Original

Vigorous Osteoinductivity Observed in Crude Bone Morphogenetic Protein Stored for 25 Years after Extraction at Room Temperature

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Abstract: Bone morphogenetic protein (BMP) is the only cytokine that induces heterotopic new bone. BMP has been classified as a subfamily of the TGF superfamily. The most important and essential aspect for the clinical application of BMP is the replenishment and reinforcement of bone defects and fragile areas with new bone. However, it is difficult to ascertain long term clinical results. In this study, we examined the osteogenic potential in mouse femoral fascia of crude BMP extracted from bovine bone that had been stored at room temperature for 25 years. The results showed vigorous osteogenesis. This study demonstrates the long-term stability of crude BMP.

Key words: BMP, Long term storage, Heterotopic new bone

Introduction

Since its discovery by Urist et al. in 19651), bone morphogenetic protein (BMP) has received attention as the only cytokine to induce heterotopic new bone2). In addition, several BMP superfamilies have been identified3,4), and they have shown a wide variety of effects in addition to their osteogenic potential.

Sampath et al. reported that BMP is the only cytokine in muscles with osteogenic potential5). From a clinical perspective, this cytokine should present adequate stability for long periods after extraction. In this study, we examined the osteogenic potential of crude BMP (cBMP) extracted from a bovine tibia approximately 25 y ago and stored at room temperature, and the material exhibited vigorous osteogenesis. This study reports the long-term stability of the cBMP osteogenic potential.

Materials and Methods

Animals, BMPs, and surgery

The cBMP used in the graft experiment was refined in 1996 using the Urist method6). After extraction, it was placed in a clear glass phial and stored in a desiccator (Sanplatec, Osaka, Japan) at room temperature for 25 y until 2021 (Fig. 1).

The cBMP was separated into 3 groups (5.0, 10, and 20 mg) and placed in gelatin capsules #5 (Matsuya, Osaka, Japan) to create transplant samples. Empty gelatin capsules were used as the control.

A total of 24 five-week-old male BALB/c mice were used. They were six animals for each group (5.0, 10, 20 mg, and control).

A 3-drug anesthetic composed of medetomidine hydrochloride (DOMITOR®, Nippon zenyaku kogyo, Fukushima, Japan), midazolam (Dormicum®, Astellas Pharma, Tokyo, Japan), and butorphanol tartrate (Vetorphale®, Meiji Seika Pharma, Tokyo, Japan) was administered intraperitoneally to induce general anesthesia7) and cBMP was transplanted into the left femoral interfascial space of the mice. For the control, an empty gelatin capsule was transplanted on the opposite side of the same mouse.

The management and research methods were approved by the animal experiment committee of the Aichi Gakuin University School of Dentistry (AGUD461), and guidelines on animal experiments were followed.

The mice were sacrificed 3 weeks after cBMP transplantation, and their thighs were removed.

Soft X-ray imaging

The thigh samples were imaged with soft X-ray film (Fuji X-ray film; FR 12×16.5 cm, Kabine) using a soft X-ray device (OMC-403, OHMIC Ltd., Shiga, Japan) at 23 kV and 3 mA for 23 s, followed by the prescribed development process, rinsing, and drying. Soft X-rays were used to calculate the area of heterotopic new bone, which was used as the amount of neonatal bone.

Micro-CT imaging

The thigh samples were imaged using a micro CT device (Scan Xmate-L090H, Comscantecno). From these images, the volume (cm3) of heterotopic new bone induced by crude BMP was calculated as the osteoinductive activity using Micro CT software (TRI/3D-BON; Ratoc System Engineering, Tokyo, Japan).

Bone volume (BV) and total volume (TV) were measured to calculate the BV/TV ratio (mm3).

Histological examination with HE staining

The heterotopic new bone was removed in a manner to include the surrounding soft tissue, and it was fixed at 4°C for 1 week with 10% neutral formalin. Subsequently, the samples were demineralized in 10%
EDTA (pH 7.2) for 21 d at 4°C. After demineralization, the samples underwent paraffin embedding according to the regular method to prepare 5–7 μm sections. The sections were stained with hematoxylin-eosin to check the status of the new bone.

**Statistical analysis**

Collected experimental data were expressed as mean and standard deviation. Statistical significance tests were performed using Tukey’s multiple comparison test. GraphPad Prism v. 6 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis. Statistical significance was set at p < 0.05.

**Results**

**Soft X-ray findings**

Soft X-rays of mouse thighs transplanted with cBMP showed new bone-like radiopaque images. Soft X-ray analysis showed heterotopic new bone in the femoral region. The area of new bone-like formation in the soft X-rays increased according to the cBMP transplantation amount (Fig. 2A, B).

![Figure 1. Transplanted cBMP](image1)

![Figure 2. A) Soft radiographs 3 weeks after cBMP transplantation. B) cBMP-induced new bone amounts measured from soft radiographs (**p < 0.001, *p < 0.05).](image2)
Figure 3. A) Micro-CT image 3 weeks after cBMP transplantation. B) cBMP-induced bone volume (BV, cm$^3$), total volume (TV, cm$^3$), and BV/TV (%) measured from micro CT images (**p < 0.01, *p < 0.05)

Figure 4. Histopathological image 3 weeks after cBMP transplantation. A) Low magnification; Bar 1 mm. B) High magnification; Bar 500 μm. M: Muscle; B: Trabecular bone; F: Fibrous tissue
μCT analysis

Heterotopic new bone was observed in the femoral region in the μCT analysis. The BV and TV of the new bone from μCT increased according to the cBMP transplantation amount. There was no difference of the BV/TV among the cBMP transplantation amount (Fig. 3A, B).

HE staining

Bone-like tissue images were observed in areas where cBMP was transplanted. Trabecular bone, bone marrow adipocyte and fibrous tissue were observed, which indicated a bone structure (Fig. 4).

Discussion

Wozney et al. successfully sequenced BMP in 1988, thereby identifying BMP2[7]. This included BMP2 and BMP4, which can form new bone on their own and are bioactive in the form of dimers[8-11]. BMP2 and BMP4 should be stored at −20°C or below[12,13]. The amounts of BMP2 and BMP4 in the cBMP used in our experiments were unknown, as well as the final BMP forms or compounds. Although further analysis is needed, the results clearly show that crude BMP stored in a desiccator at room temperature for 25 y after being refined remained bioactive. This demonstrates that BMP retains extremely stable osteoinductivity over a long period. This stability of action over 25 y is considered formative with respect to its clinical applications, as it demonstrates strong long-term osteogenic potential in a stable form[14,15].

In conclusion, the results suggest that cBMP maintained the capacity to create new bone for 25 y. This long-term stability of action can represent the first step to pharmacologically understand the stability of the action of BMP and other cytokines for long periods.

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Conflict of Interest

The authors declare that they have no conflicts of interest with regard to the present study.

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