

Effects of the Bone Morphogenetic Protein Binding Protein spp24 (Secreted Phosphoprotein 24 kD) on the Growth of Human Lung Cancer Cells

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ABSTRACT: Bone morphogenetic proteins (BMPs) and transforming growth factor-beta (TGF- β) contribute to the growth of some skeletal metastases through autocrine stimulation. Secreted phosphoprotein 24 kDa (spp24) has been shown to bind to both BMP-2 and TGF- β and to markedly inhibit the osteogenic properties of rhBMP-2. We hypothesized that the addition of spp24 would sequester autocrine growth factors (especially BMP-2) and reduce tumor growth in a system (A549 human non-small cell lung cancer cell line) where autocrine stimulation by BMP-2 is known to be important. A549 cells were injected into two sites (subcutaneous and intraosseous) in SCID mice with and without the co-injection of BMP-2 and spp24. Tumor growth after 8 weeks was assessed through gross examination, radiological imaging, and histological analysis. Spp24 attenuated the tumor growth enhancing effects of rhBMP-2 and reduced the tumor growth when added to tumor cells that were not treated with BMP-2. We conclude that spp24 can reduce A549 cell tumor growth in both soft tissue and intraosseous environments. We hypothesize that the mechanism for this inhibition is interruption of autocrine stimulation through the sequestration of BMP-2. Spp24 can be developed into a therapeutic agent that can be employed in clinical situations where the inhibitions of BMPs and related proteins is advantageous. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 29:1712–1718, 2011

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Bone morphogenetic protein-2 (BMP-2) is a member of the transforming growth factor-beta (TGF- β) family of proteins. These proteins perform a variety of pattern specifying and morphogenic functions in the mammalian embryo^{1,2} and are critical to skeletal homeostasis in post-natal life.³ Recombinant human BMP-2 (rhBMP-2) is well accepted as an osteoinductive therapeutic which is used to promote bone healing in fracture non-unions and spine fusion in degenerative disc diseases.^{4–6} It would be expected, therefore, that they would play a significant role in pathological processes such as skeletal metastasis.

Secreted phosphoprotein 24 kD (spp24) is a bone matrix protein which binds proteins of the TGF- β family through a region that is similar to the TGF- β receptor II (TGF- β receptor II homology 1 domain or TRH1 domain).⁷ Full-length spp24 strongly inhibits BMP-2 induced bone formation when tested in an ectopic bone forming assay.⁸ Smaller forms of spp24 produced through proteolysis are less inhibitory of BMP-2 induced bone formation⁹ which has lead to the hypothesis that regulated proteolysis of spp24 is one mechanism through which the availability of BMPs is regulated in skeletal tissue.¹⁰

Cell line A549 is a well characterized human non-small cell lung carcinoma line that expresses BMP-2 and responds to this autocrine secretion with increased growth.¹¹ Previous investigations have shown that noggin, an inhibitor of BMPs, can be employed to reduce tumor growth in this model of skeletal metastasis.¹²

The hypothesis for this study was that spp24 could be used to bind to and sequester BMP-2 (and possibly other related cytokines) and through this interruption of autocrine stimulation decrease tumor growth. A material with this property would have obvious applications in the orthopedic management of metastatic disease.

METHODS

Cell Culture and Experimental Materials

The human non-small cell lung cancer (NSCLC) cell line A549 was used in this study (ATCC, Manassas, VA). A549 cells were maintained in DMEM with 10% FBS and antibiotics (Fisher Scientific, Pittsburgh, PA) in a humidified incubator with 5% CO₂ at 37°C. Cells were not used beyond 10 passages. RhBMP-2 (INFUSE[®], Medtronic Sofamor Danek, Minneapolis, MN) was obtained commercially. Recombinant spp24 was prepared as described previously.¹³

Animals and Experimental Groups

Forty, 8-week-old male SCID mice (weight 23.5 to 29.1 g, mean 27.1 g) were housed under pathogen-free conditions in

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accordance with the protocol approved by the Chancellor's Animal Research Committee (ARC) at the University of California, Los Angeles. There were four experimental groups in this study, and each group underwent implantation of cells in both a subcutaneous model and an intra-tibial injection. Group A animals received A549 cells alone as a control group. Group B animals received A549 cells + spp24 (1,000 μ g). Group C received A549 cells + rhBMP-2 (10 μ g/10 μ l). Group D received A549 cells + rhBMP-2 + spp24. There were 10 animals in each group. Obvious tumors developed in most animal subjects that received cells only or cells plus rhBMP-2. Fewer animals that received cells plus spp24 or cells plus rhBMP-2 plus spp24 developed obvious tumors. Therefore, not all negative samples were carried forward for histological and morphometric analysis (see Statistical Analysis Section, below).

Subcutaneous Implantation and Direct Measurement of Tumor Size

A549 cells (1×10^5) were suspended in 15 μ l of $1 \times$ PBS and 15 μ l Affi-Gel Blue (BioRad Laboratories, Hercules, CA) and injected into the subcutaneous space on the backs of mice. Briefly, after the SCID mice were anesthetized, then maintained via an isoflurane face mask, the overlying skin was prepped in a sterile fashion with 70% ethanol and betadine. Different materials and A549 cells were then injected into the subcutaneous space on the backs of SCID mice. Animals were killed at 8 weeks or earlier if tumor size exceeded that permitted by the ARC protocol and the tumor removed. Tumors were measured in three dimensions (length $\times$ width $\times$ depth). These measurements were repeated by three observers blinded to treatment group. Results are reported in cubic millimeters.

Intratibial Implantation and Radiographic Analysis

Tibial implantation of A549 cells plus experimental materials was performed as previously described.^{14,15} Mice were anesthetized with isoflurane, then maintained via an isoflurane face mask. The overlying skin was prepped in a sterile fashion with 70% ethanol and betadine. A 3-mm longitudinal incision was made over the patellar ligament with a number 11 scalpel blade and a 2-mm longitudinal incision was then made along the medial border of the patellar ligament to the tibial plateau. A 27.5-gauge needle was introduced through the proximal tibial plateau and into the proximal tibia. Thirty microliters of a suspension containing 1×10^5 A549 cells in 15 μ l of $1 \times$ PBS with 15 μ l of Affi-Gel Blue containing the appropriate test materials was injected into the cavity. The wound was closed with a single 5-0 Vicryl suture. At 8 weeks, prior to their sacrifice, animals were anesthetized as previously described, and radiographs were obtained using a Faxitron X-ray cabinet (Faxitron, Inc. Lincolnshire, IL). Three independent observers blinded to the treatment groups evaluated the radiographs for the presence of osteoblastic and osteolytic lesions. Radiographs were scored as follows: 0-normal; 1-lytic lesion present within the medullary canal only; 2-oblation of one cortex; 3-oblation of two cortices.

Histologic and Histomorphometric Analysis

Animals were killed at 8 weeks (or earlier if required by an excessive tumor size) and both the soft tissue tumor and the hind limb were harvested for histological analysis. Soft tissue tumors were fixed in 10% buffered formalin. The tibias were

fixed in 10% buffered formalin followed by decalcification in a solution of 10% EDTA for 2 weeks at room temperature with gentle stirring. Longitudinal sections of the proximal tibia were prepared and stained with H&E. For histologic and histomorphometric analyses, all microscopic slides were scanned with a ScanScope GL (Aperio Technologies, Inc., Vista, CA) equipped with a 20 $\times$ objective. The following parameters were considered in the qualitative analysis: amount of tumor cells between cortices from the proximal tibia; anterior and posterior cortical destruction and remodeling; new bone formation within the medullary canal; and anterior/posterior extension of bone formation outside the medullary canal. These histologic findings were graded with the following scale: 0 (none), 1 (mild), 2 (moderate), 3 (intense). Bone volume and tumor volume, expressed as a percentage of total tissue volume (%BV/TV and %TuV/TV, respectively) were quantified in whole-slide images using Spectrum software (Aperio Technologies, Inc. Vista, CA).

Statistical Analysis

To provide for the most conservative estimates of statistical significance because some variables were not normally distributed, all differences were compared using the Mann-Whitney *U*-test. Calculations were performed with SPSS (version 15, SPSS, Inc., Chicago, IL). Data are presented as the mean and the 95% confidence interval, (CI). Many subjects did not develop obvious tumors so not all specimens were subjected to morphometric and histomorphometric analysis. Obvious tumors within bone were present in 8 of 10 subjects in the cells only group and 9 of 10 of the cells plus rhBMP-2 group. Measurements from all 10 subjects in each of the four groups were included in the radiological analysis. For the tumor volume and histology and the histomorphometry analysis, the number of specimens examined was limited to the highest number of subjects with tumors in either the cells only or cells plus rhBMP-2 groups, which were seven and six, respectively. All specimens in the cells plus spp24 or cells plus rhBMP-2 and spp24 that had any visible tumors were included. That is, only negative samples were not processed for further evaluation.

RESULTS

Effects of rhBMP-2 and spp24 on Subcutaneous Tumor Formation

The effects of subcutaneous injection of A549 cells in conjunction with the specified treatment materials are illustrated in Figure 1. Addition of rhBMP-2 increased tumor volume by about fourfold, whereas addition of spp24 to the cells reduced tumor volume by about 90%. The means and 95% CI for each group were: A (cells only): 127.4 mm³ (18.7–236.1); B (cells + spp24): 12.6 mm³ (–7.4 to 32.5); C (cells + BMP): 558.4 mm³ (12.1–1104.7); D (cells + BMP + spp24): 14.3 mm³ (–8.9 to 37.5). The *p*-value for the critical comparison of A (cells only) versus B (cells + spp24) was 0.053 by Mann-Whitney *U*-test. Seven specimens from each group were included in these analyses.

Radiographic Analyses of rhBMP-2 and spp24 On Intratibial Tumor Formation

Radiographs of injected tibias were obtained at the time of death and evaluated by three independent observers

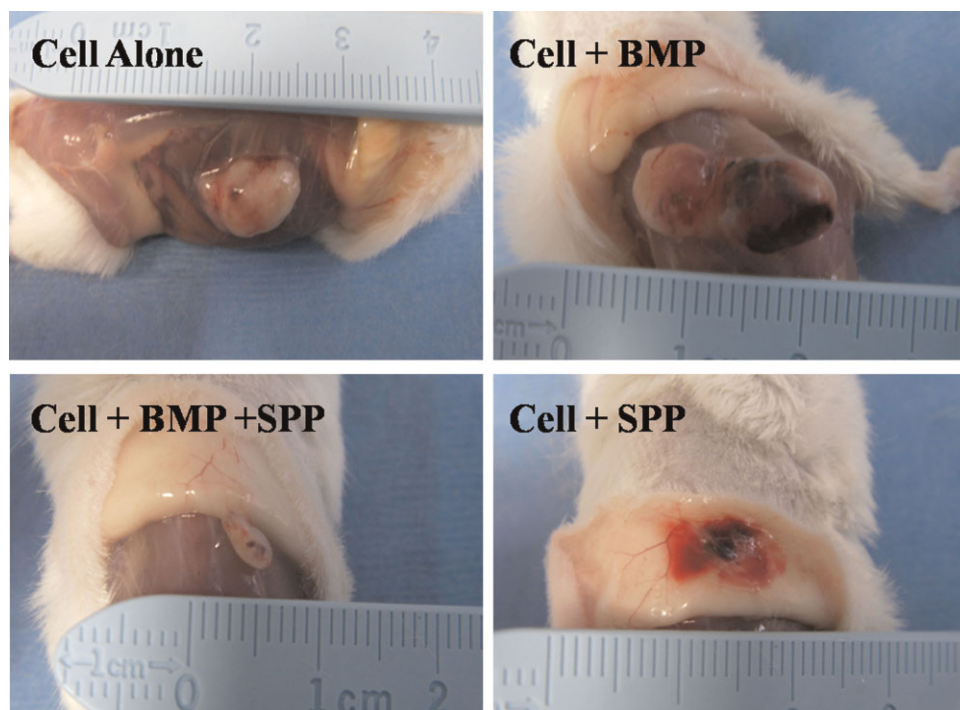


Figure 1. Subcutaneous tumor formation 8 weeks after injection of A549 human NSCLC cells co-injected with rhBMP-2, spp24, both rhBMP-2 plus spp24 or vehicle alone. *p*-Values for critical comparisons: cells + rhBMP-2 vs. cells alone ($p = 0.041$); cells + spp24 vs. cells alone ($p = 0.027$); cells + rhBMP-2 + spp24 vs. cells alone ($p = 0.028$); cells + rhBMP-2 + spp24 vs. cells + rhBMP-2 ($p = 0.048$). Images illustrate representative tumors at necropsy. For numerical analysis of measurements from all tumors, see text.

who were blinded with respect to treatment groups. There was excellent overall agreement between readers with a kappa statistic of 0.89. These results are illustrated in Figure 2. In these studies addition of BMP to the cells increase the radiological score, whereas addition of spp24 to the cells decreased the score. The means and 95% CI for each group were: A (cells only) 2.1 (1.18–3.02); B (cells + spp24): 0.5 (–0.11–1.11); C (cells + BMP): 2.4 (1.71–3.09); D (cells + BMP + spp24): 0.73 (0.20–1.26). The *p*-value for the critical comparison

of A (cells alone) versus B (cells + spp24) was 0.009 by Mann–Whitney *U*-test. Ten specimens from each group were included in these analyses.

Histologic and Histomorphometric Analyses

Specimens from the cells alone and the cells plus rhBMP-2 treatment groups (groups B and C) showed abundant masses of adenocarcinoma cells in both the subcutaneous (Fig. 3) and intratibial (Fig. 4) sites, whereas those from the cells + spp24 and the

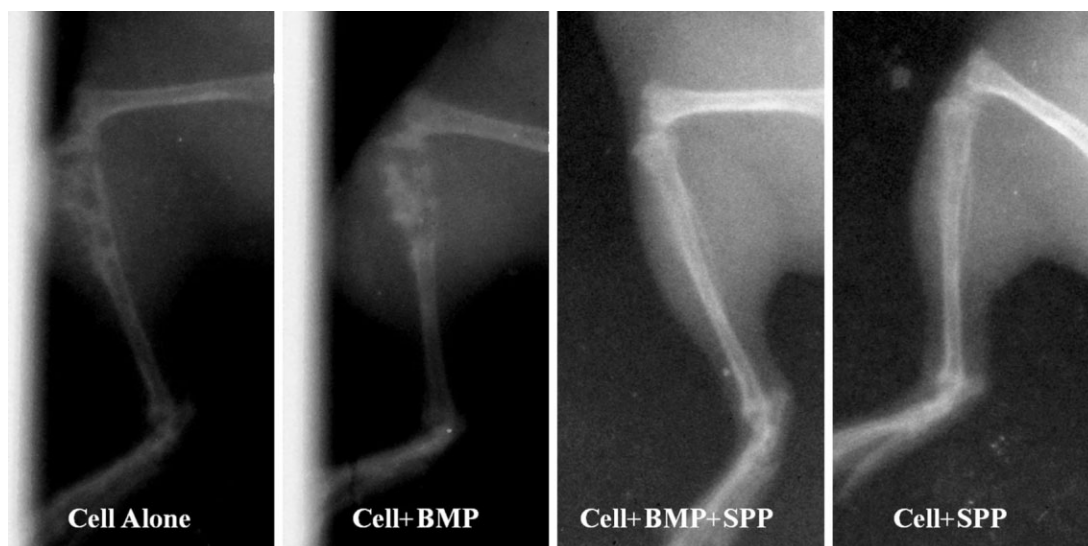


Figure 2. Radiographs illustrating intratibial tumor formation 8 weeks after injection of A549 human NSCLC cells co-injected with rhBMP-2, spp24, both rhBMP-2 plus spp24 or vehicle alone. For numerical analysis of measurements from all animal subjects, see text.

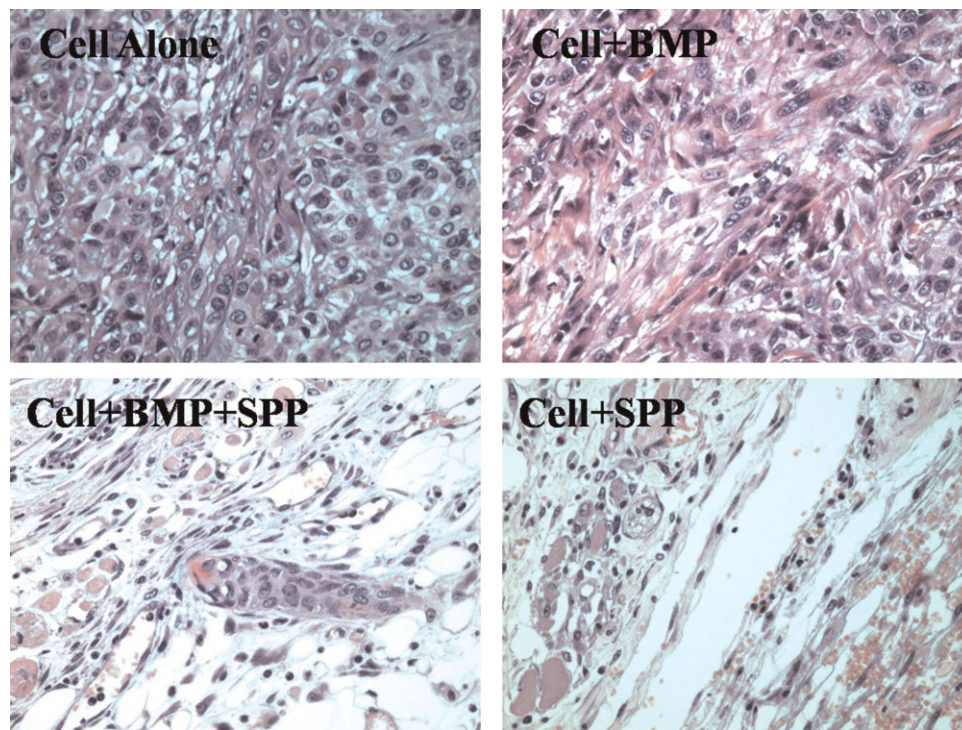


Figure 3. Representative histological sections of subcutaneous tumors 8 weeks after injection of A549 human NSCLC cells co-injected with rhBMP-2, spp24, both rhBMP-2 plus spp24 or vehicle alone. Original magnification 400 $\times$.

cells + rhBMP2 + spp24 (groups B and D) groups revealed only a few scattered tumor cells.

The histologic parameters considered in the qualitative analysis are presented in Table 1. With respect to the critical comparison of group A (cells only) versus group B (cells + spp24) there was significantly more tumor mass between cortices and more destruction of both the anterior and posterior cortices. The differences in new bone within the marrow cavity and extension of bone formation outside the medullary cavity were not statistically significant.

Table 2 shows the calculated results for the %TuV/TV (the proportion of tumor volume to total tissue volume in a longitudinal section of the proximal tibia at midline) and the %Bv/TV (the proportion of total mineralized trabeculae volume in the total tissue volume in the section) from the specimens in this study. Animals that were injected with A549 cells plus rhBMP-2 (group C) had the highest tumor volume but this value was not significantly greater than that of the A549 cells alone group (group A). Both of these groups had higher relative tumor volumes than those of either group that received spp24 but there was insufficient tumor mass in the specimens from the groups that received spp24 to allow for quantitative analysis. Six specimens from each group were included in these analyses.

DISCUSSION

Many research reports indicate that BMPs are expressed in a variety of carcinoma cell lines and

tumors originating from multiple organs including tumors of lung,^{11,16,17} prostate,^{18–20} breast,^{21,22} and kidney.²³ While not all reports have shown a proliferation enhancing effect of BMPs,^{24,25} in general, expression profiling has demonstrated that tumor cells with osteoblastic characteristics secrete cytokines in the BMP/TGF- β family that increase osteoblastogenesis and bone formation, including BMPs-2, -4, -6, and -7 and TGF- β .^{15,26,27} Osteoblastic tumor cells express mRNA and protein for BMP receptors, and exogenous BMPs stimulate tumor cell proliferation, cellular migration, and invasion in vitro and in vivo.²⁰ These results suggest, therefore, that under certain conditions, BMP/TGF- β cytokines are important in the development of metastatic osteoblastic lesions and that inhibition of their activity would effectively limit tumor growth.

Spp24 is a bone matrix protein that binds to and inhibits the osteogenic activity of BMP-2.^{8,9} This protein exists in bone tissue in several proteolytic size forms which effect BMP-2 activity differently.⁹ Interestingly, spp24 shares sequence and physico-chemical properties with pro-osteogenic proteins described decades ago by two investigators (Urist and Sen)^{9,28,29} and a BMP enhancing therapeutic peptide has been developed which is based on the sequence of the BMP/TGF- β binding region of spp24.⁷

Our studies confirmed the finding that rhBMP-2 enhances tumor growth of A549 cells in both subcutaneous and intraosseous site, as previously reported.¹² In both sites, co-injection of spp24 with rhBMP-2

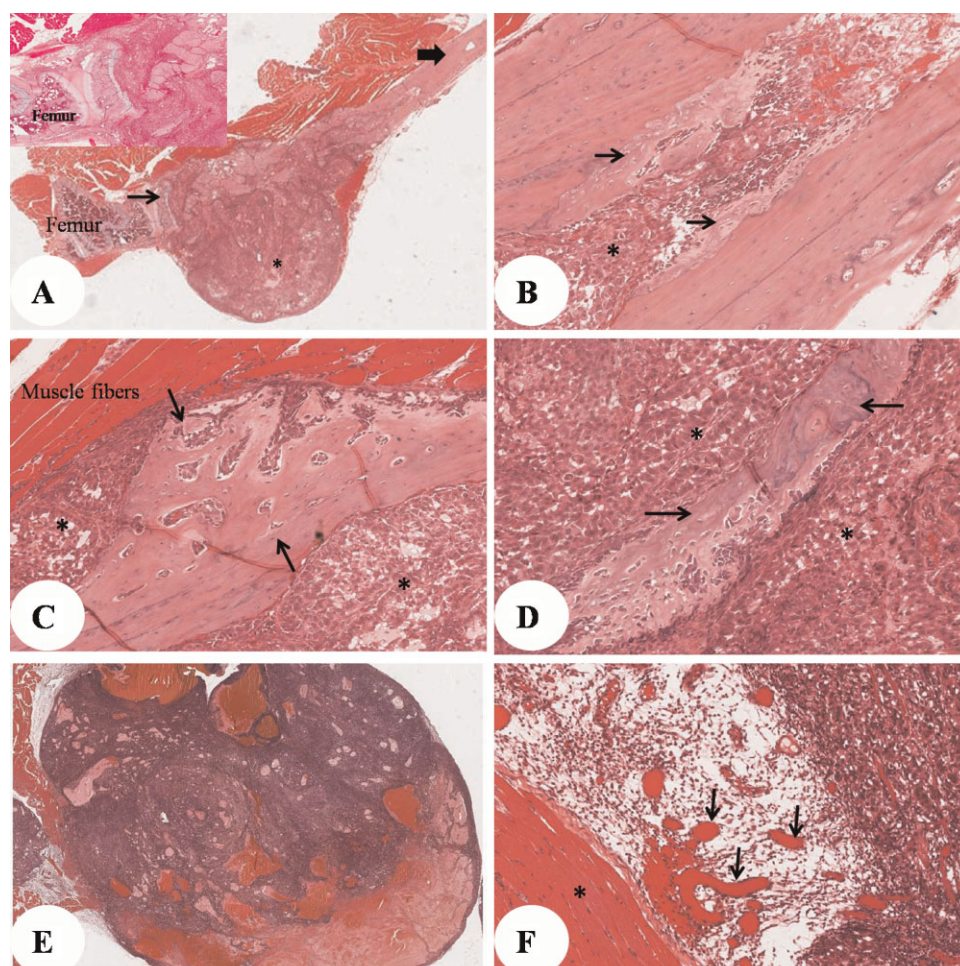


Figure 4. Histologic analyses of intratibial tumors 8 weeks after injection of A549 human NSCLC cells. Animals treated with A549 cells alone and A549 cells + rhBMP-2 showed significant tumor formation in the proximal tibia. (A) Panoramic view of a longitudinal section showing the distal femur (far left) and the proximal tibia with a huge tumor mass (asterisk) that extends outside the tibial cortex between the proximal metaphysis (arrow) and diaphysis (block arrow). Insert in upper left shows high power view of the knee joint; (B) New corticoendosteal bone formation in association with occupation of the intramedullary canal by infiltrating tumor cells (asterisk); (C) New subperiosteal bone formation (arrows) associated with tumor invasion of muscular tissue and the medullary canal (asterisks); (D) New (woven) bone formation (arrows) within the tumor mass (asterisks); (E) Hemorrhage and necrotic areas within a large tumor mass; (F) Peritumoral angiogenesis (arrows) seen between normal muscle fibers (asterisk) and tumor cells.

Table 1. Histologic Analysis of Tibiae

Treatment groups/parameters ^a	Cells only (A)	Cells + spp24 (B)	Cell + rhBMP-2 (C)	Cells + rhBMP-2 + spp24 (D)
Amount of tumor cells between cortices (1)	2.67 (2.13–3.21)	0 (0–0)	3.0 (3.0–3.0)	0 (0–0)
Anterior cortex destruction (2)	2.33 (1.06–3.60)	0 (0–0)	3.0 (3.0–3.0)	0 (0–0)
Posterior cortex destruction (3)	2.33 (1.79–2.88)	0 (0–0)	3.0 (3.0–3.0)	0 (0–0)
New bone formation within the medullary canal (4)	0.41 (–0.10–0.93)	0 (0–0)	0.50 (–0.31–1.31)	0 (0–0)
Extension of bone formation outside the medullary canal (5)	0.58 (0.07–1.10)	0 (0–0)	0.83 (0.40–1.26)	0 (0–0)

Values shown as mean (95% CI). *p*-values for pairwise comparisons with Mann–Whitney *U*-test:

Parameter (1): A vs. B: 0.002; A vs. C: 0.394; A vs. D: 0.002; B vs. C: 0.002; C vs. D: 1.0.

Parameter (2): A vs. B: 0.015; A vs. C: 0.394; A vs. D: 0.015; B vs. C: 0.002; C vs. D: 1.0.

Parameter (3): A vs. B: 0.002; A vs. C: 0.065; A vs. D: 0.002; B vs. C: 0.002; C vs. D: 1.0.

Parameter (4): A vs. B: 0.180; A vs. C: 0.937; A vs. D: 0.180; B vs. C: 0.180; C vs. D: 1.0.

Parameter (5): A vs. B: 0.065; A vs. C: 0.394; A vs. D: 0.065; B vs. C: 0.015; C vs. D: 1.0.

^aParameters were graded as follows: 0 (none), 1 (mild), 2 (moderate), 3 (intense).

Table 2. Quantitative Histomorphometric Analysis of Tibiae

Treatment group/ parameter	Cells only (A)	Cells + spp24 ^a (B)	Cells + rhBMP-2 (C)	Cells + rhBMP-2 + spp24 ^a (D)
%TuV/TV (1)	35.6 (20.6–50.6)	Insufficient	58.3 (41.9–74.8)	Insufficient
%BV/TV (2)	3.11 (0.062–6.17)	Insufficient	1.30 (0.23–2.37)	Insufficient

%TuV/TV, tumor volume/total volume; proportion of tumor volume to total tissue volume in a longitudinal section of the proximal tibia at midline; %BV/TV, Bone volume/total volume; proportion of total mineralized new formed bone in the total section. *p*-values from comparisons from Mann–Whitney *U*-test: Parameter (1): A vs. C: 0.065. Parameter (2): A vs. C: 0.485.

^aThere was insufficient tumor mass in specimens from these groups to permit quantitative analysis.

ameliorated the effect of the BMP-2. Furthermore, co-injection of spp24 (without BMP) with A549 cells markedly inhibited tumor growth in vivo at both soft tissue and the intraosseous sites. In this regard, spp24 had an effect that was similar to that of noggin^{11,12} and anti-BMP-2 antibody.¹¹ We would hypothesize that this was due to sequestration of endogenous BMPs which are required for autocrine stimulation though we cannot completely rule out a less specific effect. However, spp24 did not inhibit the proliferation of AML12 mouse hepatocytes which we used as a control cell line and tested with in vitro studies (data not shown).

The current therapies for metastatic cancer in bone include hormone treatment (which is ineffective against androgen- or estrogen-independent tumors), external beam radiation (with corticosteroids or surgical decompression of spinal metastases), radioisotopes, prophylactic surgery for osteolytic lesions with impending fracture, bisphosphonates or RANK/RANKL system inhibitors (e.g., osteoprotegerin) for osteolytic bone lesions, and growth factor, growth factor receptor, or cell adhesion protein antagonists.³⁰ The limitations of these approaches include toxicity, lack of specificity (osteolytic vs. osteoblastic lesions), cost, and the potential for adverse side effects.³⁰ The therapeutic potential of broad-spectrum BMP/TGF- β cytokine growth factor antagonists, such as spp24, has not been fully exploited. If the pro-osteogenic effects of TGF- β /BMP cytokines on metastatic osteoblastic lesions in bone can be inhibited with a highly-specific, inexpensive, non-toxic native binding protein, such as spp24, it could lead to the development of a new class of therapeutic agents that could reduce morbidity and improve the quality of life for cancer patients.

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