cultured with or without BMP4 for 24 hours. The lysates were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against SMAD1, phosphorylated SMAD1/5/8, and α-tubulin.*

We found that some cell lines express *BMP4* mRNA and BMP receptor mRNA, which suggests that BMP4 functions *via* an autocrine mechanism in these cells. *BMP4* mRNA expression has been reported in acute promyelocytic leukemia (7), myeloma (8), and brain tumor cells (6). BMP4 and BMP4 receptors may therefore play currently unknown roles *via* autocrine mechanisms in malignant cells.

We confirmed that exposure to BMP4 mediates signal transduction in the cell lines by showing that BMP4 treatment promoted the phosphorylation of the SMAD1/5/8 protein complex. We also showed that BMP4 treatment up-regulated the expression of *ID1/ID2* genes, which are the target genes of BMP signaling. ID1 and ID2 proteins are known to play a role in cell growth and differentiation (9). However, we were not able to clarify the significance of the up-regulation of the *ID* gene expression on cell growth mechanisms, because the up-regulation was found to be independent from promotion or suppression by BMPH. Further investigation is therefore required.

Overall, our study demonstrated that BMP4 treatment suppressed the clonogenicity of the selected leukemia/lymphoma cell lines. In contrast, it has been reported that BMP4 stimulation promotes the maintenance of normal hematopoietic stem cells (1). Taken together, these findings suggest that regulation of BMP4 signaling may be a useful therapeutic approach for leukemia/lymphoma, if appropriate cases are selected. For that purpose, the effects of BMP4 on the growth of leukemia/lymphoma cells from patients and the precise molecular mechanisms of the effects of BMP4 must be investigated.

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