Acta vet. scand. 1998, *39*, 165-171.

Extraction and Characterization of Native Canine Bone Morphogenetic Protein (cBMP) Qualified with Osteoinductivity

By *J. Oksanen*[1], *A. Marttinen*[1], *S. Paatsama*[2], and *T.S. Lindholm*[1]

[1]Surgical Bioimplants Center, Institute of Medical Technology, University of Tampere, and [2]Department of Clinical Sciences Faculty of Veterinary Medicine, University of Helsinki, Finland.

Oksanen J, Marttinen A, Paatsama S, Lindholm TS: Extraction and characterization of native canine bone morphogenetic protein (cBMP) qualified with osteoinductivity. Acta vet.scand. 1998, 39, 165-171. – Bone morphogenetic protein (BMP) was extracted from canine bone matrix, partially purifed and tested for osteoinductivity. A radiographically and histologically detectable ectopic bone formation was induced by 6.0 mg canine (cBMP) in muscle pouch of BALB mouse at 21 days post implantation. Characterization of the cBMP preparation by a gel filtration chromatography defined that the material consisted of proteins or protein complexes with molecular weights from 4 to 120 kD. Isoelectric focusing showed that the molecules were acidic with isoelectric points of 4.6-5.6.

bone remodeling; mouse; purification.

Introduction

During the last decade, research in the field of bone growth and repair has largely focused on bone morphogenetic proteins (BMPs), originally identified 30 years ago when intramuscularly implanted demineralized bone matrix induced new bone formation was reported by *Urist* (1965). Bone morphogenetic proteins are hydrophobic matrix glycoproteins, many of which are able to induce mesenchymal cells to the specific pathway of an osteoprogenitor cell line and initiate bone generation both through a process of endochondral bone formation (*Reddi* 1992) and directly without a cartilage precursor (*Wozney* 1994).

Being a differentiative factor, BMP stimulates DNA synthesis and cell replication both in vivo and in vitro in mesenchymal cell line, but has no effect on a specialized cell line (*Canalis et al.* 1985). Specific characterization of BMP was first reported 10 years ago when cDNA clones corresponding to BMP-1, BMP-2 and BMP-3 were identified (*Wozney et al.* 1988) and presently, human cDNAs encoding 15 novel proteins, named BMP-1 through BMP-15, have been isolated (*Dube & Celeste* 1996, *Riley et al.* 1996). BMP-2 through BMP-8 are members of TGF-β superfamily containg 7 cysteine domain in the carboxy terminal end (*Wozney* 1989).

The molecular weight of native BMP fractions are between 17.5 kD and 36 kD, but there may also be other associated fractions of proteins which potentiate osteoinductive capacity in the BMP biological complex (*Wozney et al.* 1988). The structural similarities between BMPs from various animal species have been demonstrated (*Sampath & Reddi* 1983). For instance, there is

only a two-amino-acid discrepancy between recombinant canine and human BMP-7 (*Ishibashi et al.* 1993). However, bone inductive capacity between native BMPs from different origins shows large variances (*Jortikka et al.* 1993a).

BMPs have been used as promising tools in treatment of bone defects, including major injuries and trauma, non unions, chronic infections, bone neoplasms and skeletal deformities (*Johnson et al.* 1988, *Lindholm et al.* 1994, *Paatsama et al.* 1996). Clinical trials with native and recombinant BMPs have been succesfully accomplished although occasionally the activity of recombinant proteins has been weaker than with native preparations (*Wozney* 1989). The native BMP contains a mixture of different BMPs combined with other growth factors allowing their synchronized action (*Wozney* 1989). There are also reports suggesting that recombinant BMPs have the same efficacy as native BMPs (*Cook et al.* 1994).

Native BMPs from different animal species as well as recombinant human (rh) BMP-2 and rhBMP-7 (OP-1) have been succesfully used to induce bone formation in a variety of different animal models with excellent healing capacity (*Cook et al.* 1994, *Johnson et al.* 1988, *Kirker-Head et al.* 1995, *Nilsson & Urist* 1991).

Using a tangential flow system modified by our laboratory, a high recovery of native BMP with biological activity was obtained after extraction of bovine, human, ovine, porcine and reindeer bone matrix (*Jortikka et al.* 1993a). As one part of research on native-origin BMPs, cBMP was also involved in our study using the same extractive method (*Jortikka et al.* 1993b). Investigation of cBMP is important because the canine skeleton is considered to have a similar remodeling activity and developed haversian system as human skeleton (*Kirker-Head et al.* 1995, *Wozney* 1992).

The purpose of this study was to show some basic properties of cBMP from our preliminary results. Since there are only a few reports related to the application of BMP in veterinary medicine in contrast to established clinical application of BMP in human medicine, research on cBMP might encourage into further clinical application of BMP in veterinary orthopaedics.

Materials and methods

Seven kilograms of canine diaphyseal bones were stored deep frozen (–80 °C) for one month. Periosteum and bone marrow tissue was mechanically removed from the bones. After sawing into pieces, the bones were frozen in liquid nitrogen, then ground to a powder of 0.5-1.0 mm in diameter.

The pulverized bone was demineralized with 0.6 N HCL at 4 °C for 72 h and extracted with 4 M guanidine hydrochloride solution for 48 h. Thereafter, dissolved fractions were first filtered through a 0.65 μm filter by a tangential flow system (Minitan, Millipore, USA) and refiltered through an ultrafiltration membrane with a cut-off point of 10 kD. The filtered GuHCl solution was dialyzed against water for 24 h and the water-insoluble material collected and redissolved in 4 M GuHCl solution. This solution was again dialyzed against 7 volumes of 0.25 M citrate buffer with pH = 3.1 to remove gelatine peptides. The precipitate was washed with a large volume of cold water and lyophilized. Seventy mg of partially purified canine bone morphogenetic protein was obtained by the end of the process.

The osteoinductivity of cBMP in vivo was assayed using three 35-day old BALB mice under inhalation anesthesia with Trothane (I. S. C. Chemicals Ltd., England). Loaded in gelatin capsules, lyophilized partially purified cBMP in a dose of 1.0 mg, 6.0 mg and 10.0 mg of BMP were implanted into the left side of a thigh muscle pouch. Capsuled bovine serum albumin

in a corresponding dose was implanted into the right side of thigh muscle pouches as controls. The mice were killed 21 days after implantation with carbon dioxide and the bilateral hindlimbs including the implants were sampled. Soft radiograms were taken using mammography equipment (20 KV, 100 mA, 0.06 s.). Specimens were dissected from the muscle of the hindlimbs and fixed with 10% neutral formalin. After demineralization, histologic sections 7 mm in thickness were prepared and stained with combined hematoxyline-eosin and azure II for microscopic evaluation.

To characterize partially purified cBMP by HPLC gel filtration, 5.0 mg of cBMP was dissolved in 2.0 ml of GuHCl and applied to a 300 mm long Sepharyl 200 HPLC gel filtration column (Pharmacia Diagnostics, Sweden). Isoelectric focusing was performed by determining the isoelectric points as follows: the cBMP preparation was dissolved in 6.0 M urea to the concentration of 800 μg/ml. Then 50 μl of the solution was applied to a polyacrylamide isoelectric focusing gel with a pH gradient from 3.5 to 9.5. The gel was stained with Coomassie brilliant blue G-250 (Serva, Germany).

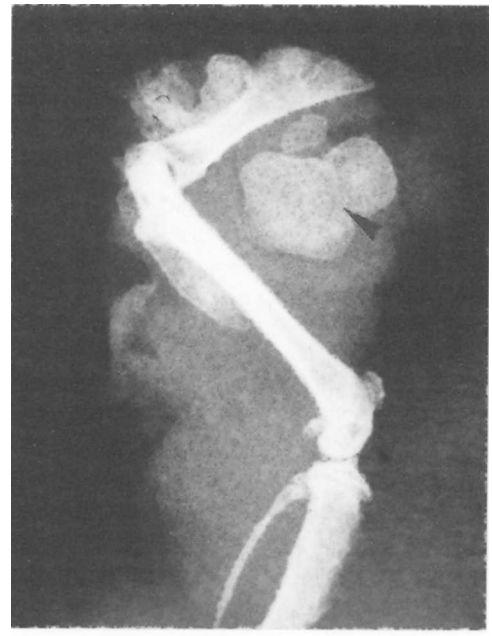

Figure 1. A roentgenogram presenting ossicle island of new bone in a thigh muscle pouch 21 days after implantation of 6 mg cBMP. Ossification mainly occurred in the central part and a sharp margin exists between ectopic new bone and muscle surrounding.

Results

A radiologically detectable ectopic bone formation was observed in the muscle pouches of the mice with 6.0 mg and 10.0 mg of partially purified cBMP after 3 weeks of implantation (Fig. 1). Histologically new woven bone was identified. A sharp margin of newly formed bone against the muscle was noticed. No difference in the amount of ossified bone between mouse with 6.0 mg and the mouse with 10.0 mg cBMP was observed. An ossicle island consisting mostly of woven bone and a spotty patch of cartilage was found microscopically in the implanted site of mice with 6.0 mg and 10.0 mg cBMP. The marrow tissue seemed to generate in the middle of the ossicle island. As a sign of immunological reaction lymphocytes infiltrated around the ectopic formed bone were noticed (Fig. 2). The dose of 1.0 mg of partially purified cBMP was not efficient enough to induce bone formation observed radiologically or histologically. Implanted gelatin capsules with bovine albumin did not provoke calcified tissue nor new bone formation.

Gel filtration chromatography defined that partially purified cBMP was composed of 3 identified peaks with a molecular weight ranged from 4 to 120 kD. The dominant component was located in the region of low molecular weight from 4 to 12 kD in the spectrum. One minor peak was individually distributed in the region with molecular weight from 70 to 120 kD (Fig.

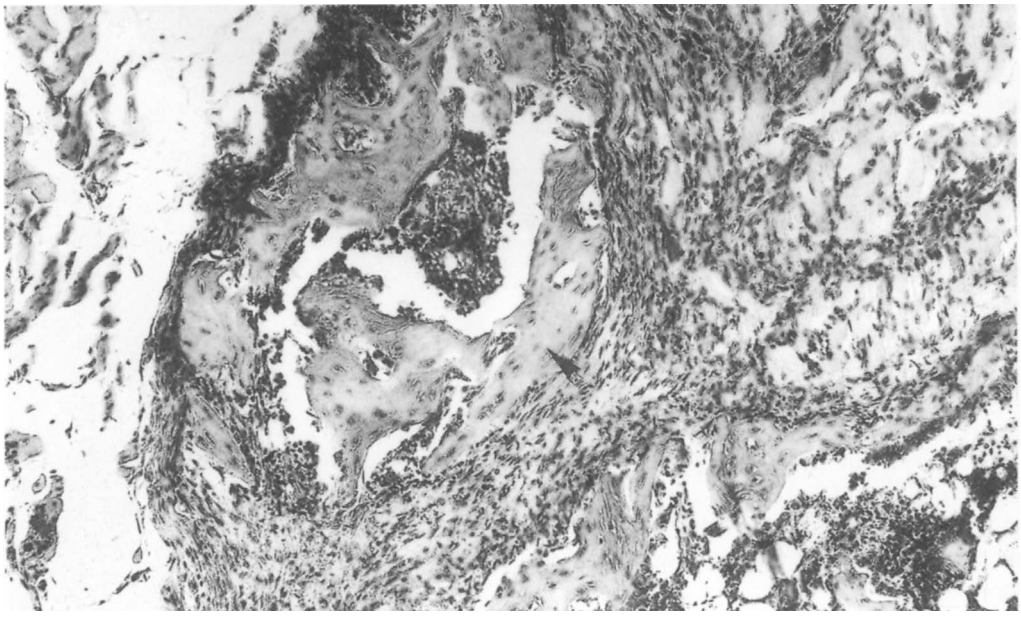

Figure 2. A microhistogram of histologically identified new woven bone in the mouse muscle pouch implanted with 6.0 mg cBMP. Some basiphilic cells infiltration around newly-formed bone were noticed (hematoxylin-eosin and azur, original magnification X 160).

3). Isoelectric focusing showed 6 detectable bands on the gel. The isoelectric points ranged from 4.6 to 5.6 (Fig. 4).

Discussion

Ectopic bone induction in vivo, especially in mice, has been used as a convenient and reliable bioassay for osteoinductive activity produced by BMPs. Morphogenetic activity of canine BMP has been demonstrated by implanting reparative dentine combined to allogenic BMP (*Nakashima* 1990). We showed that the partially purified BMP extracted by 4 M GuHCl and purified by tangential flow filtration possessed osteoinductive capacity. Implanting 6.0 mg rather than 1.0 mg of cBMP induced visible bone formation radiologically and histologically in 21 days. Compared to different origins of native BMP extracted and purified by established methods in our laboratory (*Jortikka et al.* 1993b), the osteoinductivity of cBMP was much lower than reindeer (0.6 mg), closer to ovine BMP (5.1 mg) and bovine (2.5 mg) BMP whereas higher than porcine BMP (8.0 mg) combined to gelatin capsule and implanted into a mouse muscle pouch (*Jortikka et al.* 1993a). Less osteoinductivity of dog bone matrix was also verified by *Schwarz et al.* (1989) in comparison to rat bone matrix when implanted in nude rats. Since there was a relatively large amount of ossified ectopic bone formation initiated by 6.0 mg of cBMP, we assumed that the minimal dose of the partially purified cBMP used in this experiment should be somewhere between 1.0 mg and 6.0 mg.

Chromatographically, partially purified cBMP was characterized as an acid protein mixture containing three major fractions with molecular

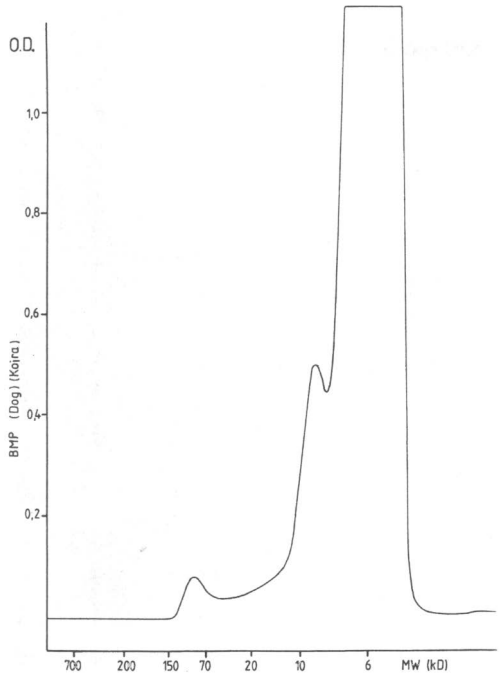

Figure 3. The spectrum of gel filtration chromatography of the canine BMP. Two major peaks showed the fractions with molecular weight from 4 to 7.5 and from 7.6 to12KD and one minor peak showed the fraction with a molecular weight from 70-120 KD.

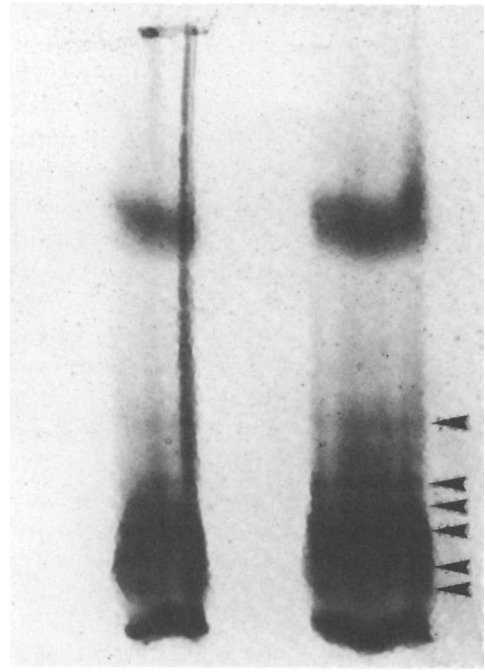

Figure 4. Isoelectric focusing electrophoresis of the canine BMP. Left column was isoelectric point marks in reference. Right column described isoelectric points of at least 6 detectable fractions: Frac. 1: 4.66, Frac. 2: 4.71, Frac. 3: 4.99, Frac. 4: 5.04, Frac. 5: 5.10 and Frac. 6: 5.65.

weights ranging from 4-7.5, 7.6-12 and 70-120 kD, respectively. Two dominant fractions were included with molecular weights from 4 to 12 kD, which were not identical to BMPs with molecular weight between 17 and 36 kD (Wozney, 1989). Most of the bioactive form of BMP was composed of several associated acidic polypeptides.

Partially purified cBMP was used in treatment of italian greyhound with a radio-ulnar fracture without union for 6 weeks previously reported by *Paatsama et al.* (1996). cBMP combined with biocoral carrier was implanted into the fracture gap and 2 weeks after the operation the dog began to use the leg. A month later the roentgenograms provided fracture healing induced by cBMP (*Paatsama et al.* 1996). *Johnson et al.* (1988) reported that 100 mg of bovine BMP combined with noncollagenous proteins and autogenous cancellous grafts induced a good recovery in the ulnar defects of 29 dogs. In the report of *Cook et al.* (1994, 1996) recombinant BMP-7 (OP-1) induced healing in canine ulnar defects were noticed. When tested mechanically nearly the intact limb strength was achieved with BMP treated defects. The results with the defects treated with BMP were remarkably greater than defects treated with autogenous bone (*Cook et al.* 1994, *Cook and Rueger* 1996).

Conclusions

The tangential flow system used in extraction and purification of cBMP was a reproducible and effective method (*Jortikka et al.* 1993b). A good recovery of partially purified cBMP (70 mg) was obtained from 7 kilograms of deep frozen canine bone. Compared to other purification methods (*Urist et al.* 1979), the simplicity of this method makes it possible to prepare moderate amounts of partially purified cBMP for both research purposes and application to clinical cases in veterinary practice. Partially purified cBMP induced a new bone formation amounts equal to some other mammals. A new bone formation induced by BMP is greater than bone formation induced by autogenous grafts used as a golden standard. New innovations in a field of BMP research open great opportunities in treatment of large bone defects and nonunions. Enhanced healing of the fractures and remodeling to growth disturbances are potential uses of BMPs in veterinary orthopaedics.

References

Canalis E, Centrella M, Urist MR: Effect of partially purified bone morphogenetic protein on DNA synthesis and cell replication in calvarial and fibroblast cultures. Clin Orthop 1985, *198,* 289-296.

Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC, Whitecloud TS: The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. J Bone Joint Surg 1994, *76 A/6,* 827-838.

Cook SD, Rueger DC: Osteogenic protein-1, biology and applications. Clin Orthop 1996, *324,* 29-38.

Dube JL, Celeste AJ: Human bone morphogenetic protein-15, a new member of the transforming growth factor-β superfamily. Abstr 18th Ann Meet An Soc Bone Mineral Res 1996.

Ishibashi K, Sasaki S, Akiba T, Maromo F: Expression of bone morphogenetic protein 7 mRNA in MDCK cells. Bioch and Biophys Res Commun 1993, *193,* 235-239.

Johnson EE, Urist MR, Finerman GAM: Bone morphogenetic protein augmentation grafting of resistant femoral nonunions. Clin Orthop 1988, *230,* 257-265.

Johnson EE, Urist MR, Schmalzried TP, Chotovichit A, Huang HK, Finerman GA: Autogeneic cancellous bone grafts in extensive segmental ulnar defects in dogs. Clin Orthop 1989, *243,* 254-265.

Jortikka L, Marttinen A, Lindholm TS: Partially purified reindeer (*Rangifer tarandus*) bone morphogenetic protein has a high bone-forming activity compared with some other artiodactyls. Clin Orthop 1993a, *297,* 33-37.

Jortikka L, Marttinen A, Lindholm TS: Purification of monocomponent bovine bone morphogenetic protein in a water-soluble form. Ann Chirurg 1993b, *82,* 25-30.

Kirker-Head CA, Gerhart TN, Schelling SH, Hennig GE, Wang E, Holtrop ME: Longterm healing of bone using recombinant human morphogenetic protein 2. Clin Orthop 1995, *318,* 222-230.

Lindholm TC, Lindholm TS, Marttinen A, Urist MR: Bovine bone morphogenetic protein (bBMP/NCP) – induced repair of skull trephine defects in pigs. Clin. Orthop 1994, *301,* 263-270.

Nakashima M: The induction of reparative dentine in the amputated dental pulp of the dog by bone morphogenetic protein. Arch of oral biol 1990, *35(7),* 493-497.

Nilsson OS, Urist MR: Immune inhibition on of repair of canine skull trephine defects implanted with partially purified bovine morphogenetic protein. Int Orthop 1991, *15,* 257-263.

Paatsama S, Lindholm S, Oksanen J, Axelson P: Die Anwendung Knochenmorphogenetischen Proteins bei verzögerter Frakturheilung, Pseudarthrose und bei wegen Ellbogengelenkerkrankungen durchgeführter ulnaosteotomie. (Clinial application of bone morphogenetic protein in delayed union, pseudoarthosis and in ulnar osteotomy performed for treatment of elbow disease in dogs). Tierärztl Prax 1996, *24,* 164-168.

Reddi AH: Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr Opin cell biol 1992, *4,* 850-855.

Riley EH, Lane JM, Urist MR, Lyons KM, Lieberman JR: Bone morphogenetic protein-2. Clin Orthop 1996, *324,* 39-46.

Sampath TK, Reddi AH: Homology of bone-inductive proteins from human, monkey, bovine and rat extracellular matrix. Proc Natl Acad Sci USA 1983, *80,* 6591-6595.

Schwarz N, Dinges H, Schiesser A, Reddi H, Schlag G: Dog bone less osteogenetic than rat bone,

bone-matrix transplants in nude rats. Acta Orthop Scand 1989, *60(6),* 693-695.

Urist MR: Bone: Formation by autoinduction. Science 1965, *150(3698),* 893-899.

Urist MR, Mikulski A, Lietze A: Solubilized and insolubilized bone morphogenetic protein. Proc Natl Acad Sci USA 1979, *76,* 1828-1832.

Wozney JM: Bone morphogenetic proteins. Progress in growth factor research 1989, *1,* 267-280.

Wozney JM: The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 1992, *32(2),* 160-167.

Wozney JM: Molecular biology of the bone morphogenetic proteins. Bone grafts, derivates and substitutes 1994, chapter *20,* 397-412.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA: Novel regulators of bone formation: Molecular clones and activities. Science 1988, *242,* 1528-1534.

Sammanfattning

Extrahering och karakterisering av nativt morfogenetiskt protein (cBMP) från hundben med verifierad osteoinduktivitet.

Benmorfogenetiskt protein (cBMP) extraherades från hundben, renades partiellt och testades för osteoinduktivitet. En röngenologiskt och histologiskt påvisbar ektopisk benybildning inducerades med 6 mg hund (c) BMP i muskulaturen hos BALB möss 21 dagar efter implantationen. Karakteriseringen av cBMP preparatet med gelfiltrations- kromatografi visade att preparatet bestod av proteiner eller proteinkomplex med molekylvikter mellan 4 till 120 kD. Isoelektrisk focusering visade att molekylerna var sura och hade isoelektriska punkter från 4.6-5.6.

(Received March 24, 1997; accepted February 12, 1998).

Reprints may be obtained from: J. Oksanen, MD, Institute of Medical Technology, P.O. Box 607, FIN-33101 Tampere. E-mail: Jarkko.Oksanen@uta.fi, tel: +358-3-2157733, fax: 358-3-2157710.