Anti-Cancer Drugs 1998, 9, pp. 327–331

Research paper

Antiproliferative effects of recombinant human bone morphogenetic protein-2 on human tumor colony-forming units

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Bone morphogenetic protein-2 (BMP-2) is a differentiation factor for normal osteoblasts. BMP-2 is structurally related to transforming growth factor-β which inhibits cell proliferation and enhances apoptosis. A recent study has shown the presence of BMP-2 receptors on several cancer cell lines. In this study, we attempted to determine if recombinant human BMP-2 (rhBMP-2) can modulate the proliferation of human tumor colony-forming units taken from 113 patients. Tumor cells were cultured in soft agar and continuously exposed to three concentrations of rhBMP-2 (10, 100 and 1000 ng/ml) for 14 days in the capillary cloning system. There were 65 evaluable specimens, including 17 breast cancers, 15 ovarian cancers, 14 non-small cell lung cancers and five prostate cancers. Importantly, rhBMP-2 did not stimulate the tumor cell proliferation. A significant inhibition (50% or less survival of tumor colony-forming units) was seen in 16 of 65 specimens (24.6%) at 1000 ng/ml, including five of 14 non-small cell lung cancers, five of 17 breast tumors and two of 15 ovarian tumors. A concentration–response relationship was observed ($p<0.001$ by Mantel-extension test). The results of this study encourage further evaluation of the antiproliferative effects of rhBMP-2 against human cancers. [© 1998 Lippincott-Raven Publishers.]

Key words: Antineoplastic agents, growth factors, human tumor cloning assay.

Introduction

Bone morphogenetic proteins (BMPs) are differentiation factors for normal osteoprogenitor cells.[1] These proteins were initially extracted from bone matrix and were shown to induce ectopic bone formation when injected into animal muscle.[2] Recently, several BMPs (including BMP-2 through BMP-7) have been cloned and purified as recombinant protein.[3,4] rhBMP-2 has been shown to differentiate osteoprogenitor cells into osteoblasts *in vitro*[5–8] and induce new bone formation *in vivo*.[4,9] In addition, mRNA for BMP-2 is detected in various mouse embryonic tissue.[10] However, the biological function of BMP-2 in extraskeletal tissue remains unknown.

Based on amino acid homology, BMP-2 belongs to the transforming growth factor (TGF)-β superfamily, including TGF-β, activin and Müllerian inhibiting substance.[11] Activities associated with this family include cell proliferation, differentiation and apoptosis.[12] TGF-β inhibits the proliferation of normal epithelial cells, hematopoietic cells and some cancer cells.[13] This growth inhibition is partially due to arrest of the cell cycle and the enhancement of apoptosis.[13–15] To our knowledge, there is no study concerning the effects of BMP-2 on the proliferation of extraskeletal cancer cells. However, a recent study has shown the presence of BMP-2 receptors in several cancer cell lines, although it is not known whether these receptors are functional.[16]

Since rhBMP-2 could become a therapeutically important molecule by encouraging bone growth, particularly in patients with cancers who have severe osteoporosis, it is important to determine the effect of rhBMP-2 on the growth of tumor cells. Because BMP-2 receptors were observed on some tumor cells, it is possible that rhBMP-2 could also have some antiproliferative effects. Therefore, in this study, we evaluated the effect of rhBMP-2 on the proliferation of tumor

This work was supported in part by a grant from Genetics Institute and the Cancer Therapy and Research Foundation of South Texas.

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cells taken from patients, using a human tumor colony-forming unit assay.[17,18]

Materials and methods

rhBMP-2 and supplies

The sources were as follows: rhBMP-2 (Genetics Institute, Cambridge, MA); McCoy's 5A medium, sodium pyruvate, glutamine, L-serine, asparagine, penicillin and streptomycin (Gibco/BRL, Gaithersburg, MD); CMRL 1066 medium (Irvine Scientific, Irvine, CA); fetal calf serum (JRH Biosciences, Lenexa, KS); horse serum and orthosodium vanadate (Sigma St Louis, MO); agar and soy broth (Difco, Detroit, MI).

Tumor samples

This study was conducted in accordance with institutional and Federal guidelines for written informed consent. Tumor specimens were collected by sterile procedures from patients undergoing procedures done as part of a diagnostic work-up or as part of treatment for their disease. No surgical procedures were performed solely to obtain specimens for drug sensitivity testing. All samples were granted exempt research status by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. Solid specimens were minced in McCoy's 5A medium and repeatedly passed through a stainless steel mesh. Malignant effusions with heparin were centrifuged and passed through a 25-gauge needle. All specimens were suspended in enriched McCoy's 5A medium containing 5% horse serum, 10% fetal calf serum, 2 mM sodium pyruvate, 2 mM glutamine, 35 μg/ml L-serine, 900 U/ml penicillin and 90 μg/ml streptomycin. The number of viable cells for plating was determined by a Trypan blue dye exclusion method.

Human tumor cloning assay

The human tumor capillary cloning assay was performed using the soft agar system as previously reported.[17,18] The cells to be cloned were suspended in 0.3% agar in enriched CMRL 1066 supplemented with 15% heat inactivated horse serum, 100 U/ml penicillin, 2 mg/ml streptomycin, 2 mM glutamine, 2 U/ml insulin, 0.6 mg/ml asparagine and 10 mM HEPES buffer. The concentrations of rhBMP-2 were adjusted to 10, 100 and 1000 ng/ml in the cell suspension to allow for 14 day continuous exposure. The concentrations of rhBMP-2 were selected based on the previous experiments with osteoblastic differentiation,[5-8] because there is to date no available data on pharmacokinetics of rhBMP-2 in patients. Utilizing capillary action, 100 μl of the mixture (containing the medium, agar and 20 000 cells per 100 μl) was placed in each capillary tube. The ends of the capillary tube were sealed with clay. Six capillary tubes were prepared for each data point. Each experiment included an untreated control without rhBMP-2 and a positive control with orthosodium vanadate to assure the presence of a single-cell suspension.[19] The tubes were placed in a 37°C incubator and removed on day 14 for counting of the number of colonies in each tube. The number of colonies (defined as more than 50 cells) formed in the six preparation treated tubes was compared to the number of colonies formed in the six control tubes and the percent colonies surviving at that concentration of rhBMP-2 was calculated.

Statistical analysis

An experiment was considered evaluable for analysis when the untreated control had an average of three or more colonies and the vanadium control had colony formation 30% or less of the untreated control.[17,18]

Table 1. Growth of tumor cells in the capillary cloning system

Tumor type	Tested specimens	Evaluation specimens[a] [*n* (%)]
Non-small cell lung cancer	34	14 (41.1)
Breast cancer	26	17 (65.3)
Ovarian cancer	17	15 (88.2)
Prostate cancer	7	5 (71.4)
Melanoma	4	4 (100)
Brain tumor	2	2 (100)
Bladder tumor	2	1 (50)
Colon cancer	2	1 (50)
Kidney tumor	2	1 (50)
Gallbladder cancer	1	1 (100)
Liver tumor	1	1 (100)
Thyroid cancer	1	1 (100)
Lymphoma	1	1 (100)
Unknown primary site	3	1 (33.3)
Others[b]	10	0
Total	113	65 (57.5)

[a]Evaluable specimen is defined as one where the untreated control yielded an average of more than three colonies/tube and the vanadium control yielded an average colony number that was 30% or less of the untreated control.
[b]Including small cell lung cancer, benign tumor ($n = 3$, each), uterine, head and neck, stomach cancers, and sarcoma ($n = 1$, each).

The inhibition and stimulation of colony formation was defined as 50% or less and 150% or more of the untreated control, respectively. No effect was reported when the result was 51–149% of the untreated control. The difference between evaluable and non-evaluable groups was tested by Student's *t*-test or χ^2 test. The overall effect of rhBMP-2 on a set of tumor specimens was expressed as percentage and 95% CI. The dose-response relationship of rhBMP-2 was evaluated by Mantel-extension test[20] and a two-tailed $p<0.05$ was considered to indicate statistical significance.

Results

Out of 113 specimens, 65 (57.5%) were evaluable (Table 1) and 48 (42.5%) were non-evaluable. There was no difference in age, sex, biopsy site or prior therapy between the evaluable and non-evaluable groups (Table 2).

Of the 48 non-evaluable samples, 37 had an average of less than three colonies in the untreated control with or without acceptable vanadium control and 11 demonstrated adequate colony numbers in the untreated control but unacceptable vanadium controls. None of the 37 specimens with inadequate growth in the control plates demonstrated tumor colony formation in the presence of rhBMP-2.

The 65 evaluable specimens included 17 breast cancers, 15 ovarian cancers and 14 non-small cell lung cancers. As can be seen in Table 3, rhBMP-2 showed significant inhibition in 16 of 65 specimens (24.6%, 95% CI: 14.1–35.1) at 1000 ng/ml, including five of 14 non-small cell lung cancers (35.7%, 95% CI: 10.6–60.8), five of 17 breast cancers (29.4%, 95% CI: 7.7–51.1) and two of 15 ovarian cancers (13.3%, 95% CI: 0–

Table 2. Characteristics of evaluable and non-evaluable groups in major tumor types[a]

Tumor type	Age (mean ± SD)	Sex (M/F)	Biopsy site (primary/metastatic)	Prior therapy [*n* (%)]
All tumors				
evaluable (n = 65)	56 ± 17	22/43	33/32	17 (26.1)
non-evaluable (n = 48)	56 ± 12	21/27	20/28	16 (33.3)
Non-small cell lung cancer				
evaluable (n = 14)	66 ± 9	10/4	6/8	4 (28.6)
non-evaluable (n = 20)	61 ± 9	14/6	8/12	7 (35.0)
Breast cancer				
evaluable (n = 17)	49 ± 17	0/17	9/8	6 (35.3)
non-evaluable (n = 9)	49 ± 12	0/9	4/5	4 (44.4)
Ovarian cancer				
evaluable (n = 15)	64 ± 15	0/15	3/12	3 (20.0)
non-evaluable (n = 2)	76 ± 6	0/2	0/2	1 (50.0)

[a]There is no significant difference in age, sex, biopsy site or prior therapy between the two groups.

Table 3. Relationship between concentration of rhBMP-2 and tumor growth inhibition[a]

Tumor type	Evaluable specimens	No. of specimens (%) yielding tumor growth inhibition at different concentrations (ng/ml) of rhBMP-2[b]		
		10	100	1000
Non-small cell lung cancer	14	0	1 (7.1)	5 (35.7)
Breast cancer	17	1 (5.9)	1 (5.9)	5 (29.4)
Ovarian cancer	15	1 (6.6)	1 (6.6)	2 (13.3)
Prostate cancer	5	0	1 (20)	0
Melanoma	4	0	0	0
Others	10[c]	0	0	4 (40.0)[d]
Total	65	2 (3.0)	4 (6.1)	16 (24.6)

[a]The relationship is evaluated by Mantel-extension test ($p<0.001$).
[b]Inhibition is defined as 50% or less survival.
[c]Including brain cancer (n = 2), bladder, colon, gallbladder, kidney, liver, lymphoma, thyroid and unknown primary (n = 1, each).
[d]Inhibition is observed in bladder, brain, kidney tumors and lymphoma (n = 1, each).

30.5). Growth inhibition by rhBMP-2 demonstrated a concentration-response relationship ($p < 0.001$ by Mantel-extension test). rhBMP-2 inhibited breast and ovarian tumor colony-forming units at all concentrations tested, and inhibited non-small cell lung cancers at concentrations of 100 and 1000 ng/ml. The inhibitory effect of rhBMP-2 was not related to the biopsy site (primary/metastatic) or prior therapy. Importantly, none of the evaluable specimens showed growth stimulation with rhBMP-2.

Discussion

In the present study, we have demonstrated that rhBMP-2 inhibited the growth of some human tumor colony-forming units and did not stimulate the formation of tumor colony-forming units. A trend toward a concentration-response relationship for rhBMP-2 having an antiproliferative activity at high concentration was observed. The sample size we studied was small but the data suggests that rhBMP-2 may have potential for use as an antineoplastic or a growth-modulating agent. Furthermore, it appears that rhBMP-2 did not stimulate the formation of tumor colony-forming units in tumor cells taken directly from patients.

The signaling pathway for BMP-2 has been defined. BMP-2 receptors are transmembrane serine/threonine kinases, composed of type I and type II receptors.[12,21,22] BMP-2 first binds a type II receptor and this complex is recognized by the type I receptor.[12] The signal of these receptors induces phosphorylation of *Mothers against decapentaplegic-related protein* (MADR), which then binds DNA in the nucleus and has transcriptional activity.[12,23,24]

Recently, interesting evidence has been reported regarding the relationship between BMP-2 and certain malignancies. About 90% of pancreas cancers show alleic loss at chromosome 18q. DPC4 (deleted in pancreatic cancer, locus 4) is a candidate tumor suppressor gene at the chromosome 18q21,1 location.[25] The DPC4 gene product is structually similar to MADR[12,25] and also contains transcriptional activity.[24] These results support the contention that rhBMP-2 could inhibit tumor cell growth.

BMP-2 receptors are different from TGF-β receptors, despite the structural similarity of the two ligands. BMP-2 receptors are not bound by TGF-β and their distribution is different from that of TGF-β.[16,26] BMP-2 receptors are present on normal and tumor cells of epithelial and mesenchymal origin except for hematopoietic cells.[16] In contrast, TGF-β receptors are observed on normal and tumor cells of epithelial, mesenchymal and hematopoietic origin.[26]

Some investigators have reported the relationship between BMP-2 and tumor progression.[27-29] In osteosarcoma, BMP-2 receptors are present on HuO-3N1 cell line[16] and BMP-2 can be detected immunohistochemically in some clinical specimens.[27] Yoshikawa *et al.* have reported that BMP production from osteosarcoma is closely related to the frequency of bone metastasis and survival of patients.[28] Also, PC-3 prostate cancer cells, which originate from a site of bone metastasis, have BMP-2 receptors and mRNA for BMP-2.[16,29] Our present study did not include any evaluable samples of osteosarcoma or samples that had metastasized to bone. Further studies are needed to elucidate the connection, if any, of BMP-2 production by tumors and the progression of primary and metastatic bone tumors.

In conclusion, rhBMP-2 did not cause stimulation of colony formation of extraskeletal tumor cells taken directly from patients. rhBMP-2 inhibited ovarian and breast cancer colony-forming units at all concentrations tested and inhibited non-small cell lung cancers at concentrations of 100 and 1000 ng/ml. Our study results encourage further evaluation of antiproliferative effects of rhBMP-2 against human tumors.

Acknowledgments

The authors thank Peggy Durack for excellent assistance in collecting the data and preparing this manuscript.

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(Received 27 January 1998; accepted 19 February 1998)