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Controlled release of rhBMP-2 from collagen minipellet and the relationship between release profile and ectopic bone formation

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Abstract

The purpose of this study was to examine the effects of various additives on the profiles of rhBMP-2 release from minipellet, which is a sustained release formulation for protein drugs using collagen as a carrier, and to examine the influence of varying release profiles on ectopic bone formation. When the amount of rhBMP-2 remaining in the preparation after subcutaneous implantation to mice was examined, it was found that the addition of sucrose, glucose, PEG4000, alanine (Ala) or acacia in a concentration of 20% (w/w) to the minipellet with 5% (w/w) of rhBMP-2 did not accelerate the drug release in a noticeable manner, while the addition of sodium chondroitin sulfate, glutamic acid (Glu) or citric acid accelerated the release of rhBMP-2 markedly. When two types of minipellets (a fast release type added with 20% Glu and 20% Ala and a slow release type without additives) containing varying amounts of rhBMP-2 were implanted subcutaneously to mice, the soft X-ray observation, histological examination and measurement of calcium formation 3 weeks after implantation revealed extensive ectopic bone formation in mice implanted with the fast release type preparation. Ectopic bone formation was dose-dependent. The result of this study exhibited that the effects of controlled release formulation of rhBMP-2 on bone formation vary depending on their release profiles, and suggested that combination of initial burst and sustained release was effective for bone formation. It was also shown that minipellet is useful as a controlled release formulation which can release rhBMP-2 to areas around the implanted site with various release profiles.

Keywords: Bone morphogenetic protein (BMP); Collagen; Minipellet; Controlled release; Ectopic bone formation

1. Introduction

We have been studying a delivery system called "minipellet" (MP) (Fujioka et al., 1995a), which is a matrix type cylindrical solid preparation for injection made of collagen (Rubin et al., 1969; Miyata et al., 1992). MP has been shown to be useful for controlled release of various protein drugs such as interferon (Fujioka et al., 1995b), nerve growth factor (Takemoto et al., 1998), interleukin-2 (Fujiwara et al., 1991), fibroblast growth factor (Inui et al., 1998) and granulocyte colony-stimulating factor (Maeda et al., 2003).

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)-β superfamily and are known well as factors forming bone and cartilage (Wozney et al., 1988). It has been shown that BMPs induce the differentiation of undifferentiated mesenchymal cells into osteoblasts and chondrocytes (Yamaguchi et al., 2000). It has been shown experimentally that recombinant human BMP-2 (rhBMP-2) can induce ectopic bone formation at various sites in many animal species (Aspenberg and Turek, 1996;

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Uludag et al., 1999; Okubo et al., 2000). At present, rhBMP-2 is clinically used for the treatment of fractures and spinal fusion procedures (Riedel and Valentin-Opran, 1999; Boden et al., 2000).

Since BMPs induce bone formation through their local action, it is necessary to deliver BMPs directly with a carrier to the site where bone formation is needed. Various materials, including inorganic materials (Boden et al., 1999; Ijiri et al., 1997), synthetic polymers (Winn et al., 1999; Saito et al., 1999; Whang et al., 1998; Renier and Kohn, 1997), natural materials (Gerhart et al., 1993; Boyne et al., 1997), and its combination (Yokota, 2003) have been tested as promising BMP carriers. Many of the delivery systems designed for BMPs and other bone inducing factors were primarily designed to replace the carrier with bone, i.e., they used the carrier as a scaffold for bone formation (Uludag et al., 1999; Whang et al., 1998; Hedberg et al., 2002; Lee et al., 2002). On the other hand, as for bone formation at areas around the implantation site, not as a scaffold, there is few report, and few published studies is concerning the effects of the release of BMPs from the carrier on bone formation in surrounding tissue. However, it was reported that local administration of solid depot rods, which used hyaluronan as a carrier, induced new bone formation effectively (Kim et al., 2002), and the usefulness of promoting the diffusion of rhBMP-2 to areas around the implanted site has also been suggested. Therefore, MP, a cylindrical solid preparation, is expected to be a useful carrier as a local delivery system for rhBMP-2.

MP can contain drugs for a wide range of concentration from minimal level to 50% (w/w) or higher. For this reason, MP allows various local concentration of rhBMP-2 including high concentration by implantation of the MP with compact size. Since MP is a cylindrical solid preparation with a diameter of 0.9 mm or less, it can be implanted by an ordinary injection technique. This means that MP can be administered noninvasively, without requiring incision of the affected part. On the other hand, since rhBMP-2 can induce ectopic bone formation in various tissues, it involves the risk of inducing excessive bone formation in unneeded sites (Takaoka et al., 1996) due to outflow of liquid preparation. Since MP is very hard and flexible, it does not involve the risk of leakage of its contents at sites other than the targeted site and thus allows reliable avoidance of ectopic bone formation associated with dosing. Furthermore, MP can be manufactured in a reliable manner, without using heat or organic solvents (Fujioka et al., 1995a). Therefore, MP was considered as an appropriate controlled release formulation of rhBMP-2 on bone formation in the areas around the implantation site.

The present study was undertaken to examine the potential of MP as a controlled release preparation designed for efficient induction of bone formation in areas around the implantation site. For formulations like MP which are expected to induce bone formation in areas around the site of administration, it is essential to examine the relationship between drug release profiles and effects on bone formation. Therefore, in the present study, we first explored methods of controlling the release of rhBMP-2 from MP by using various additives. In particular, since rhBMP-2 is hardly soluble under neutral conditions, it was necessary to establish a new method of release control for rhBMP-2, which was different from conventional methods. Then, using MP with varying release profiles and varying amounts of rhBMP-2, we evaluated the effects of different rhBMP-2 release profiles on bone formation in areas around the implantation site using subcutaneous ectopic bone formation model in mice as an indicator of efficacy.

2. Materials and methods

2.1. 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. rhBMP-2 was a product of Wyeth (Cambridge, MA, USA) in the form of an aqueous solution (pH 3 water). Normal male mice (BALB/c) 5 weeks of age were purchased from Charles River Japan (Kanagawa, Japan), and they were used after 1 week of quarantine. During the experiment, animals were allowed free access to feed and water.

2.2. Preparation of minipellets

Tables 1 and 2 show the samples prepared. They were prepared as follows. Aqueous solu-

Table 1
Composition of rhBMP-2 minipellets

Sample #	rhBMP-2 (%, w/w)	Additive (%, w/w)	Diameter (mm)	Length (mm)
Add-free	5	–	0.78	5
SUC-5	5	Sucrose (5)	0.72	5
Glc-5	5	Glucose (5)	0.73	5
PEG-5	5	PEG (MW = 4000) (5)	0.77	5
Ala-5	5	Alanine (5)	0.76	5
Aca-5	5	Acacia (5)	0.79	5
CS-5	5	Chondoroitin sulfate Na (5)	0.74	5
Glu-5	5	Glutamic acid (5)	0.78	5
CA-5	5	Citric acid (5)	0.72	5
SUC-20	5	Sucrose (20)	0.72	5
Glc-20	5	Glucose (20)	0.70	5
PEG-20	5	PEG (MW = 4000) (20)	0.93	5
Ala-20	5	Alanine (20)	0.88	5
Aca-20	5	Acacia (20)	0.81	5
CS-20	5	Chondoroitin sulfate Na (20)	0.86	5
Glu-20	5	Glutamic acid (20)	0.91	5
CA-20	5	Citric acid (20)	0.83	5
Glu-10	5	Glu (10)	0.84	5
Glu-10/Ala-10	5	Glu (10), Ala (10)	0.80	5
Glu-10/Glc-10	5	Glu (10), Glc (10)	0.77	5
Glu-20/Ala-10	5	Glu (20), Ala (10)	0.87	5
Glu-20/Glc-10	5	Glu (20), Glc (10)	0.85	5

tion of rhBMP-2 (5.5 mg/ml (pH 3 water); diluted with pH 3 water if needed), 2% (w/w) aqueous solution of atelocollagen, distilled water, and according to the composition, aqueous solution of additive (5 mg/ml) were combined and agitated well. The mixture was freeze-dried and combined with distilled water, to yield collagen gel containing rhBMP-2. The gel was filled in a syringe, and was then extruded through a nozzle (1.6 mm in inner diameter). The cylindrical product was dried and cut into appropriate lengths to yield MP containing rhBMP-2.

Table 2
Composition of rhBMP-2 minipellet

Sample #	rhBMP-2 (%, w/w)	Additive (%, w/w)	Diameter (mm)	Length (mm)
BMP-20(−)	20	–	0.87	10
BMP-20(+)	20	Glutamic acid (20), Alanine (20)	0.85	10
BMP-5(−)	5	–	0.88	10
BMP-5(E10)	5	Glutamic acid (10), Alanine (20)	0.86	10
BMP-5(+)	5	Glutamic acid (20), Alanine (20)	0.89	10
BMW-0.5(−)	0.5	–	0.90	10
BMP-0.5(+)	0.5	Glutamic acid (20), Alanine (20)	1.03	10
BMP-0.05(−)	0.05	–	0.91	10
BMP-0.05(+)	0.05	Glutamic acid (20), Alanine (20)	1.06	10
BMP-0.005(−)	0.005	–	0.84	10
BMP-0.005(+)	0.005	Glutamic acid (20), Alanine (20)	0.86	10
BMP-0.0005(−)	0.0005	–	0.86	10
BMP-0.0005(+)	0.0005	Glutamic acid (20), Alanine (20)	0.93	10
BMP-0(−)	–	–	0.85	10
BMP-0(+)	–	Glutamic acid (20), Alanine (20)	0.85	10

2.3. Measuring rhBMP-2 contents

Each sample, 5 mm in length, was combined with 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 h at room temperature and left standing overnight at 5 °C. The obtained solution was subjected to reverse-phase high performance liquid chromatography (HPLC), as mentioned below, to determine the amount of rhBMP-2 contained in the MP. In case of the MP containing chondroitin sulfate (CS), the solution was treated with chondroitinase before subjected to HPLC. HPLC was performed using a C4 column (0.46 cm i.d. × 5 cm, mean particle size 5 μm; Vydac, CA, USA). Water containing trifluoroacetic acid (0.1%, w/v) served as mobile phase A, and a 90:10 mixture (v/v) of acetonitrile and water containing trifluoroacetic acid (0.1%, w/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 min and then increased linearly to 60% in 14 min. The column temperature was set at 30 °C. Detection was made by fluorescence (Ex: 287 nm, Em: 348 nm). For treatment with chondroitinase, 50 μl of MP solution, 30 μl of 0.2 M Tris–HCl–0.06 M sodium acetate (pH 8.0) and 10 μl of chondroitinase ABC protease free (1 unit per vial; Seikagaku Corporation, Tokyo, Japan) dissolved in 130 μl of 0.2 M Tris–HCl–0.06 M sodium acetate (pH 8.0) were added and the mixture was incubated at 37 °C for 1 h and then added with 10 μl of 1 M HCl.

2.4. In vitro release test

Samples containing 5 or 20% (w/w) of rhBMP-2, shown in Table 2, were immersed in 1 ml of 0.3 M phosphate buffer (pH 6.2) containing 5% (v/v) PEG400 [release test solution] and incubated at 37 °C. One, 4 and 7 days after the start of the test, the release test fluid was exchanged with new solution. The amount of rhBMP-2 released was measured by HPLC, and release profiles were determined ($n = 5$). The amount of rhBMP-2 remaining in the samples 7 days after the start of the test was measured by the above-mentioned method used for measuring rhBMP-2 contents.

2.5. Time course of the residual amount of rhBMP-2 in the formulation after subcutaneous implantation to mice

Samples listed in Table 1 were implanted using an indwelling needle to the right side of the subcutaneous tissue on the back of ether-anesthetized mice (avoiding the area around the spinal vertebrae) ($n = 2$). Seven days after implantation, the animals were sacrificed by excess ether, and the implanted formulation was harvested. The amount of rhBMP-2 remaining in the formulation was measured by the above-mentioned method used for measuring of rhBMP-2 contents. In the same way, samples containing 5 or 20% (w/w) of rhBMP-2, shown in Table 2, were implanted and the residual rhBMP-2 amount was measured 3 and 7 days after implantation ($n = 5$).

2.6. Soft X-ray after subcutaneous implantation into mice

Samples containing 0, 0.005, 0.5 or 20% (w/w) of rhBMP-2, shown in Table 2, were implanted using an indwelling needle, to the right side of the subcutaneous tissue on the back of ether-anesthetized mice (avoiding the area around the spinal vertebrae) ($n = 4$). Three weeks after implantation, the animals were sacrificed with excess ether, and the implanted formulation was harvested together with surrounding tissue. It was fixed in phosphate-buffered saline (PBS) containing 10% (v/v) formalin and then subjected to soft X-ray.

2.7. Histological findings after subcutaneous implantation into mice

After taking soft X-ray, the samples were embedded in paraffin and made into histological sections. The sections were stained with hematoxylin and eosin (H&E) and observed under a light microscope. The stage of mature bone formation was rated on a three-point scale: + (standard), 2+ (about twice the mature bone formed areas compared to + cases) or 3+ (about twice the area compared to 2+ cases).

2.8. Calcium formation after subcutaneous implantation into mice

Samples, shown in Table 2 except for sample BMP-5(E10), were implanted subcutaneously into mice for

3 weeks, in the same method for the soft X-ray study ($n = 4$). The harvested samples with surrounding bony tissue were immersed in 5 ml of 0.6 M HCl and left standing at room temperature for 2 days. The concentration of calcium in this HCl solution was measured with a calcium quantification kit (Calcium C-test Wako, Wako Pure Chemical Industries, Osaka, Japan) to yield the amount of calcium formed. In addition, mice were subcutaneously injected with 100 μl of 60 μg/ml rhBMP-2 solution (6 μg dose group), 100 μl of 500 μg/ml rhBMP-2 solution (50 μg dose group) and 100 μl of 5 mg/ml rhBMP-2 solution (500 μg dose group) ($n = 4$) to compare the effect on calcification with rhBMP-2 minipellets. The amount of calcium formed in this group of mice was measured using the above-mentioned method.

3. Results

3.1. Effects of additives on rhBMP-2 release

For the samples listed in Table 1, the effects of additives on the release of rhBMP-2 from MP were evaluated, using the amount of rhBMP-2 remaining in the sample after subcutaneous implantation into mice for 7 days as an indicator of release profile. Fig. 1a shows the results for samples containing 5% (w/w) rhBMP-2 and 5% (w/w) additive. Fig. 1b shows the results for samples containing 5% BMP (w/w) and 20% (w/w) additive. For the samples containing 5% (w/w) additive, the amount of rhBMP-2 remaining in the formulation 7 days after implantation was 70% or more of the initial concentration and did not differ markedly depending on the type of additive used. For the samples containing 20% (w/w) additive, the amount of rhBMP-2 remaining 7 days after implantation differed greatly depending on the type of additive, recording 30% or less of the initial concentration when the additive was sodium CS, glutamic acid (Glu) or citric acid (CA) and 80% or more when the other additives were used.

Fig. 1c shows the results for samples which contained Glu (which accelerated the release of rhBMP-2 in the above-mentioned experiment) with or without Ala or glucose (Glc) (which did not accelerate the release of rhBMP-2 in the above-mentioned experiment). The release profiles of Glu-added samples were not altered by the addition of Ala or Glc.

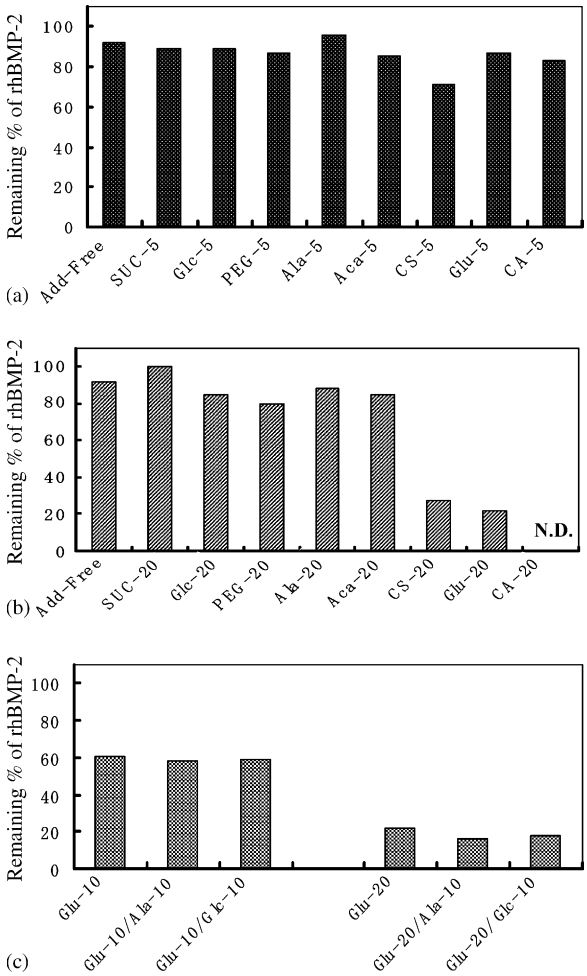

Fig. 1. Amounts of rhBMP-2 remaining in rhBMP-2 minipellets containing various additives 7 days after subcutaneous implantation to mice. (a) Sample with 5% additive, (b) sample with 20% additive, (c) sample with glutamic acid. The mean is shown at each point ($n = 2$). N.D.: not detected.

3.2. In vitro release profile of rhBMP-2

For samples containing 5 or 20% (w/w) of rhBMP-2 (three samples containing Glu which accelerates the release of rhBMP-2 and two additive-free samples), shown in Table 2, the release profiles and the remaining drug in the period of 7 days were examined in vitro. The results are shown in Fig. 2. For the MPs contain-

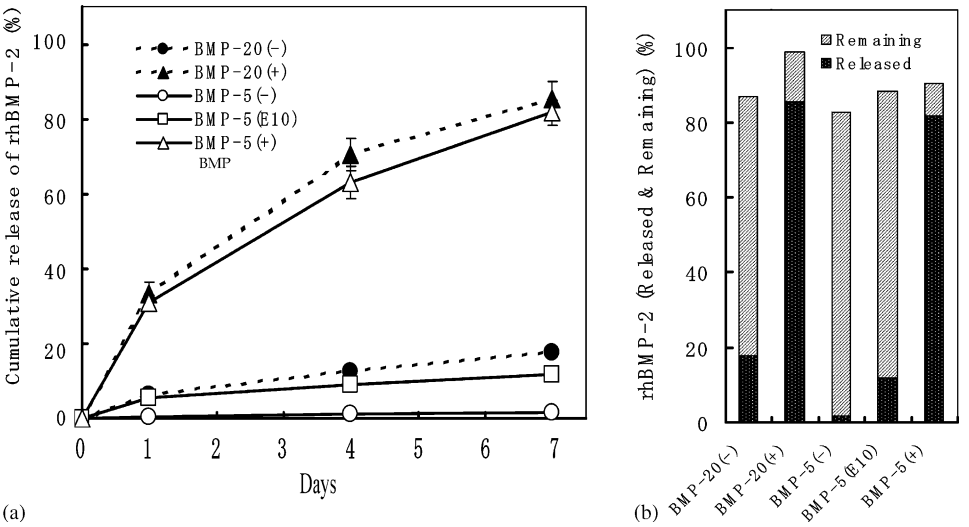

Fig. 2. In vitro release profiles of rhBMP-2 minipellet (a), and cumulative release and residual drug (b). The mean ± S.D. is shown at each point ($n = 5$).

ing 5% (w/w) rhBMP-2, the cumulative drug release during the first 7 days was 1.6 ± 0.8%, 11.9 ± 1.0% and 81.7 ± 3.3% when the percentage of Glu added was 0% (w/w), 10% (w/w) and 20% (w/w), respectively. For the MPs containing 20% (w/w) rhBMP-2, the cumulative drug release during the first 7 days was 17.7 ± 1.4% and 85.5 ± 4.5% when the percentage of Glu added was 0% (w/w) and 20% (w/w), respectively (Fig. 2a). Thus, the release of rhBMP-2 became faster as the amount of Glu added increased. The total of the cumulative release drug and the remaining drug in the period of 7 days was 83% or higher (Fig. 2b).

3.3. Time course of the amount of rhBMP-2 remaining in the formulation after subcutaneous implantation to mice

For the MPs containing 5 or 20% (w/w) of rhBMP-2, the time course of the amount of rhBMP-2 remaining in the formulation after subcutaneous implantation to mice was examined. The results are shown in Fig. 3. For the MPs containing 5% (w/w) of rhBMP-2, the percentage of rhBMP-2 remaining at 7 days after implantation was 77.1 ± 11.7%, 36.9 ± 6.5% and 9.2 ± 3.3% when the percentage of Glu added was 0% (w/w), 10% (w/w), 20% (w/w), respectively. For the MPs containing 20% (w/w) of rhBMP-2, that value was 82.5 ± 3.5% and 0% when the percentage of Glu added was 0% (w/w) and 20% (w/w), respectively. Thus, the amount of rhBMP-2 remaining in the formulation tended to decrease as the amount of Glu added increased. When samples containing the same additive were compared, the release profiles did not differ markedly depending on the amount of rhBMP-2 initially contained.

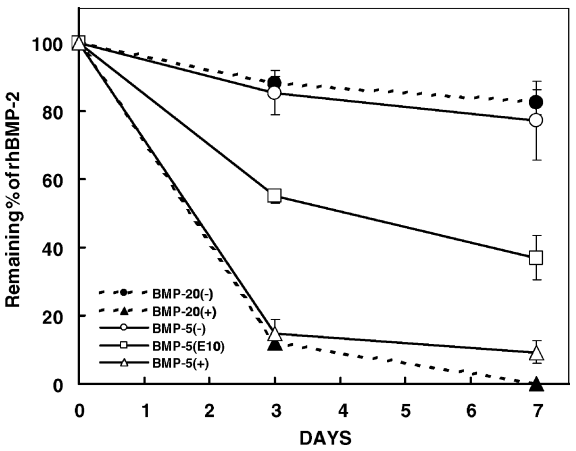

Fig. 3. rhBMP-2 retention profiles after subcutaneous implantation of rhBMP-2 minipellet into mice. The mean ± S.D. is shown at each point ($n = 5$).

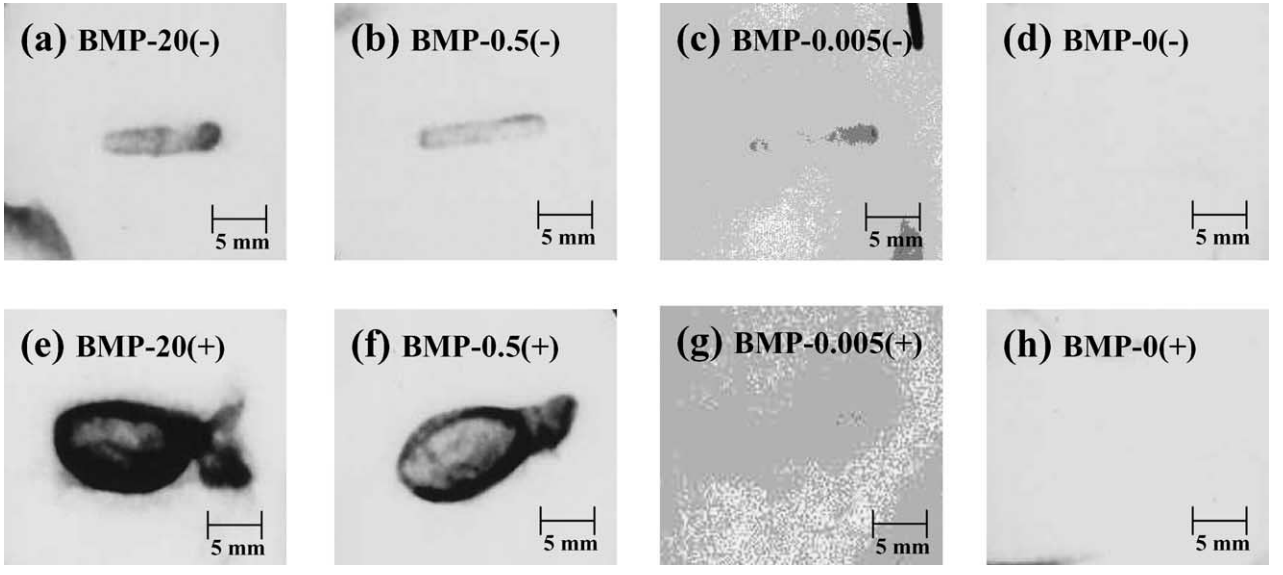

Fig. 4. Soft X-ray of the area around the implanted site 3 weeks after subcutaneous administration of rhBMP-2 minipellet to mice. (a) 20% rhBMP-2, slow release type, (b) 0.5% rhBMP-2, slow release type, (c) 0.005% rhBMP-2, slow release type, (d) without rhBMP-2, slow release type, (e) 20% rhBMP-2, fast release type, (f) 0.5% rhBMP-2, fast release type, (g) 0.005% rhBMP-2, fast release type, (h) without rhBMP-2, fast release type.

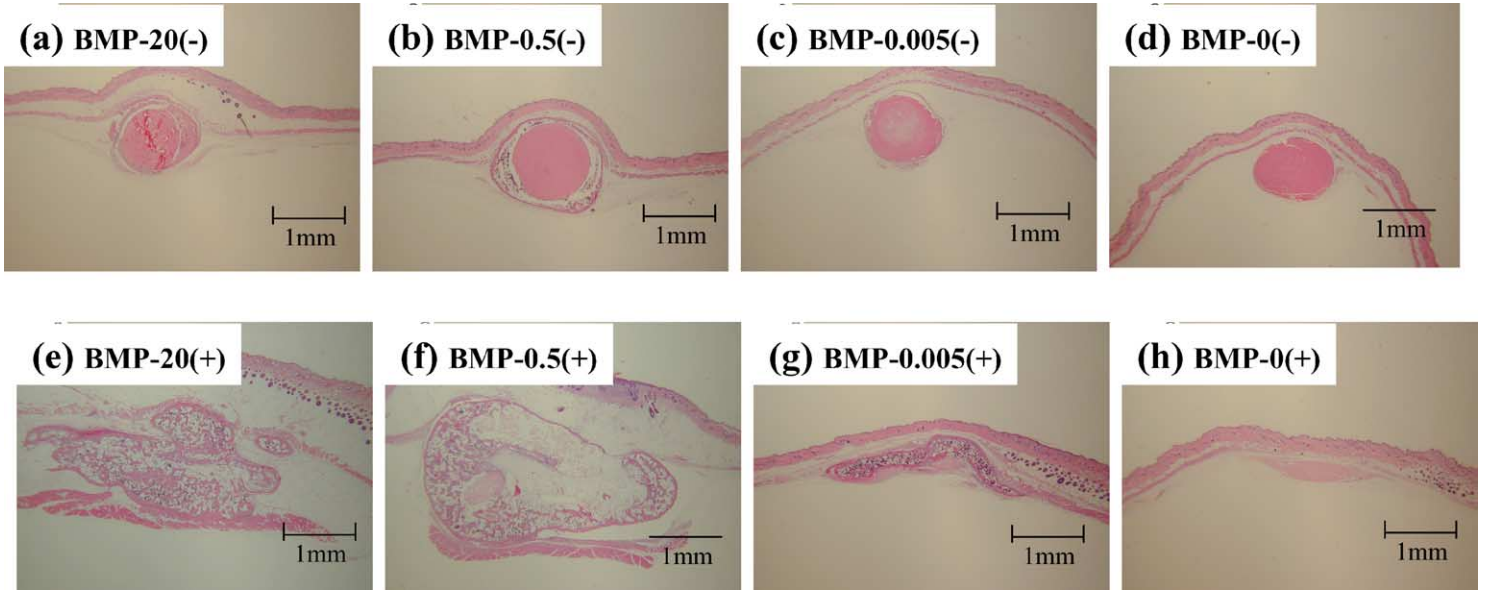

Fig. 5. Histological examination of H&E stained specimens 3 weeks after subcutaneous implantation of rhBMP-2 minipellet to mice: cross-sectional view of a minipellet sample. (a) 20% rhBMP-2, slow release type, (b) 0.5% rhBMP-2, slow release type, (c) 0.005% rhBMP-2, slow release type, (d) without rhBMP-2, slow release type, (e) 20% rhBMP-2, fast release type, (f) 0.5% rhBMP-2, fast release type, (g) 0.005% rhBMP-2, fast release type, (h) without rhBMP-2, fast release type.

3.4. Soft X-ray after subcutaneous implantation into mice

For the slow release type MPs (additive-free with 0.005, 0.5 or 20% (w/w) of rhBMP-2), the fast release type MPs (Glu and Ala-added with 0.005, 0.5 or 20% (w/w) of rhBMP-2) and each type of rhBMP-2 free placebo MPs, shown in Table 2, the capability of inducing ectopic bone formation after subcutaneous implantation into mice was examined by soft X-ray. The results are shown in Fig. 4. Placebo samples did not induce new bone formation, irrespective of the presence or absence of additives, when evaluated on soft X-rays. Each MP containing rhBMP-2 induced new bone formation. Slow release type samples induced bone formation in a shape resembling the shape of the formulation. Fast release type samples induced extensive bone formation around the implanted site. The intensity of bone formation, as evaluated on soft X-ray, was dependent on the amount of rhBMP-2 contained in the formulation and was higher with the fast release type than with the slow release type.

3.5. Histological examination after subcutaneous implantation to mice

The tissue specimens were made into H&E-stained histological specimens after soft X-ray observation. The histological findings are shown in Fig. 5. The results of scoring of mature bone formation are shown in Fig. 6. rhBMP-2 minipellets induced the formation of bone tissue. In the same manner as soft X-ray, the formation of bone tissue was dependent on the amount of rhBMP-2 contained in the formulation. Bone formation was more abundant with the fast release type than with the slow release type when the rhBMP-2 content was the same.

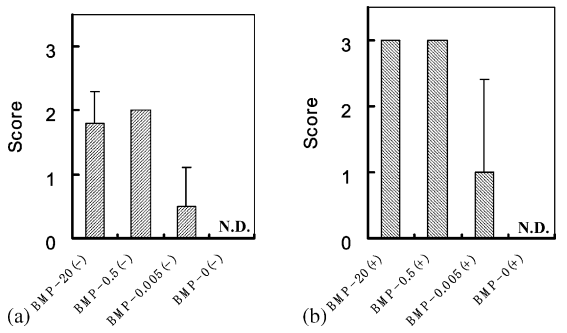

Fig. 6. Scores of mature bone formation histologically evaluated using H&E stained specimens 3 weeks after subcutaneous implantation of rhBMP-2 minipellet into mice. The mean is shown at each point ($n = 4$). N.D.: not detected.

3.6. Calcium formation after subcutaneous implantation to mice

Fig. 7 shows the amount of total calcium contained in the recollected formulations and formed tissue around the implanted site 3 weeks after subcutaneous implantation of MPs, shown in Table 2 except for BMP-5(E10), and rhBMP-2 solution. Calcification was seen for samples containing 0.005% (w/w) or more of rhBMP-2. The amount of calcium was dependent on the amount of rhBMP-2 contained for both the fast release type and the slow release type. When the amount of rhBMP-2 contained was the same, calcium formed was higher with the fast release type than with the slow release type. Administration of rhBMP-2 solution (6 μg dose group) did not lead to bone formation. For BMP-0.05(−) and (+), both of which were rhBMP-2 minipellets (dose of rhBMP-2: 5 μg/pellet/head), the amount of calcium formed was 0.106 ± 0.046 mg and 0.275 ± 0.097 mg, respectively. The amount of calcium formed was 0.090 ± 0.045 mg after administration of rhBMP-2 solution (50 μg dose group). However, for BMP-0.5(−) and (+) which were rhBMP-2 minipellets (23 and 32 μg/pellet/head, respectively, similar dose to the rhBMP-2 solution), the amount of calcium formed was greater (0.117 ± 0.020 mg and 0.488 ± 0.128 mg, respectively). In the same manner, the amount of calcium formed was 0.129 ± 0.096 mg after administration of rhBMP-2 solution (500 μg dose group). However, for BMP-5(−) and (+) which were rhBMP-2 minipellets (384 and 346 μg/pellet/head, respectively, similar dose to the rhBMP-2 solution), the amount of calcium formed was greater (0.166 ± 0.042 mg and 0.786 ± 0.265 mg, respectively). For rhBMP-2 minipellets, the logarithm of the dose had a linear relationship to the amount of calcium formed as shown in Fig. 7d. The amount of calcium formed per unit rhBMP-2 dose increased as the content of rhBMP-2 became smaller.

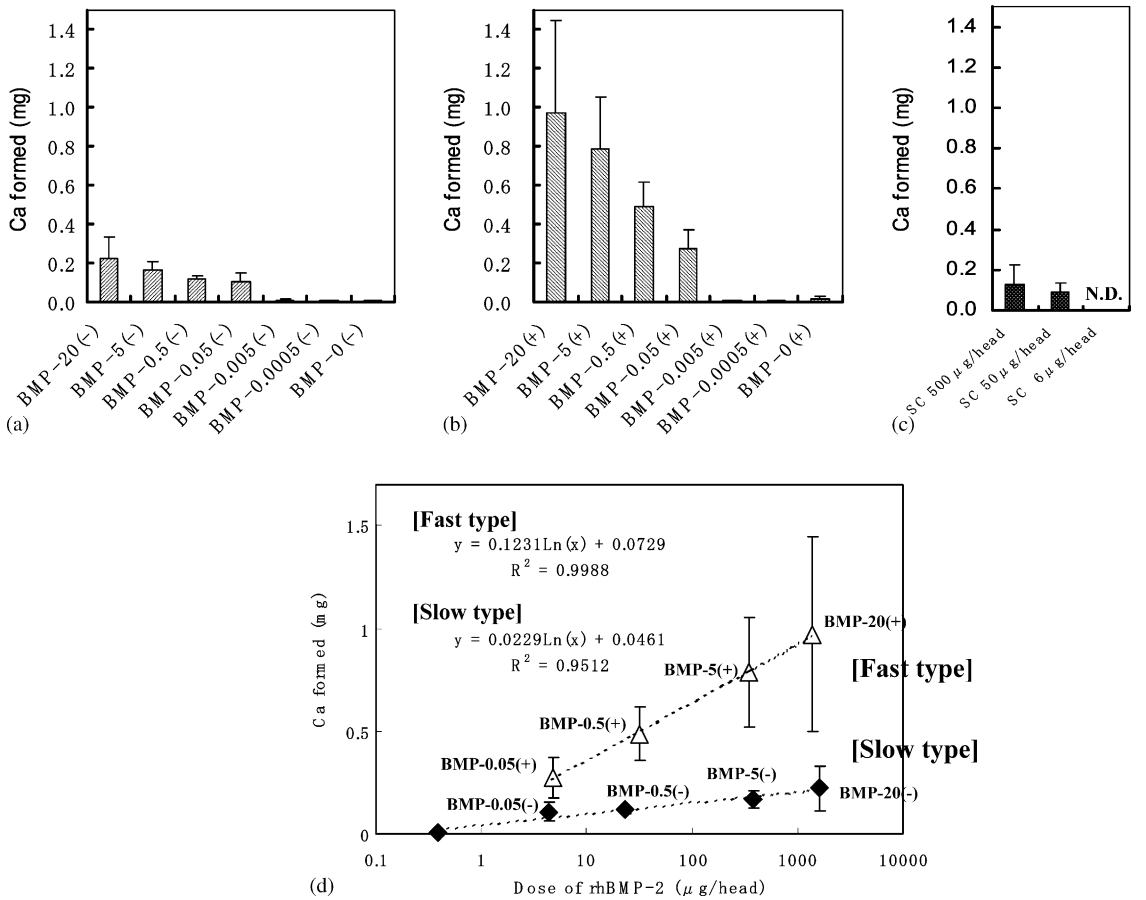

Fig. 7. Amounts of calcium formed 3 weeks after subcutaneous administration of rhBMP-2 minipellet or solution. (a) Slow release type of rhBMP-2 minipellet, (b) fast release type of rhBMP-2 minipellet, (c) rhBMP-2 solution, (d) relationship between rhBMP-2 content in minipellet and the amount of calcium formed. The mean ± S.D. is shown at each point ($n = 4$). N.D.: not detected.

4. Discussion

In case of MP containing such proteins as interferon (IFN) or rhG-CSF (Fujioka et al., 1995b; Maeda et al., 1999, 2003), the release of the protein was accelerated by additives. In the present study of MP containing rhBMP-2, which was implanted subcutaneously into mice (Fig. 1), 90% or more of the rhBMP-2 remained in the additive-free MP when measured 7 days after implantation, and the release of the protein was little accelerated by the addition of sugar (sucrose, glucose), PEG4000 (a solid amphiphilic surfactant), alanine (neutral amino acid) or acacia in a concentration of 20% to MP. On the other hand, the addition of sodium CS, Glu or CA in a percentage of 20% each resulted in marked acceleration of drug release (Fig. 1b). As stated above, we found that the use of CS, Glu and CA as additives makes it possible to control the release of rhBMP-2 from MP, although this control is not possible by the methods previously used for MPs with IFN or rhG-CSF.

The release of IFN from MP was accelerated depending on the amount of human serum albumin (HAS) added to the MP (Maeda et al., 1999). This may be attributable to the following factors. First, since IFN is highly soluble in water, IFN is likely to be dissolved in water which invades the formulation after implantation. Second, the addition of HSA reduces the density of collagen, and HSA (a water-soluble additive) is dissolved after implantation. As a result,

the diffusivity of IFN across collagen fibers increased depending on the amount of HSA added. On the other hand, the release of rhBMP-2 was not accelerated by additives, excluding some exceptional cases (Fig. 1a and b). The reason for the difference between IFN and rhBMP-2 is that rhBMP-2 is hardly soluble even when water has invaded the MP because of the very low solubility of rh BMP-2 under neutral conditions. This means that even when some additive is added to MP with rhBMP-2, in order to widen the pathway of drug diffusion by means of dissolution of the additive during the drug release process in the same way as MP with IFN as mentioned-above, rhBMP-2 itself remained undissolved and does not diffuse. However, the addition of CS, Glu or CA in a percentage of 20% resulted in marked stimulation of rhBMP-2 release. As stated later, this stimulation seems to result from enhanced disintegration of the formulation and elevated solubility of rhBMP-2 after administration, due to the effects of these additives. When the shape of the formulation recollected 7 days after implantation into mice was examined, the original shape had been retained for rhBMP-2 minipellets which showed no acceleration of drug release, while the original shape had been lost for the rhBMP-2 minipellets with CS, Glu, or CA, which showed accelerated drug release. Acceleration of drug release seems to be attributable to dispersion of matrix with disintegration of the formulation and increase in surface area, which results in the dissolution of rhBMP-2. Moreover, when Glu or CA was added, the formulation was temporarily acidic for an initial period. Therefore, the solubility of rhBMP-2 was probably elevated by the addition of Glu or CA, leading to accelerated release of rhBMP-2 since rhBMP-2 is more soluble under acidic conditions. In this respect, it has been reported that in case of MP containing IFN, the formulation is not disintegrated after administration (Aisaka et al., 1996), but IFN is released satisfactorily in vivo since IFN is highly soluble and diffusible.

The disintegration in vivo of above-mentioned MP, containing additives such as CS, Glu and CA, is explained by the following mechanism. Since MP is composed of densely arranged collagen fibers (Maeda et al., 1996, 1999), the collagen fibers keep their high density after administration, thus contributing to control of protein release. On the other hand, the addition of CS to MP results in loss of the dense arrangement of collagen fibers (Maeda et al., 2003). For this reason, MP containing CS swells more greatly during the release process, making disintegration of MP more easily. Since MP with Glu or CA becomes acidic after administration, collagen fibers can not keep tight arrangement and the formulation becomes to soften, thus making it more likely for MP to undergo disintegration in vivo.

The addition of Glu was found to allow control of drug release dependent on its amount added. Even when Glu was added together with Ala or glucose which had not accelerated drug release when added independently, the acceleration of drug release was dependent on the amount of Glu alone and was not affected markedly by Ala or glucose (Fig. 1c). When Ala or glucose was added to MP independently, the disintegration of the formulation after implantation was not observed, and the release of rhBMP-2 from MP was not affected by such additives. This explains why rhBMP-2 release from MP with Glu and Ala or glucose did not differ from that from MP with Glu alone. On the other hand, since the addition of Ala or glucose together with Glu made the shape of the formulation good and made the surface of the formulation smoother compared to the addition of Glu alone (data not shown), this combination of additives is useful for MP, which is a dosage form to be injected in almost the same manner as liquid injection.

In the past, only protein drugs with relatively high solubility were applied to MP, and the release of these drugs could be controlled relatively easily by using various additives. However, in case of rhBMP-2 used in the present study, it was not possible to control the drug release simply by using the conventional simple method involving additives. The present study demonstrated that for the purpose of controlling the release of low-solubility proteins, it is useful to improve the solubility of the proteins by altering the disintegrating potential of the formulation or to directly elevate the solubility of the proteins by the use of additives.

The capability to induce ectopic bone formation after subcutaneous implantation into mice was evaluated for the fast release type MP (with Glu) and the slow release type MP (without additive), by means of soft X-ray, histological examination and measurement of calcium formed. Bone formation as evaluated by soft X-ray (Fig. 4), histological examination (Figs. 5 and 6) and measurement of calcium formed (Fig. 7)

was seen for MPs with more than 0.005% of rhBMP-2 loading ratio. When multiple formulations containing the same amount of rhBMP-2 in this range were compared, bone formation evaluated by soft X-ray and histological examination was more extensive and intense for the fast release type (releasing almost all rhBMP-2 within 1 week) than for the slow release type (releasing about 20% of rhBMP-2 in 1 week), and the amount of calcium formed was greater for the fast release type. Moreover, the rhBMP-2 solution with the same dose of rhBMP-2 as those MPs induced less calcium formation compared to MPs.

Uludag et al. (1999) studied several carriers such as collagen sponge, and reported that the initial burst of rhBMP-2 from the implant and subsequent consistent release effectively induced bone formation. Also in the present study of MP, a similar tendency was noted. That is, the bone forming effect was higher with the fast release type than with the slow release type, indicating that high initial release leads to more bone formation. On the other hand, aqueous solution of rhBMP-2, which records the peak local concentration of rhBMP-2 soon after administration and is hardly retained locally thereafter, resulted in less bone formation compared to MP. This indicates that not only the initial level of rhBMP-2 but also the local retention of rhBMP-2 is important for achieving adequate bone formation.

When evaluated by soft X-ray (Fig. 4), the slow release type MP resulted in the formation of bone assuming the shape of the MP, while the fast release type MP, which is more likely to disintegration, resulted in more extensive bone formation. When the fast release type MP was implanted, the disintegration of the formulation accelerated the release of rhBMP-2 from the formulation as mentioned above, elevating the initial rhBMP-2 level around the implanted site. Furthermore, since rhBMP-2 can diffuse together with the carrier, rhBMP-2 seems to be maintained and retained on the carrier. These factors seem to explain why bone formation was more extensive and intense with the fast release type MP. Formulations possibly suitable for intraosseous administration (Kim et al., 2002) include some of liquid type formulations, in addition to MP. However, if liquid formulation is administered, outflow of the administered liquid from the local site is possible, resulting in the possibility for unfavorable ectopic bone formation (Takaoka et al., 1996) to take place. On the other hand, MP, the solid formulation, is retained locally within the bone, continuing to supply rhBMP-2 to surrounding bone tissue. This formulation is useful in increasing bone density and is hence regarded as a formulation suitable for the prevention of fracture. In the present evaluation of ectopic bone formation following subcutaneous administration, extensive ectopic bone formation was noted around the administered site. When administered intraosseously, this formulation is expected to elevate the bone density around the site of administration, instead of inducing ectopic bone formation observed in the subcutaneous model of this study.

When formulations containing varying amounts of rhBMP-2 were compared, the amount of calcium formed was dose-dependent for both the fast and slow release types (Fig. 7). The amount of calcium formed had a linear relationship to the logarithm of the amount of rhBMP-2 contained in MP. As the dose level became lower, the efficiency of bone formation per unit rhBMP-2 dose increased. Although no quantitative relationship between bone formation and dose level or local rhBMP-2 level can be established from this study alone, it will be possible to determine an optimum local rhBMP-2 concentration profile for bone formation if the relationship between release profiles from MP and bone formation is further analyzed.

5. Conclusion

Minipellets containing rhBMP-2, using collagen as a carrier, were prepared as a controlled release formulation of rhBMP-2. Their drug release profiles and capability of inducing bone formation were evaluated. The results of this study indicate that the use of Glu as an additive allows the rhBMP-2 release profiles to be changed. It was also shown that the bone forming effect varies depending on the release profiles. Since minipellets can contain high concentrations of rhBMP-2, they allow local administration of concentrated rhBMP-2. Furthermore, since the drug release from minipellets can be controlled by additives, it will be possible to change the rhBMP-2 concentration around the implanted site. Minipellets with these features will be useful in examining the relationship between drug release profiles and efficacy. Their ap-

plication to treatment of fractures and prophylactic drug therapy is expected.

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