BONE INDUCTION IN MONKEYS BY BONE MORPHOGENETIC PROTEIN

A TRANS-FILTER TECHNIQUE

SHIMPEI MIYAMOTO, KUNIO TAKAOKA, KEIRO ONO

From Osaka University Medical School, Japan

We investigated bone induction by bone morphogenetic protein in primates, comparing it with that seen in rodents.

Twelve Millipore diffusion chambers containing 5 mg of semipurified bone morphogenetic protein were implanted into the dorsal muscles of 12 young, adult crab-eating monkeys (*Macaca fascicularis*) and were retrieved six weeks later. In six of nine unbroken chambers, new bone with haematopoietic marrow had been induced on the host-side surface of the filter. The incidence of trans-filter bone induction in the monkeys was almost equal to that observed in mice, and the new bone yield was approximately half as much as in mice.

Our results show that bone morphogenetic protein can induce new bone formation in primates within six weeks, and support the hope that it will be useful as a substitute for bone graft in man.

J Bone Joint Surg [Br] 1993;75-B:107-10.

Received 30 January 1992; Accepted 13 July 1992

Demineralised bone matrix has been reported to induce ectopic bone formation in rodents when implanted into allogeneic hosts (Urist 1965). Bone morphogenetic protein (BMP), a peptide or group of peptides, is responsible for this bone induction (Urist 1980). Its molecular structure has recently been reported and some BMPs have been successfully produced using DNA recombination techniques (Wang et al 1990). BMP has

the biological capacity to induce the differentiation of mesenchymal-type cells into cartilage, which is then replaced by bone through a process of enchondral ossification (Urist 1965, 1980), and may have a clinical potential as a bone-graft substitute in orthopaedic and maxillofacial surgery.

Discussion continues, however, about bone induction by BMP (Solomon 1991) because its biological action has been investigated predominantly in rodents, which are more susceptible to bone formation (Connor 1983), and it is uncertain whether it is effective in higher animals (Schwarz et al 1991), especially primates. Hosny and Sharawy (1985) found limited bone induction by allogeneic demineralised bone powder in adult rhesus monkeys. Aspenberg, Lohmander and Thorngren (1988) also reported failure of bone induction after intramuscular implantation of demineralised bone matrix in adult squirrel monkeys and in the human thigh. More recently, Ripamonti (1991) reported that chemically treated allogeneic bone matrix induced ectopic bone formation in adult baboons. This took several months, however, as compared with several weeks in rodents (Aspenberg and Andolf 1989). These reports made it doubtful whether BMP would be useful for inducing bone in human patients.

This led us to study the presence and speed of bone induction by BMP in primates. To make our investigation more precise, we used a diffusion chamber system (Goldhaber 1961; Heiple et al 1968) to examine trans-filter bone induction.

MATERIALS AND METHODS

Preparation of BMP. BMP was partially purified from a BMP-producing murine osteosarcoma (Dunn and Anderson 1963; Amitani, Nakata and Stevens 1974) as previously described (Takaoka et al 1988, 1993 in press). Samples of the tumour were minced, homogenised with deionised water and centrifuged. The precipitate was defatted with chilled acetone and dried, and the resultant powder extracted with 4 M guanidine-HCl for 48 hours

S. Miyamoto, MD, PhD, Assistant Professor
K. Takaoka, MD, PhD, Associate Professor
K. Ono, MD, PhD, Professor and Chairman
Department of Orthopaedic Surgery, Osaka University Medical School,
1-1-50 Fukushima, Fukushima-ku, Osaka 553, Japan.

Correspondence should be sent to Dr S. Miyamoto.

©1993 British Editorial Society of Bone and Joint Surgery
0301-620X/93/1509 \$2.00

at 4°C. After further fractionation of the extract with chilled acetone, the supernatant was dialysed against 10 mM phosphate buffer (pH 7.4). The precipitate collected from the dialysis tubing by sedimentation was redissolved in 0.1 M glycine-HCl buffer/10 mM NaCl solution (pH 2.4), and loaded on to a Sepharose CL-6B column (Pharmacia, Uppsala, Sweden). The peak fraction with bone-inducing activity eluted from the column was collected, dialysed against deionised water and lyophilised. This material was dissolved in 4 M guanidine-HCl and applied to a Sephacryl-S-200 gel column (Pharmacia, Uppsala, Sweden). A BMP-active fraction obtained from gel filtration was then dialysed against deionised water and lyophilised; this fraction was used as the semipurified BMP preparation.

Preparation of the diffusion chambers. Twenty-four diffusion chambers were constructed in a uniform manner using a diffusion-chamber assembly tool (Millipore, Massachusetts, USA). Lucite rings, 2 mm thick, with an external diameter of 14 mm and a central hole of 12 mm, and Millipore HA filters, 150 µm thick, 0.45 µm pore size, were used. All the tools and materials were sterilised by ethylene oxide gas, and construction was under sterile conditions. The chamber was made by sealing the filter to one side of the ring with Millipore MF cement No 1. After 5 mg of semipurified BMP fraction had been placed in the chamber, the filter was sealed on the other side. Twelve chambers contained 5 mg of semipurified BMP; the other 12 were left empty to be used as controls.

Experimental protocol. The experiments were performed under the guidance of the Primate Society of Japan for the care and use of laboratory primates. Twelve, young, adult crab-eating monkeys (*Macaca fascicularis*; 6 males and 6 females; mean weight, 3.6 ± 0.4 kg; range, 3.2 to 4.3) were anaesthetised with intramuscular ketamine hydrochloride (20 mg/kg body-weight). After preparation of the skin, one BMP-containing diffusion chamber was implanted into the left latissimus dorsi muscle and one control chamber into the right, in each monkey. After surgery, 50 mg of dibekacin sulphate were given by intramuscular injection as prophylaxis against infection. Six weeks later, the animals were anaesthetised again and the diffusion chambers retrieved.

The 48 Millipore filters (two from each chamber) with any tissue adherent to them were detached from the rings and examined radiologically and histologically for bone formation. Radiography used soft X-rays at 16 kV and 1.6 mA with an exposure time of 60 seconds. Twenty-four filters detached from one side of each of the 24 chambers were fixed in 10% formaldehyde solution, decalcified in formic acid and embedded in paraffin. Serial sections, 5 µm thick, were stained with haematoxylin and eosin or toluidine blue and examined by light microscopy.

The other 24 filters, from the other side of the 24 chambers, were used to quantify the newly formed bone mass. The harvested tissue on each filter was defatted

with a solution of trichloroethylene and methanol (1:1 v/v) for 24 hours and then dried. Decalcification was in 5 ml of 0.6 N HCl for 24 hours and the calcium content in the HCl supernatant was then measured with a kit (Calcium C-test Wako, Wako Pure Chemical Industries, Osaka, Japan).

In a positive control series in rodents, we used the same type of diffusion chamber also containing 5 mg of the semipurified BMP and implanted them under the fasciae of the dorsal muscles in 12 ten-week-old male ICR strain mice. These diffusion chambers were also retrieved six weeks after implantation. Two Millipore filters with adherent tissue were resected from one ring and examined by the same methods used for the monkey experiment.

RESULTS

Our results are summarised in Table I.

In the monkeys, three of the 12 diffusion chambers containing BMP were discarded because the filters were broken and had allowed invasion by fibrous tissue. Of the remaining nine, six chambers had hard tissue anchored on the host-side surface of the filter. Radiography showed that this was a mesh-like network of calcified tissue (Fig. 1). Histological examination indicated that it was newly formed bone with haematopoietic marrow (Figs 2a, b). In some areas there was cartilage under the bone layer, in contact with the filter (Fig. 2c). The other three intact chambers containing BMP and all the control chambers were surrounded by fibrous tissue, with no bone or cartilage on the surface of the filters.

Table I. Incidence of bone induction and new bone yield (mean $\pm$ SD) after implantation of diffusion chambers containing BMP into monkeys and mice

	Incidence*	New bone yield (mg)†
Monkeys	6/9	1.11 ± 0.46
Mice	6/8	2.10 ± 0.81

* incidence is represented as the number of chambers with bone/the number of unbroken chambers retrieved from the host

† as calcium content of induced bone; unpaired Student's *t*-test; difference $p < 0.05$

In the mice, four of the 12 diffusion chambers were broken and discarded accordingly. In six of the remaining eight chambers, new bone had been induced on the host side of the filters.

The diffusion chambers containing 5 mg of semipurified BMP induced new bone containing 1.11 ± 0.46 mg (mean $\pm$ SD) of calcium in the monkeys, as against 2.10 ± 0.81 mg in the mice (Table I). The yield of new bone was statistically greater in the mice (unpaired Student's *t*-test; $p < 0.05$), but the incidence of bone induction

Fig. 1

Radiograph of a 14 mm diameter Millipore filter with adherent tissue from a chamber containing BMP retrieved from a monkey. There is a mesh-like network of mineralised trabeculae.

showed no statistically significant difference between the monkeys and the mice (Fisher's exact probability test).

DISCUSSION

We have shown that BMP can induce ectopic bone formation in adult crab-eating monkeys. It seemed that soluble BMP in the chambers gradually diffused across the Millipore filter, attracted responding mesenchymal-type cells and caused them to differentiate into osteoblasts (or chondrocytes which were then replaced by osteoblasts by enchondral ossification), forming new bone on the host side of the filter (Urist et al 1977). The yield of new bone was greater in the mice than in the monkeys, but there was no statistically significant difference in the incidence of bone induction.

We conclude that, although primates are less susceptible to bone formation than rodents, the tissues of

Fig. 2a

Fig. 2b

Fig. 2c

Photomicrographs of histological sections of the tissues induced six weeks after implantation of a diffusion chamber containing BMP into a monkey. Figure 2a - Newly formed bone is seen on the outer surface of the filter (haematoxylin and eosin $\times 80$). Figure 2b - High-power view shows osteocytes and osteoblasts within and on the organised bone matrix (haematoxylin and eosin $\times 320$). Figure 2c - In some areas there is cartilage under the bone layer in contact with the filter (toluidine blue $\times 320$).

crab-eating monkeys do respond to the bone-inducing signals of BMP. We agree with Ripamonti (1991) that primates do possess bone-inducing protein in their bone matrix as well as the crucial set of responding mesenchymal-type cells capable of differentiation into osteoblastic cell lines. In his experiment, however, it took three to nine months to induce bone formation in baboons. This is very slow when compared with the six weeks of our study.

There are two possible reasons for the failure of bone induction in primates reported by Aspenberg et al (1988) and the delay observed by Ripamonti (1991). The first is that their implants, which were demineralised bone matrix and autolysed, antigen-extracted allogeneic bone respectively, contained too small amounts of the bone-inducing protein. Since primates are less susceptible to bone formation than rodents, this low content of BMP may have been insufficient to induce bone formation or to hasten bone induction. The semipurified BMP that we used contained a much greater amount of bone-inducing protein and succeeded in inducing ectopic bone formation in primates within six weeks.

The second possible reason may have been the 'carrier' of BMP. We found that the intramuscular implantation of semipurified BMP alone failed to induce ectopic bone formation in monkeys (data not reported) while semipurified BMP enclosed in diffusion chambers, allowing the sustained release of soluble BMP, *did* so on the outer surface of the filters. We have previously reported that implantation of semipurified BMP alone in rodents failed to evoke a bone-inductive response because of the rapid outward diffusion of the protein from the implantation site (Takaoka et al 1988; Nakahara et al

1989). BMP requires a carrier or delivery system to allow its sustained release in order to express its biological potency (Takaoka et al 1988; Miyamoto et al 1992, 1993 in press). In addition, an inappropriate carrier may itself impede bone induction (Miyamoto et al 1992). In the classical experiments of bone induction in rodents, bone matrix effectively constituted the carrier component, which was absorbed by the host tissue and was replaced in situ by new bone (Urist 1965, 1980). It is not known whether bone matrix is an appropriate carrier of BMP in primates and it is possible that the bone matrix used by Aspenberg et al (1988) and Ripamonti (1991) was not a suitable carrier.

Our study has provided evidence that BMP can induce ectopic bone formation in primates within six weeks. If a sufficient amount of pure recombinant human BMP (Wang et al 1990) becomes available, and can be combined with a carrier suitable for bone induction in humans, then the combination could become an important substitute for bone graft in orthopaedic surgery.

The authors thank Mr Yoichiro Oohara for his surgical assistance and monitoring of the monkeys.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

REFERENCES

- Amitani K, Nakata Y, Stevens J. Bone induction by lyophilized osteosarcoma in mice. *Calcif Tissue Res* 1974; 16:305-13.
- Aspenberg P, Andolf E. Bone induction by fetal and adult human bone matrix in athymic rats. *Acta Orthop Scand* 1989; 60(2):195-9.
- Aspenberg P, Lohmander LS, Thorgren K-G. Failure of bone induction by bone matrix in adult monkeys. *J Bone Joint Surg [Br]* 1988; 70-B:625-7.
- Connor JM. *Soft tissue ossification*. Berlin, etc: Springer-Verlag, 1983:108-16.
- Dunn TB, Andervont HB. Histology of some neoplasms and non neoplastic lesions found in wild mice maintained under laboratory conditions. *J Nat Cancer Inst* 1963; 31:873-901.
- Goldhaber P. Osteogenic induction across Millipore filters in vivo. *Science* 1961; 133:2065-7.
- Heiple KG, Herndon CH, Chase SW, Wattleworth A. Osteogenic induction by osteosarcoma and normal bone in mice. *J Bone Joint Surg [Am]* 1968; 50-A:311-25.
- Hosny M, Sharawy M. Osteoinduction in rhesus monkeys using demineralized bone powder allografts. *J Oral Maxillofac Surg* 1985; 43:837-44.
- Miyamoto S, Takaoka K, Okada T, et al. Evaluation of polylactic acid homopolymers as carriers for bone morphogenetic protein. *Clin Orthop* 1992; 278:274-85.
- Miyamoto S, Takaoka K, Okada T, et al. A new biodegradable synthetic carrier for bone morphogenetic protein: polylactic acid - polyethylene glycol block copolymer. *Clin Orthop* 1993; in press.
- Nakahara H, Takaoka K, Koezuka M, et al. Periosteal bone formation elicited by partially purified bone morphogenetic protein. *Clin Orthop* 1989; 239:299-305.
- Ripamonti U. Bone induction in nonhuman primates: an experimental study on the baboon. *Clin Orthop* 1991; 269:284-94.
- Schwarz N, Schlag G, Thurnher M, et al. Fresh autogeneic, frozen allogeneic, and decalcified allogeneic bone grafts in dogs. *J Bone Joint Surg [Br]* 1991; 73-B:787-90.
- Solomon L. Bone grafts. *J Bone Joint Surg [Br]* 1991; 73-B:706-7.
- Takaoka K, Nakahara H, Yoshikawa H, et al. Ectopic bone induction on and in porous hydroxyapatite combined with collagen and bone morphogenetic protein. *Clin Orthop* 1988; 234:250-4.
- Takaoka K, Yoshikawa H, Hashimoto J, et al. Purification and characterization of a bone-inducing protein from a murine osteosarcoma. *Clin Orthop* 1993; in press.
- Urist MR. Bone: formation by autoinduction. *Science* 1965; 150:893-9.
- Urist MR, Granstein R, Nogami H, Svenson L, Murphy R. Transmembrane bone morphogenesis across multiple-walled diffusion chambers. *Arch Surg* 1977; 112:612-9.
- Urist MR. Bone transplants and implants. In: Urist MR, ed. *Fundamental and clinical bone physiology*. Philadelphia, etc: JB Lippincott, 1980:331-68.
- Wang EA, Rosen V, D'Alessandro JS, et al. Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci USA* 1990; 87:2220-4.