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RESEARCH PAPER

Profile of rhBMP-2 Release from Collagen Minipellet and Induction of Ectopic Bone Formation

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ABSTRACT

For a cylindrical controlled-release formulation using collagen as a carrier, called the minipellet (MP), which contains rhBMP-2, the relationship between the diameter of MPs and rhBMP-2 release profiles was investigated, and its effect in inducing bone formation was evaluated. Samples with three different diameters were tested for each of the following formulations: MP without additives, MP with 10% (w/w) glutamic acid (Glu) and 20% (w/w) alanine (Ala), and MP with 20% (w/w) Glu and 20% (w/w) Ala. The results of the in vitro release test and the amount of rhBMP-2 remaining in the MPs after subcutaneous implantation into mice were compared among different samples. It was found that the addition of Glu accelerated release of rhBMP-2 effectively. Release was accelerated as the diameter of MP became smaller and the amount of Glu added increased. The amount of calcium formed in 3 weeks after subcutaneous implantation into mice was dose-dependent. The amount of calcium formed per unit rhBMP-2 dose tended to increase as the diameter of MP became smaller and the amount of Glu added became greater; calcification was thus associated with release rate. These results indicate that MPs with smaller diameters induce bone formation more efficiently. For use in the treatment of fracture, etc., MP is considered to be a suitable dosage form, which can be administered noninvasively.

Key Words: Bone morphogenetic protein (BMP); Collagen; Minipellet; Controlled release; Ectopic bone formation.

INTRODUCTION

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)-β superfamily and are known well as factors inducing bone and cartilage formation.[1] It has been shown that BMPs induce differentiation of undifferentiated mesenchymal cells into osteoblasts and chondrocytes.[2] Among

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recombinant BMPs shown to have the potential of inducing ectopic bone formation, rhBMP-2 and rhBMP-7 (OP-1) have been actively studied, and their clinical application has been advanced.[3–5] Since rhBMP-2 and rhBMP-7 induce bone formation through local effects, it is necessary to deliver them directly with a carrier to the site where bone formation is needed. For the purpose of inducing bone formation effectively, various carriers for these BMPs have been studied.[6–9] These studies focused on developing a type of implant device designed to effectively serve as a scaffold for bone formation.[10,11] These types of devices induce or supplement formation of bone, the morphological features of which are affected by the shape of the devices used, and these devices can thus treat fractures or bone defects. However, since these devices require surgery for their application, it is desirable to develop a formulation of BMPs that can be administered noninvasively for use in the treatment of nontraumatic fracture, etc.

The minipellet (MP),[12] which uses collagen[13] (a material used extensively in medical treatment) as a carrier, is a cylindrical, solid preparation. The MP can be administered almost in the same way as a conventional injection using an applicator designed for MPs. In addition, MPs can contain a high content of rhBMP-2. Thus, it is possible to topically administer a high dose of rhBMP-2 in a noninvasive manner. We previously carried out an additive screening test, then designed a method for controlling the release of rhBMP-2 from the MP using glutamic acid (Glu) and alanine (Ala), and found that the effect in inducing bone formation is dependent on drug release profile.[14] The rhBMP-2 is barely soluble under neutral conditions but easily soluble under acidic conditions. Therefore, the solubility of rhBMP-2 is elevated by the addition of Glu, leading to the accelerated release of rhBMP-2, since the formulation is temporarily acidic during the initial period of implantation. Moreover, since collagen fibers cannot maintain a tight arrangement and the formulation softens under acidic conditions, the MP disintegrates and the release of rhBMP-2 is accelerated. On the other hand, the addition of Ala improves the MP's shape. Therefore, the combination of the additives, Glu and Ala, is useful for the MP. For formulations like MPs, which are targeted at inducing bone formation in areas around the site of administration by means of diffusion of rhBMP-2 to surrounding tissue rather than by serving as the scaffold for bone formation, it is essential to determine the relationship between bone-inducing effect and drug release profile.

The present study was carried out to examine the relationship between formulation factors, additives, and diameter of MPs, and drug release profiles and its effect on inducing bone formation, using calcification in subcutaneous ectopic bone formation model in mice as an indicator.

MATERIALS AND METHODS

Materials

The collagen used in this study was atelocollagen, a product of KOKEN (Tokyo, Japan). Atelocollagen was prepared by enzymatically removing telopeptide from cattle dermal collagen with pepsin treatment. The rhBMP-2 was a product of Wyeth (Cambridge, MA) in the form of an aqueous solution. L-glutamic acid and L-Alanine were guaranteed reagents and were purchased from Nacalai Tesque (Kyoto, Japan).

Animals

The mice used in this study were normal male mice (BALB/c) purchased at 5 weeks of age from Charles River Japan (Kanagawa, Japan). They were

Table 1. Composition of rhBMP-2 minipellet.

Sample #	rhBMP-2 (%, w/w)	Additive (%, w/w)	Diameter (mm)
A/Free-L	5	–	0.88
B/Free-M	5	–	0.64
C/Free-S	5	–	0.45
D/Glu10-L	5	Glutamic acid (10), alanine (20)	0.86
E/Glu10-M	5	Glutamic acid (10), alanine (20)	0.62
F/Glu10-S	5	Glutamic acid (10), alanine (20)	0.39
G/Glu20-L	5	Glutamic acid (20), alanine (20)	0.89
H/Glu20-M	5	Glutamic acid (20), alanine (20)	0.70
I/Glu20-S	5	Glutamic acid (20), alanine (20)	0.42

used after 1 week of quarantine. During the experiment, animals were allowed free access to feed and water. The experiments conformed to the Guidelines for Animal Experiments of Formulation Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd.

Sample Preparation

Table 1 shows the samples prepared. Aqueous solution of rhBMP-2 (5.5 mg/mL), 2% (w/w) aqueous solution of atelocollagen, distilled water, and aqueous solution of additive filtrated through 0.22 μm membrane (5 mg/mL, depending on the composition) were combined uniformly and agitated well. The mixture was freeze-dried and combined with distilled water to yield collagen gel containing rhBMP-2. A syringe was filled with this gel, which was then extruded from a nozzle with three inner diameters (1.6, 1.1, and 0.7 mm). The thus obtained cylindrical products with different diameters were dried and cut into appropriate lengths to yield MPs containing rhBMP-2.

Quantitative Analysis of rhBMP-2

Reverse-phase high performance liquid chromatography (HPLC) was performed for quantitative analysis of rhBMP-2, using a C4 column (0.46 cm I.D. × 5 cm, mean particle size 5 μm; Vydac, CA). Water containing (0.1%, v/v) trifluoroacetic acid (sequencing grade, from Beckman coulter, CA) served as mobile phase A, and a 90:10 mixture (v/v) of acetonitrile (HPLC grade, from Nacalai tesque, Kyoto, Japan) and water containing trifluoroacetic acid (0.1%, v/v) was used as mobile phase B. The flow rate of the mobile phase was 1.5 mL/min. Mobile phase B was flowed at 25% of the flow rate for 2 minutes and then increased linearly to 60% in 14 minutes. The column temperature was set at 30°C. Detection was made by fluorescence (Ex: 287 nm, Em: 348 nm).

Measurement of rhBMP-2 Content

Each sample was immersed in 2.5 mL of 0.05% (w/v) aqueous solution of Tween 20 at pH 2 adjusted with HCl. The mixture was agitated for 6 hours at room temperature and left to stand overnight at 5°C. The thus obtained solution was subjected to HPLC, to determine the amount of rhBMP-2 contained in the MP.

In Vitro Release Test

Each sample was immersed in 1 mL of 0.3 M phosphate buffer (pH 6.2) containing 5% (v/v) polyethelene glycol (PEG) 400 (the release test solution) and incubated at 37°C (n=5). One, 4 and 7 days after the start of the test, the release test solution was exchanged with new solution. The test points were selected to clarify the difference of release profiles among samples. The amount of rhBMP-2 released was measured by HPLC, and release profiles were determined.

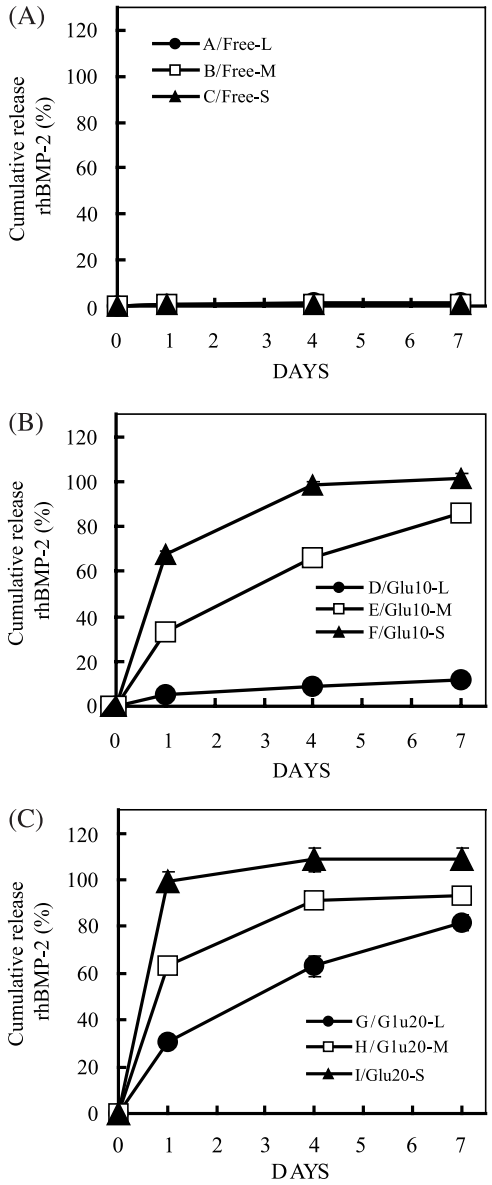

Figure 1. In vitro release profiles from minipellets containing 5% of rhBMP-2 (% of ''cumulative amount of rhBMP-2 released/total loading in the sample''): (A) sample without additives, (B) sample with 10% glutamic acid and 20% alanine, (C) sample with 20% glutamic acid and 20% alanine. The mean±SD is shown at each point (n=5).

In Vivo Release Evaluation

Each sample was implanted using an indwelling needle into the right side of the subcutaneous tissue on the back of ether-anesthetized mice (avoiding the area around the spinal vertebrae) (n=5). Three days after implantation, which was expected to exhibit the difference of the profile among the samples based on the result of the in vitro release test, the animals were sacrificed with excess ether, and the implanted samples were harvested. The amounts of rhBMP-2 remaining in the harvested samples were measured by the above-mentioned method used for measurement of rhBMP-2 content.

Calcification Analysis

Each sample was implanted subcutaneously into mice for 3 weeks, using the method similar to that used for in vivo release evaluation (n=8). Three weeks was selected as the test point based on the previous reports,[11,14] in which ectopic bone formation was markedly observed at 3 weeks after implantation. The harvested samples with surrounding bony tissue were immersed in 5 mL of 0.6 M HCl and left to stand at room temperature for 2 days. The concentration of calcium contained in the sample dissolved in HCl was measured with a calcium quantification kit (Calcium C-test Wako, Wako Pure Chemical Industries, Osaka, Japan) to determine the amount of calcium formed.

RESULTS

In Vitro Release Profile

Samples with three different diameters of each of the following formulations were subjected to a release test in vitro: MP without additives, MP with 10% (w/w) Glu and 20% (w/w) Ala, and MP with 20% (w/w) Glu and 20% (w/w) Ala. The results are shown in Fig. 1. In samples without additives, there was no apparent release, with no marked difference depending on diameter. However, in samples with Glu, a decrease in diameter resulted in a higher release rate (Sample D<E<F, G<H<I). When the diameter was the same, increase in amount of Glu added increased the release rate (Sample A<D<G, B<E<H, C<F<I).

In Vivo Release Profile

The amount of rhBMP-2 remaining in each sample 3 days after subcutaneous implantation into mice was examined. The results are shown in Fig. 2, and the sample appearance following explant from mice (Sample A, D, G) are shown in Table 2. In samples without additives, 85% or more of the initial amount of rhBMP-2 remained in the sample, with no marked difference depending on diameter. In samples with Glu, however, a decrease in diameter tended to result in smaller amounts of rhBMP-2 remaining in the sample

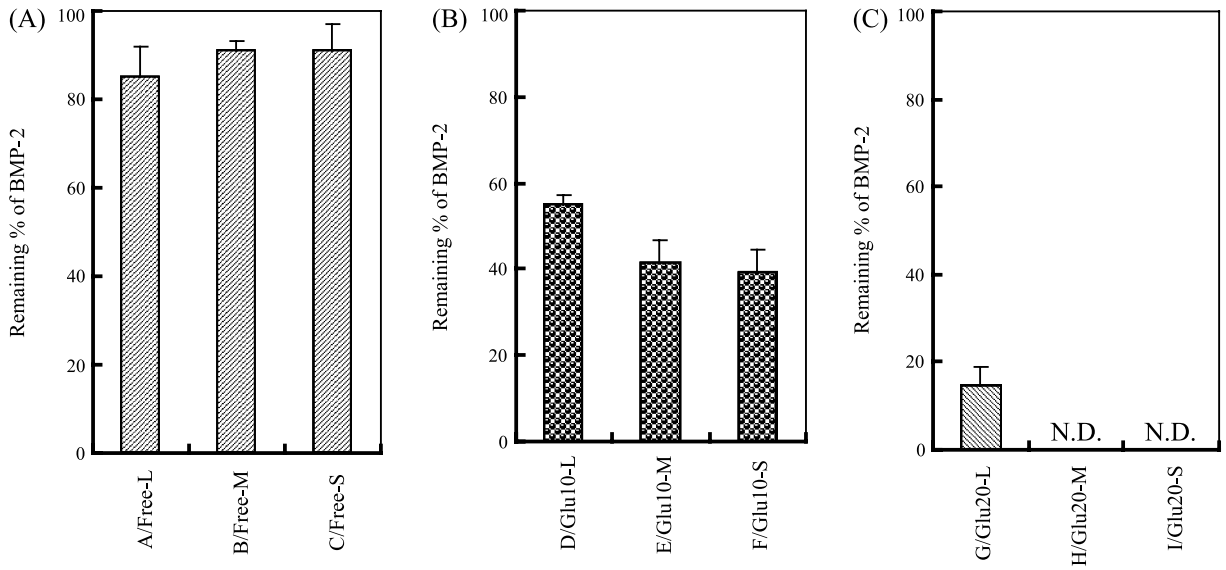

Figure 2. Remaining % of rhBMP-2 3 days after subcutaneous implantation of minipellets initially containing 5% rhBMP-2 to mice (% of ''amount of rhBMP remaining/total loading in the sample''): (A) sample without additives, (B) sample with 10% glutamic acid and 20% alanine, (C) sample with 20% glutamic acid and 20% alanine. The mean±SD is shown at each point (n=5).

(Sample D>E>F, G>H=I). When the diameter was the same, a increase in the amount of Glu added reduced the amount of rhBMP-2 remaining in the sample (Sample A>D>G, B>E>H, C>F>I). When it is postulated that the amount of rhBMP-2 lost from the sample was rhBMP-2 released, these results exhibit that the release in vivo correlates with that in vitro.

Calcification

The amount of calcium formed 3 weeks after subcutaneous implantation of each sample to mice was examined. Figure 3A shows the relationship between dose level (amount of rhBMP-2 contained in MP) and the amount of calcium formed. Figure 3B, C, and D show the amount of calcium formed per unit rhBMP-2 dose of each sample. For samples without additives, the amount of calcium formed was not dose-dependent. For samples with Glu, however, it exhibited a dose-dependent increase. Decrease in diameter resulted in more calcium formation per unit rhBMP-2 dose (Sample A<B<C, D<E<F, G<H<I). When the diameter was the same, an increase in the amount of Glu added tended to increase calcium formation (Sample A<D<G, B<E<H, F<I).

DISCUSSION

A previous study showed that when MPs of the same size were compared, release profiles did not differ markedly depending on % loading, i.e., the percentage of rhBMP-2 contained per unit weight of formulation.[14] On the other hand, it has been shown that release of interferon (IFN)[15] and G-CSF[16] was accelerated depending on the % loading. This discrepancy among previous studies appears to be associated with differences in solubility of proteins under physiological conditions. Interferon and G-CSF, which are highly soluble under physiological conditions, are immediately dissolved in water that permeates the MP after implantation, resulting in their diffusion across collagen fibers and their release from the MP.[17,18] Therefore, the diffusivity of these protein drugs is high when % loading is high, since the density of collagen fibers is low. For rhBMP-2, which is less soluble under physiological conditions, the rate of release by diffusion seems to be limited by solubility and is not dependent on % loading. The factor affecting release rate involves the affinity, such as ionic interaction between collagen and the drug and the surface area of the formulation, for example, as well as solubility of the drug, but here solubility of rhBMP-2 is believed to be a main dominant factor in the case of MPs with rhBMP-2.

In the present study, the % loading was kept constant at 5% (w/w), and three MPs differing in additives were prepared. For each MP, the release of rhBMP-2 was compared while changing the diameter of MP. In MPs without additives, change in diameter did not lead to any marked difference in release profiles in vitro or in vivo. In the case of highly soluble protein drugs, it is known that a smaller diameter leads to a greater surface area per unit protein amount in the formulation and to more rapid completion of water permeation (more rapid swelling), resulting in a higher release rate. However, in the case of rhBMP-2, which is less soluble under physiological conditions, release is limited by solubility and is probably not affected by increase in surface area. This explains the results obtained for MPs without additives shown above. For samples with Glu, an increase in the amount of Glu added and a decrease in the diameter of MPs resulted in a higher release rate. rhBMP-2 is known to be highly soluble under acidic conditions. It can therefore be presumed that in the case of MPs with Glu, the temporary increase in acidity due to permeation of water into the formulation soon after implantation probably increased the solubility of

Table 2. Sample appearance following explant from mice (after 3 days implantation).

Sample #	Sample	Tissue (encapsulation)[a]
A/Free-L	Softened, but kept shape	Surrounding the sample[b]
D/Glu10-L	Highly softened, and deformed to flat	Surrounding the sample[c]
G/Glu20-L	Dispersed	Spread over the implantation site widely[d]

[a]The stage of encapsulation was rated on a three-point scale:
[b](standard),
[c](about twice compared to +),
[d](about twice compared to ++).

rhBMP-2 and induced its diffusion through the formulation, resulting in diameter-dependent release profiles resembling the profiles for proteins like IFN and G-CSF, which are not limited by solubility. The release rate of rhBMP-2 was accelerated by Glu, depending on % loading of Glu both in vitro and in vivo, but the findings of the present study are not sufficient to understand the influence of Glu on the solubilization rate of rhBMP-2, which is related to the release rate of rhBMP-2. Furthermore, the addition of Glu to MPs makes MPs more likely to undergo disintegration after implantation. A decrease in the diameter of MPs elevates the dispersibility of the carrier collagen (data not shown) and increases the loss of the carrier, probably leading to a lower rhBMP-2-retaining effect of the carrier. These two factors, Glu-accelerated dissolution of rhBMP-2 and disintegration of MP after implantation, probably resulted in diameter-dependent changes in the release rate from MP with Glu.

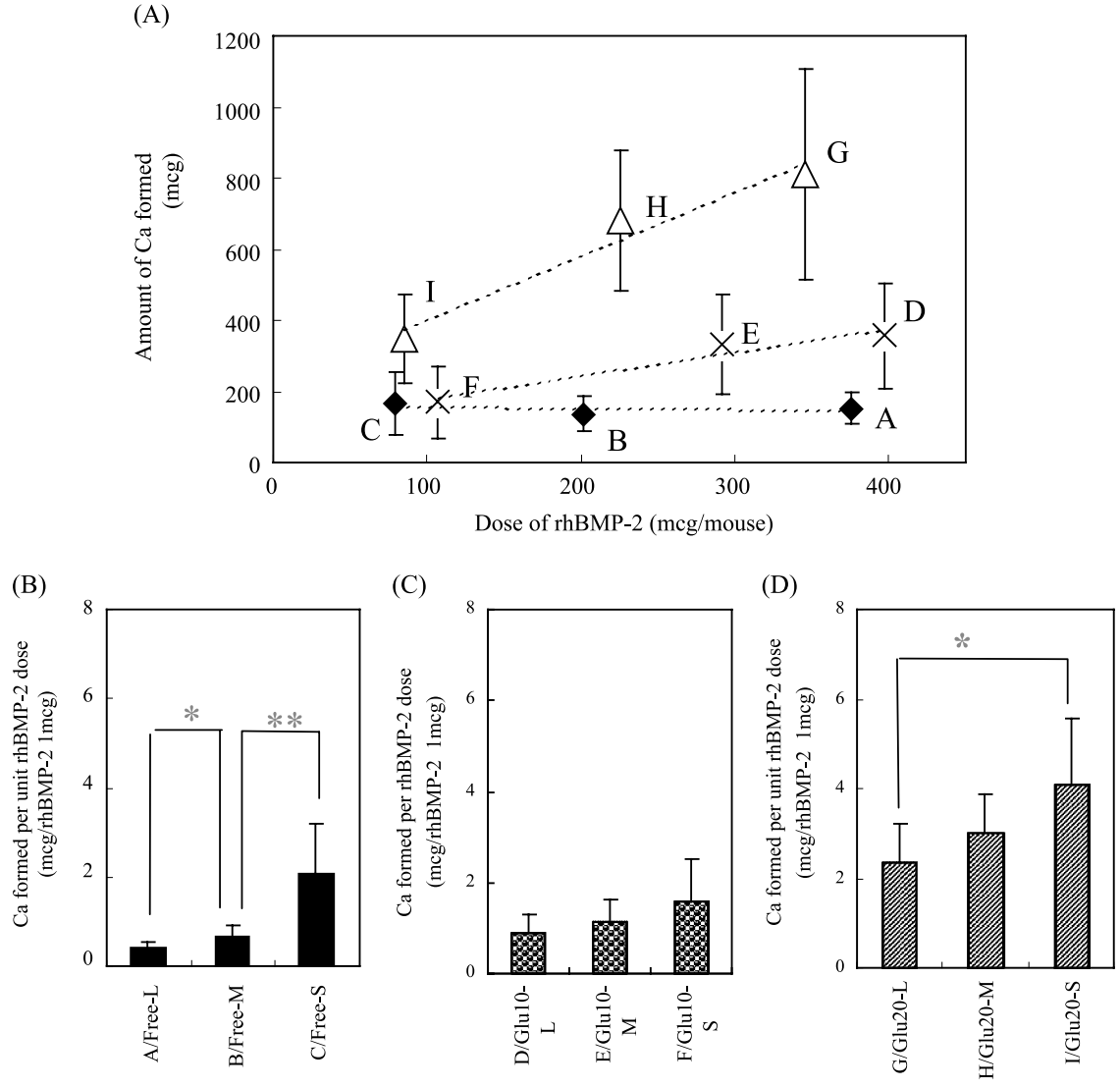

Figure 3. Amounts of calcium formed 3 weeks after subcutaneous implantation of rhBMP-2 minipellets containing 5% rhBMP-2. (A) Relationship between dose of rhBMP-2 per mouse (amount of rhBMP-2 contained in the MP implanted) and amount of calcium formed. (B–D) Amount of calcium formed per unit rhBMP-2 dose in each sample ([amount of Ca formed]/[dose of rhBMP-2] of each point in Fig. 3A): (B) sample without additives, (C) sample with 10% glutamic acid and 20% alanine, (D) sample with 20% glutamic acid and 20% alanine. The mean ± SD is shown at each point (n = 8). The significant difference was checked by Student's *t*-test using statistical software. *$p<0.05$, **$p<0.01$. (*View this art in color at www.dekker.com.*)

In a previous study using MPs with varying % loading, increase in initial rhBMP-2 release rate and decrease in dose level resulted in more efficient calcium formation per unit rhBMP-2 dose.[14] Briefly, calcium formation per unit rhBMP-2 dose after 3 weeks implantation (dose of 1.5 mg rhBMP-2/pellet/mouse) of the first release type was greater than that of the slow release type, 0.70 (μg/rhBMP-2 1μg) for first type and 0.14 (μg/rhBMP-2 1μg) for slow type. In comparison among the samples of the first release type, the calcium formation per unit rhBMP-2 with low dose was greater than that of high dose, 57 (μg/rhBMP-2 1μg) for 0.005 mg dose of rhBMP-2 per mouse and 0.7 (μg/rhBMP-2 1μg) for 1.5 mg dose of rhBMP-2. In the present study using samples of the same length, the rhBMP-2 dose level decreased as the sample diameter decreased. In samples with Glu, a decrease in diameter resulted in a higher release rate and a larger amount of calcium formed in ectopic bone per unit rhBMP-2 dose. On the other hand, the efficiency of calcium formation was relatively low in samples without additives, which featured very low release rates. These results are identical with those for formulations with varying % loading,[14] and indicate that high initial rhBMP-2 release after implantation is important for ectopic bone formation in areas around the implanted site stimulated by controlled release of rhBMP-2 from the carrier.

When an MP is to be used for treatment of fracture, etc., it can be administered noninvasively by means of injection. An MP with a smaller diameter is easier to inject. It was also shown in the present study that MPs with smaller diameters lead to more efficient bone formation. When designing formulations for clinical use, it is essential to take into account the results of evaluation in animal models with disorders. The present study revealed basic characteristics, which can be referred to when designing this kind of formulation for clinical use.

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