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Leading research paper: Osteobiology

Slow and continuous application of human recombinant bone morphogenetic protein via biodegradable poly(lactide-co-glycolide) foamspheres

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Abstract. Bone morphogenetic proteins (BMPs) are multifunctional cytokines that were originally identified as molecules that induce bone and cartilage formation *in vivo*. In order to increase the efficacy of this potent protein for application in medicine, a carrier system is needed to retain the BMP at the preferred site. Here we present and characterize a slow-release carrier system for pure human recombinant (rh)BMP. The large porous microspheres, called 'foamspheres', are biodegradable, because they consist of poly(lactide-co-glycolide) acids and release loaded rhBMP slowly and continuously. *In vivo* studies in rodents revealed that rhBMP-loaded foamspheres increased the thickness of the calvarial bone of rats by 222%. When the same amount of rhBMP was applied via a gelatine-based hydrogel, the increase in bone height was only 66%. Thus, the carrier system for rhBMP is an important factor for the efficacy of BMPs.

Key words: bone morphogenetic protein; delivery; microspheres; release system; bone regeneration; bone augmentation; hydrogel.

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In 1988[22], the first cDNAs coding for BMPs were cloned. This marked the end-point of a long search for the bone-inducing principle started after URIST had published his key discovery in this field in 1965[20]. The primary goal of all these efforts was to use the bone-inducing principle in clinical conditions for fracture healing, especially for non-unions but also for augmentation of bone. The concept for this approach was proved with native BMP preparations from human bones[8–11] and bovine bones[16–18]. The results demonstrated the potential of BMP for orthopaedic and cranio-maxillofacial surgery applications. Despite these encouraging results, there is a time gap of more than 13 years between the molecular cloning of the first BMPs and their routine medical application points to some obstacles, which can be mainly attributed to the lack of a suitable BMP-delivery system[3–5,13].

Thirteen years ago the first rhBMP produced was applied with inactivated bone collagen allografts in rodents, and

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now after a period of testing diverse carrier systems[3–5,13], clinical trials still depend on bovine collagen as a carrier. The disadvantages of bovine collagen as a rhBMP carrier include its biological origin, which implies a chance for the transmission of diseases, the occurrence of possible immune reactions, difficulties in handling wetted collagen gels, and the lack of form stability.

Another aspect that is often overlooked is the release rate of rhBMPs from the carrier. In virtually all applications, rhBMP is dried or attached to the surface of the carrier[3–5,13]. Therefore upon implantation, the entire administered protein is in contact with bodily fluids, leading to a rapid release and degradation of the rhBMP. The conclusion that the release rate of rhBMP is an important factor for its efficacy, especially at ectopic sites[23], can be drawn from experiments where rhBMP incorporated into hydrogels exhibited a more potent ability for bone formation than injected rhBMP-2. Due to the nature of these hydrogels, 60–75% of the incorporated rhBMP is released during the first 24 minutes *in vitro*. BMP expression profiles during fracture healing[14] revealed that rhBMP-2 is expressed during a period of 1–2 weeks after the fracture occurred, therefore, we wanted to create a system by which rhBMP is released over days rather than minutes, to mimic the natural rhBMP release profile. With other proteins, the release over days was achieved by encapsulation into biodegradable microspheres[2]. Therefore, porous biodegradable microspheres, termed 'foamspheres', with entrapped rhBMP were developed. This combination was more efficient in increasing the thickness of the calvarial bone than rhBMP delivered via gelatine-based hydrogels.

Materials and methods

Expression, purification, and refolding of rhBMP-2 and rhBMP-4

A BMP-2 cDNA was isolated from a human placenta library, shown to be identical to the sequence deposited in the Genbank (M22489) and used as template to generate a mature BMP-2 expression construct. The cloning and expression of the protein was performed as reported earlier[21]. After expression the cells were stored overnight at −80°C. After thawing, the cells were resuspended in 100 mM Tris-HCl pH 8.3, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and lysed by three passages through a French pressure cell at 20 000 psi. After centrifugation, rhBMP-2 was present in the pellet, which was washed extensively and dissolved by acidification to pH 2.5 with glacial acetic acid. Insoluble material was removed by centrifugation at 15 000 × g for 30 min and the supernatant dialyzed against 25 mM Tris-HCl pH 8.0, 6 M urea (TU). Insoluble material formed during dialysis was removed by centrifugation (30 min 15 000 × g) and rhBMP-2 isolated by its affinity to heparin (5 ml HiTrap® Pharmacia Biotech, New Jersey, USA), followed by a gel filtration on a HiLoad® Superdex® 200 (Pharmacia Biotech, 1.6 cm × 60 cm; 124 ml) at a flow rate of 1 ml/min. rhBMP-2 monomers were dialyzed against 1% acetic acid and dried in a speed vac (Holbrook, NY, USA). The refolding and dimerization were performed according to[1]. rhBMP-4 was expressed in Chinese hamster ovary (CHO) cells using a protein-free cell culture medium (Cytos Biotechnology GmbH and Dr Messi, Cell Culture Technologies, Zürich, Switzerland). Large-scale production cells were grown in 1 l spinner flasks in FMX-8 medium (Dr Messi, Cell Culture Technologies, Zürich, Switzerland) at 37°C, 5% CO_2, 40 r.p.m., and rotation of 720°. After 5 days, the cells were removed by centrifugation, the supernatant applied to a HiTrap® heparin column, and rhBMP-4 eluted by 2 M NaCl in TU. Further purification steps involved gel filtration (HiLoad® Superdex® 75 column, Pharmacia Biotech, New Jersey, USA, 1.6 cm × 60 cm; 124 ml), ion-exchange chromatography (Mono Q, 1 ml, FPLC, Pharmacia), and the same reversed-phase column used for the purification of bacterially derived rhBMP.

Preparation of foamspheres and hydrogel carriers

Poly(DL-lactide-co-glycolide) (PLGA) with a molecular ratio of 75:25 (lactide to glycolide; molecular weight between 90–126 000 [Sigma, MO, USA]) was chosen. The encapsulation was performed by a water-in-oil-in-water double emulsion solvent evaporation technique. Quantities of 100 μg of rhBMP and 100 mg of gelatine were dissolved in 1 ml TU and added to 400 mg poly(lactide-co-glycolide) (PLGA) dissolved in 2 ml dichloromethane. Together they were cooled on ice, and emulsified by vigorous shaking, creating a water-in-oil emulsion due to the excess of the oily phase. This water-in-oil emulsion was dispersed in 1 l of a 1% aqueous gelatine solution stirred on ice at 75 r.p.m. to form a water-in-oil-in-water double emulsion. Following the dispersion step, the stirring velocity was elevated to 250 r.p.m. and the temperature increased to 37°C over a time period of 2 h to slowly evaporate the dichloromethane. After a 2 h incubation period at 37°C, the solidified foamspheres were collected by filtration, washed with 2 l of double distilled water, and 300 ml of 70% EtOH. The foamspheres were dried overnight under the sterile hood followed by lyophilization for 24 h. For foamspheres with a rhBMP content exceeding 5 μg/30 mg foamspheres, rhBMP was added to the foamspheres in 0.01% trifluoroacetic acid and freeze-dried overnight under sterile conditions. For morphological examination, the foamspheres were mounted on double-faced adhesive tape, spotted with platinum and viewed in a Hitachi S-700 scanning electron microscope. The hydrogel carriers were made of bovine gelatine cross-linked by glutaraldehyde as described by HONG et al.[6]. The wetted gel covered an area of 1 cm^2 and had a height of 4 mm. rhBMP dissolved in 20 μl 0.01% trifluoroacetic acid was applied to the freeze-dried sterile hydrogel and freeze-dried under sterile conditions.

Encapsulation yield and release rate of rhBMP

The overall encapsulation yield and maximal release rate of rhBMP was determined in PBS-6M urea (PU), because in its presence rhBMP is readily soluble over a broad concentration range at physiological pH. A quantity of 50 mg of lyophilized foamspheres were added to 1 ml of PU, and rotated on a turning wheel at 37°C. Then 500 μl probes of the buffer were taken after 4, 7, 10, and 14 days, and replaced by the same volume of fresh buffer.

The overall yield was determined by homogenizing 50 mg foamspheres in 1 ml PU with an omni-mixer (Waterbury, CT), and rotating the subsequent mixture for 3 weeks at 37°C. To determine rhBMP content in the supernatant, defined fractions of the probes were applied to a Bio-Dot® SF microfiltration apparatus (Biorad Hercules, CA, USA) with the slot-blot device for 48 samples installed. Dilutions from a

defined rhBMP stock were employed to each blot for calibration. After the application of all probes and dilutions, they were passed through the Bio-Dot® apparatus, which blotted the proteins onto a polyvinylidene fluoride membrane. An overnight incubation with anti BMP-specific antibodies was performed after blocking the membrane with 1% bovine serum albumin in phosphate buffered saline. The incubation of the blot with a secondary anti-rabbit antibody conjugated to alkaline phosphatase allowed the rhBMP to be visualized by a tetrazoleum method. An amount of rhBMP was quantified densitometrically by using the calibration dilutions of each blot as standard.

In vivo test

On top of the calvarial bone of rats, where the periost was removed mechanically, 30 mg of foamspheres or a hydrogel were placed. The animals were sacrificed after 33 days and the calvarial bone with the implant was excised. The calvarial bone including most of the implant was fixed and embedded in poly(methyl-methacrylate). Histological sections 4.5 μm thick were prepared, Goldner-Trichrome stained, and examined for bone height, cell type, morphology, and stromal details by using bright-field light microscopy. Histology also allowed us to attribute changes in calcium content to bone formation. For the quantification of bone formation in implanted foamspheres, a probe was taken just after excision of the calvarial bone from foamsphere material located at least 1 mm above the original calvarial bone. This was possible because after 33 days, the ossified or non-ossified foamspheres still extended 2–3 mm above the original calvarial bone and ensured that no contamination of the probe with original calvarial bone occurred. For quantification of bone formation by the measurement of the calcium content, the probe was weighted, and homogenized with an omni-mixer (Waterbury, CT, USA) in 1.5 ml of 3 mM $NaHCO_3$, 150 mM NaCl. After centrifugation (1000 × g for 15 min) the supernatant was used for alkaline phosphatase determination, and the pellet was resuspended three times with 1 ml of 10 mM Tris-HCl pH 7, and mixed at room temperature for 1 h. After the final wash, the pellets were extracted overnight with 1 ml 0.5 M HCl. Calcium in the extract was quantified by atomic absorption spectroscopy.

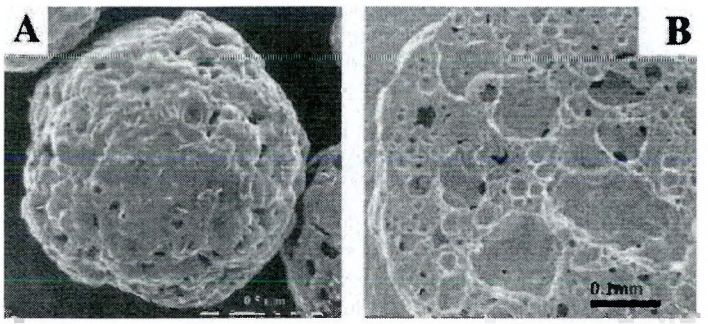


Fig. 1. Outer and inner morphology of foamspheres. To illustrate the porosity of the foamspheres, (A) intact or (B) cross-sectioned foamspheres were mounted, spotted with platinum and viewed in a scanning electron microscope. Size scales are indicated.

Statistical analysis

Student's *t*-test was implemented by a commercially available software package (SSPE, Chicago, Ill, USA). All values are represented as means ± standard deviation.

Results

A polypeptide representing the mature part of BMP-2 plus a N-terminal extension by a single methionine was expressed in *Escherichia coli*. The purity of rhBMP-2 judged on SDS-PAGE exceeded 99%. Ectopic bone formation induced in rats by our rhBMP preparations proved the bioactivity of rhBMP-2 and -4. Quantitative information on the specific biological activity was obtained by the determination of alkaline phosphatase activity induced in C3H10T1/2 cells. When these cells were subjected to 1 μg rhBMP-2/ml over 4 days, the alkaline phosphatase activity increased by a factor of 23–26 compared to untreated cells, which is similar to the data reported from CHO-derived glycosylated rhBMP-2[12].

Foamsphere production and characterization

Of the particles produced by the double-emulsion solvent evaporation technique, 90% had a diameter between 0.5–2.5 mm, which is significantly larger than the average microsphere. Therefore, we created the new term 'foamsphere', which best describes the porosity and morphology of our delivery system (Fig. 1). In initial experiments, the loading of foamspheres with CHO-derived rhBMP-4 was tested and the loading efficiency and release kinetic compared to bacterially derived rhBMP-2 and rhBMP-4. As there were no differences in the error of our measurements between both rhBMP preparations, we decided to use bacterially derived rhBMP-2 as model protein for the release studies, which we could produce in higher quantities. Averaged over 10 independent foamsphere productions, 10.8 ± 4.8% of the rhBMP-2 employed in the double-emulsion process was entrapped in the foamspheres. The release kinetic was determined from three foamsphere preparations (Fig. 2) with a loading efficiency for rhBMP-2 of 12.2 ± 2.3%. As shown in Fig. 2, all rhBMP-2 delivered via foamspheres was released over 10 days. The daily release of rhBMP was approximately linear.

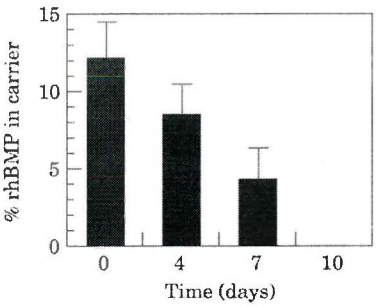


Fig. 2. In vitro release of rhBMP from foamspheres. Loading and release of rhBMP-2 averaged from three independent foamsphere preparations was determined over time. % rhBMP in carrier refers to the amount of rhBMP used initially for the loading of the foamspheres. All values represent the means ± standard deviations. 50 mg of lyophilized foamspheres were added to 1 ml of PBS-6M urea and the appearance of rhBMP in the supernatant was determined to calculate the amount of rhBMP retained in the foamspheres. After 10 days, the sum of released rhBMP and the amount of rhBMP initially entrapped in the foamspheres was identical within the error of the measurements.

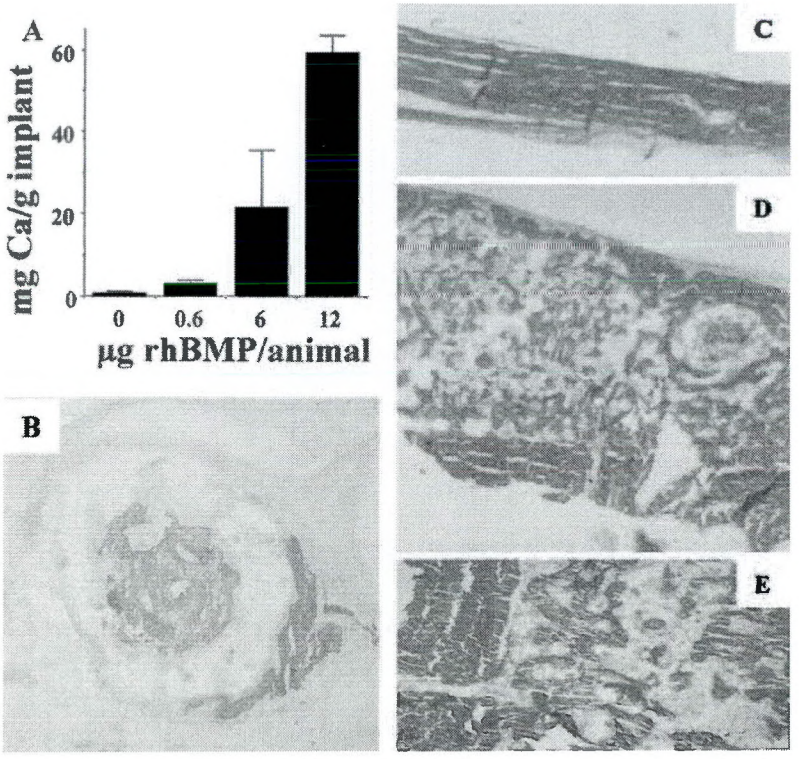


Fig. 3. Calcium content of rhBMP-2-loaded foamspheres and associated histological sections. An increase of the calcium content of foamspheres placed on top of the calvarial bone of rats correlated with the amount of applied rhBMP (A). The calcium content was determined 33 days after implantation from a probe taken from implanted (foamsphere) material located at least 1 mm above the original calvarial bone. Each value represents the mean ± standard deviation from 4–8 probes. Histological section of a foamsphere where 0.6 µg rhBMP was applied to the animal (B). On the right, a histological section of an untreated calvarial bone (no carrier, no rhBMP) (C) is compared to a calvarial bone of an animal treated with foamspheres containing 12 µg rhBMP (D), which is also shown in a 5 × magnification (E). All the implants were Goldner-Trichrome stained where calcified bone appears green and osteoid red.

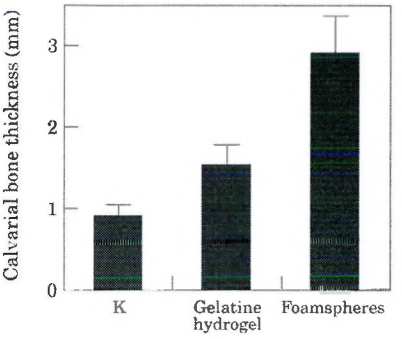


Fig. 4. Augmentation of the calvarial bone with rhBMP-2-loaded carriers. 12 µg rhBMP-2 was applied via foamspheres or via gelatine-based hydrogels. The animals were sacrificed 33 days after application and the thickness of the calvarial bone was determined from the Goldner-Trichrome-stained histosections. Results from untreated (control) bones, where no rhBMP and no carrier was applied, is shown along with gelatine- and foamspheres-treated calvarial bones with 12 µg rhBMP-2 applied. The foamspheres that had a higher retention of rhBMP-2 clearly led to greater augmentation of the calvarial bone. The columns represent the means ± standard deviation.

In vivo test of the rhBMP-loaded foamspheres

33 days after implantation, the foamspheres placed on top of the calvarial bone of rats covered an area between 1.0–1.5 cm^2. As shown in Fig. 3A, the mean calcium content of foamspheres determined from 4–8 animals applied with 0, 0.6 or 12 µg rhBMP-2 depended on the amount of rhBMP-2 applied ($P<0.004$). For eight animals tested with 6 µg rhBMP-2, the value for P equalled 0.029. Histology revealed that no ossification occurred in the absence of rhBMP-2. If the amount of applied rhBMP-2 was below 1 µg, bone formation was induced in and around foamspheres (Fig. 3B) without leading to a complete ossification of the implant. In contrast, when foamspheres were loaded with 12 µg rhBMP-2 (Fig. 3D), extra bone covered the entire foamsphere implant. Although the shapes of dissolved foamspheres were still recognizable, the entire carrier material had disappeared without leaving any fibrous tissue behind (Fig. 3E). In order to demonstrate the efficacy of our foamspheres as rhBMP-2 carrier for bone augmentation, we compared them to gelatine-based hydrogels. As shown in Fig. 4, 12 µg rhBMP-2 applied via hydrogels increased the maximal calvarial bone thickness from 0.9 ± 0.1 mm ($n=6$) without rhBMP to 1.5 ± 0.8 mm ($n=6$; $P=0.04$) with rhBMP. If the same amount of rhBMP was applied via foamspheres, the maximal thickness of the calvarial bone reached 2.9 ± 0.8 mm ($n=6$; $P=0.002$).

Discussion

Numerous delivery systems for rhBMPs have been tested, but none have demonstrated sustained delivery of rhBMP-2 for local application. In order to develop a system by which bioactive rhBMP is released over days rather seconds or minutes, we encapsulated pure rhBMPs into PLGA. The first trials with encapsulation of BMP in PLGA were performed with partially purified bovine BMP preparations[7]. However, the determination of the retention of impure, bovine BMP proved to be difficult because only the total protein released was measured. Since the BMP only represented a small fraction of the total protein, extrapolating the release data for BMP only from these numbers was not possible. Interestingly, the numbers for the release of crude bovine BMP and our results with pure rhBMP-2 were identical. This implies that BMP release is governed by the degradation of the encapsulation material, which was identical in both studies. In terms of loading efficiency, however, the results were very different. Only 5–15% of pure rhBMP but 91% of impure native BMP[7] was encapsulated in the foamspheres, which points to the possibility of modulating BMP loading efficiency by the addition of proteins, which are present in native BMP preparations, to pure rhBMP preparations.

rhBMP release kinetic from diverse hydroxyapatites and calcium phosphates was shown to be biphasic. $t_{1/2}$ of the

second phase of rhBMP release determined *in vivo* ranged from 2–17 days[19]. Gelatine based-hydrogels, which we used as reference material in the bone augmentation model, showed $t_{1/2}$ values determined *in vivo* of 15 days[23]. *In vitro* with no diffusion barrier in place, the apparent $t_{1/2}$ of gelatine-based hydrogels was below 25 minutes[23] and much shorter than the 5 days determined for the foamspheres in a similar setting. An additional benefit of the foamspheres is that the rhBMP release could be further modulated by the choice of the PLGA. Besides the extended *in vitro* $t_{1/2}$ for rhBMP release achieved by foamspheres, the quality of unreleased rhBMP was also different. rhBMP was added to the outside of all the other carriers[3–5,13]. Therefore, upon implantation, even the unreleased rhBMP is subject to bodily fluids and degradation as it is located on the surface of the diverse materials. In contrast, rhBMP delivered by foamspheres is encapsulated by a shell of PLGA that prevents rhBMP degradation and increases the chance that rhBMP released from foamspheres has retained its bioactivity. The efficiency of rhBMP delivery systems cannot be evaluated from *in vitro* or *in vivo* $t_{1/2}$ values exclusively. It also needs *in vivo* studies, where bone formation is compared in either a bone defect or augmentation model.

The advantage of slower release of rhBMP-2, especially at ectopic sites, has been demonstrated with gelatine-based hydrogels[23]. Interestingly, the same rhBMP-2-loaded hydrogels evaluated in an orthotopic bone regeneration assay were less effective than rhBMP-2 delivered in solution[6], most likely due to the slow degradation of the carrier material. In our bone augmentation assay, where bone is generated orthotopically as well, slow release of rhBMP by foamspheres led to larger amounts of bone than by the use of hydrogels, which gave faster release of rhBMP. Therefore, slower release at orthotopic sites can also be advantageous. If rhBMP-loaded foamspheres were used in calvarial defects in rabbits, they performed worse than the faster degradable gelatine hydrogels. It may be that the rate of degradation of the foamspheres was much slower than the local healing rate in the rabbit, leading to a physical blocking of the healing process within the defect.

For clinical application of rhBMP, the perfect application would involve a synchronized release of rhBMP and degradation of the delivery system that was matched to the generation of new bone in the defect. One major variable in this system is the number of mesenchymal stem cells, which not only decreases with age but also varies in individuals of the same age[15]. Especially for this aspect, a slow-release rhBMP delivery system is an advantage as was shown in the rabbit skull model[6]. In the defects where rhBMP-2 was applied with gelatine-based hydrogels, the number of osteoblasts in the defect and at the dura mater side was significantly elevated compared to defects where rhBMP-2 was applied in liquid form. This result indicates that a slow release of rhBMP supports the chemotactic attraction of bone-forming cells, which is needed, even at orthotopic sites, if the number of mesenchymal stem cells is limited. In conclusion, the apparent differences in the efficiencies of rhBMP carrier combinations for diverse applications could mean that in future, not a single but an array of rhBMP carriers with diverse rhBMP release and carrier degradation kinetics should be available to guarantee optimal rhBMP applications for all clinical situations.

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Leading research paper: Osteobiology

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Abstract. Bone morphogenetic proteins (BMPs) are multifunctional cytokines that were originally identified as molecules that induce bone and cartilage formation *in vivo*. In order to increase the efficacy of this potent protein for application in medicine, a carrier system is needed to retain the BMP at the preferred site. Here we present and characterize a slow-release carrier system for pure human recombinant (rh)BMP. The large porous microspheres, called 'foamspheres', are biodegradable, because they consist of poly(lactide-co-glycolide) acids and release loaded rhBMP slowly and continuously. *In vivo* studies in rodents revealed that rhBMP-loaded foamspheres increased the thickness of the calvarial bone of rats by 222%. When the same amount of rhBMP was applied via a gelatine-based hydrogel, the increase in bone height was only 66%. Thus, the carrier system for rhBMP is an important factor for the efficacy of BMPs.

Key words: bone morphogenetic protein; delivery; microspheres; release system; bone regeneration; bone augmentation; hydrogel.

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In 1988[22], the first cDNAs coding for BMPs were cloned. This marked the end-point of a long search for the bone-inducing principle started after URIST had published his key discovery in this field in 1965[20]. The primary goal of all these efforts was to use the bone-inducing principle in clinical conditions for fracture healing, especially for non-unions but also for augmentation of bone. The concept for this approach was proved with native BMP preparations from human bones[8–11] and bovine bones[16–18]. The results demonstrated the potential of BMP for orthopaedic and cranio-maxillofacial surgery applications. Despite these encouraging results, there is a time gap of more than 13 years between the molecular cloning of the first BMPs and their routine medical application points to some obstacles, which can be mainly attributed to the lack of a suitable BMP-delivery system[3–5,13].

Thirteen years ago the first rhBMP produced was applied with inactivated bone collagen allografts in rodents, and

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now after a period of testing diverse carrier systems[3–5,13], clinical trials still depend on bovine collagen as a carrier. The disadvantages of bovine collagen as a rhBMP carrier include its biological origin, which implies a chance for the transmission of diseases, the occurrence of possible immune reactions, difficulties in handling wetted collagen gels, and the lack of form stability.

Another aspect that is often overlooked is the release rate of rhBMPs from the carrier. In virtually all applications, rhBMP is dried or attached to the surface of the carrier[3–5,13]. Therefore upon implantation, the entire administered protein is in contact with bodily fluids, leading to a rapid release and degradation of the rhBMP. The conclusion that the release rate of rhBMP is an important factor for its efficacy, especially at ectopic sites[23], can be drawn from experiments where rhBMP incorporated into hydrogels exhibited a more potent ability for bone formation than injected rhBMP-2. Due to the nature of these hydrogels, 60–75% of the incorporated rhBMP is released during the first 24 minutes *in vitro*. BMP expression profiles during fracture healing[14] revealed that rhBMP-2 is expressed during a period of 1–2 weeks after the fracture occurred, therefore, we wanted to create a system by which rhBMP is released over days rather than minutes, to mimic the natural rhBMP release profile. With other proteins, the release over days was achieved by encapsulation into biodegradable microspheres[2]. Therefore, porous biodegradable microspheres, termed 'foamspheres', with entrapped rhBMP were developed. This combination was more efficient in increasing the thickness of the calvarial bone than rhBMP delivered via gelatine-based hydrogels.

Materials and methods

Expression, purification, and refolding of rhBMP-2 and rhBMP-4

A BMP-2 cDNA was isolated from a human placenta library, shown to be identical to the sequence deposited in the Genbank (M22489) and used as template to generate a mature BMP-2 expression construct. The cloning and expression of the protein was performed as reported earlier[21]. After expression the cells were stored overnight at −80°C. After thawing, the cells were resuspended in 100 mM Tris-HCl pH 8.3, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and lysed by three passages through a French pressure cell at 20 000 psi. After centrifugation, rhBMP-2 was present in the pellet, which was washed extensively and dissolved by acidification to pH 2.5 with glacial acetic acid. Insoluble material was removed by centrifugation at 15 000 × g for 30 min and the supernatant dialyzed against 25 mM Tris-HCl pH 8.0, 6 M urea (TU). Insoluble material formed during dialysis was removed by centrifugation (30 min 15 000 × g) and rhBMP-2 isolated by its affinity to heparin (5 ml HiTrap® Pharmacia Biotech, New Jersey, USA), followed by a gel filtration on a HiLoad® Superdex® 200 (Pharmacia Biotech, 1.6 cm × 60 cm; 124 ml) at a flow rate of 1 ml/min. rhBMP-2 monomers were dialyzed against 1% acetic acid and dried in a speed vac (Holbrook, NY, USA). The refolding and dimerization were performed according to[1]. rhBMP-4 was expressed in Chinese hamster ovary (CHO) cells using a protein-free cell culture medium (Cytos Biotechnology GmbH and Dr Messi, Cell Culture Technologies, Zürich, Switzerland). Large-scale production cells were grown in 1 l spinner flasks in FMX-8 medium (Dr Messi, Cell Culture Technologies, Zürich, Switzerland) at 37°C, 5% CO_2, 40 r.p.m., and rotation of 720°. After 5 days, the cells were removed by centrifugation, the supernatant applied to a HiTrap® heparin column, and rhBMP-4 eluted by 2 M NaCl in TU. Further purification steps involved gel filtration (HiLoad® Superdex® 75 column, Pharmacia Biotech, New Jersey, USA, 1.6 cm × 60 cm; 124 ml), ion-exchange chromatography (Mono Q, 1 ml, FPLC, Pharmacia), and the same reversed-phase column used for the purification of bacterially derived rhBMP.

Preparation of foamspheres and hydrogel carriers

Poly(DL-lactide-co-glycolide) (PLGA) with a molecular ratio of 75:25 (lactide to glycolide; molecular weight between 90–126 000 [Sigma, MO, USA]) was chosen. The encapsulation was performed by a water-in-oil-in-water double emulsion solvent evaporation technique. Quantities of 100 μg of rhBMP and 100 mg of gelatine were dissolved in 1 ml TU and added to 400 mg poly(lactide-co-glycolide) (PLGA) dissolved in 2 ml dichloromethane. Together they were cooled on ice, and emulsified by vigorous shaking, creating a water-in-oil emulsion due to the excess of the oily phase. This water-in-oil emulsion was dispersed in 1 l of a 1% aqueous gelatine solution stirred on ice at 75 r.p.m. to form a water-in-oil-in-water double emulsion. Following the dispersion step, the stirring velocity was elevated to 250 r.p.m. and the temperature increased to 37°C over a time period of 2 h to slowly evaporate the dichloromethane. After a 2 h incubation period at 37°C, the solidified foamspheres were collected by filtration, washed with 2 l of double distilled water, and 300 ml of 70% EtOH. The foamspheres were dried overnight under the sterile hood followed by lyophilization for 24 h. For foamspheres with a rhBMP content exceeding 5 μg/30 mg foamspheres, rhBMP was added to the foamspheres in 0.01% trifluoroacetic acid and freeze-dried overnight under sterile conditions. For morphological examination, the foamspheres were mounted on double-faced adhesive tape, spotted with platinum and viewed in a Hitachi S-700 scanning electron microscope. The hydrogel carriers were made of bovine gelatine cross-linked by glutaraldehyde as described by Hong et al.[6]. The wetted gel covered an area of 1 cm^2 and had a height of 4 mm. rhBMP dissolved in 20 μl 0.01% trifluoroacetic acid was applied to the freeze-dried sterile hydrogel and freeze-dried under sterile conditions.

Encapsulation yield and release rate of rhBMP

The overall encapsulation yield and maximal release rate of rhBMP was determined in PBS-6M urea (PU), because in its presence rhBMP is readily soluble over a broad concentration range at physiological pH. A quantity of 50 mg of lyophilized foamspheres were added to 1 ml of PU, and rotated on a turning wheel at 37°C. Then 500 μl probes of the buffer were taken after 4, 7, 10, and 14 days, and replaced by the same volume of fresh buffer.

The overall yield was determined by homogenizing 50 mg foamspheres in 1 ml PU with an omni-mixer (Waterbury, CT), and rotating the subsequent mixture for 3 weeks at 37°C. To determine rhBMP content in the supernatant, defined fractions of the probes were applied to a Bio-Dot® SF microfiltration apparatus (Biorad Hercules, CA, USA) with the slot-blot device for 48 samples installed. Dilutions from a

defined rhBMP stock were employed to each blot for calibration. After the application of all probes and dilutions, they were passed through the Bio-Dot® apparatus, which blotted the proteins onto a polyvinylidene fluoride membrane. An overnight incubation with anti BMP-specific antibodies was performed after blocking the membrane with 1% bovine serum albumin in phosphate buffered saline. The incubation of the blot with a secondary anti-rabbit antibody conjugated to alkaline phosphatase allowed the rhBMP to be visualized by a tetrazoleum method. An amount of rhBMP was quantified densitometrically by using the calibration dilutions of each blot as standard.

In vivo test

On top of the calvarial bone of rats, where the periost was removed mechanically, 30 mg of foamspheres or a hydrogel were placed. The animals were sacrificed after 33 days and the calvarial bone with the implant was excised. The calvarial bone including most of the implant was fixed and embedded in poly(methyl-methacrylate). Histological sections 4.5 μm thick were prepared, Goldner-Trichrome stained, and examined for bone height, cell type, morphology, and stromal details by using bright-field light microscopy. Histology also allowed us to attribute changes in calcium content to bone formation. For the quantification of bone formation in implanted foamspheres, a probe was taken just after excision of the calvarial bone from foamsphere material located at least 1 mm above the original calvarial bone. This was possible because after 33 days, the ossified or non-ossified foamspheres still extended 2–3 mm above the original calvarial bone and ensured that no contamination of the probe with original calvarial bone occurred. For quantification of bone formation by the measurement of the calcium content, the probe was weighted, and homogenized with an omni-mixer (Waterbury, CT, USA) in 1.5 ml of 3 mM $NaHCO_3$, 150 mM NaCl. After centrifugation (1000 × g for 15 min) the supernatant was used for alkaline phosphatase determination, and the pellet was resuspended three times with 1 ml of 10 mM Tris-HCl pH 7, and mixed at room temperature for 1 h. After the final wash, the pellets were extracted overnight with 1 ml 0.5 M HCl. Calcium in the extract was quantified by atomic absorption spectroscopy.

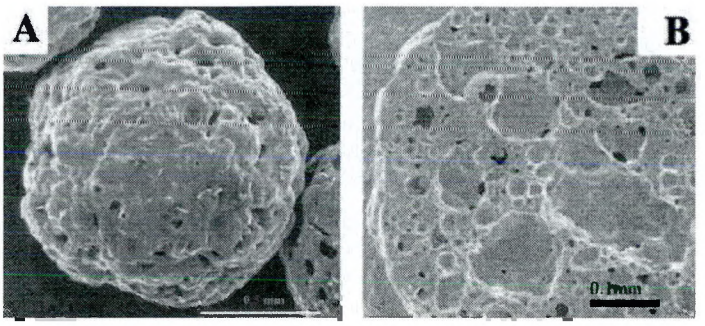


Fig. 1. Outer and inner morphology of foamspheres. To illustrate the porosity of the foamspheres, (A) intact or (B) cross-sectioned foamspheres were mounted, spotted with platinum and viewed in a scanning electron microscope. Size scales are indicated.

Statistical analysis

Student's *t*-test was implemented by a commercially available software package (SSPE, Chicago, Ill, USA). All values are represented as means ± standard deviation.

Results

A polypeptide representing the mature part of BMP-2 plus a N-terminal extension by a single methionine was expressed in *Escherichia coli*. The purity of rhBMP-2 judged on SDS-PAGE exceeded 99%. Ectopic bone formation induced in rats by our rhBMP preparations proved the bioactivity of rhBMP-2 and -4. Quantitative information on the specific biological activity was obtained by the determination of alkaline phosphatase activity induced in C3H10T1/2 cells. When these cells were subjected to 1 μg rhBMP-2/ml over 4 days, the alkaline phosphatase activity increased by a factor of 23–26 compared to untreated cells, which is similar to the data reported from CHO-derived glycosylated rhBMP-2[12].

Foamsphere production and characterization

Of the particles produced by the double-emulsion solvent evaporation technique, 90% had a diameter between 0.5–2.5 mm, which is significantly larger than the average microsphere. Therefore, we created the new term 'foamsphere', which best describes the porosity and morphology of our delivery system (Fig. 1). In initial experiments, the loading of foamspheres with CHO-derived rhBMP-4 was tested and the loading efficiency and release kinetic compared to bacterially derived rhBMP-2 and rhBMP-4. As there were no differences in the error of our measurements between both rhBMP preparations, we decided to use bacterially derived rhBMP-2 as model protein for the release studies, which we could produce in higher quantities. Averaged over 10 independent foamsphere productions, 10.8 ± 4.8% of the rhBMP-2 employed in the double-emulsion process was entrapped in the foamspheres. The release kinetic was determined from three foamsphere preparations (Fig. 2) with a loading efficiency for rhBMP-2 of 12.2 ± 2.3%. As shown in Fig. 2, all rhBMP-2 delivered via foamspheres was released over 10 days. The daily release of rhBMP was approximately linear.

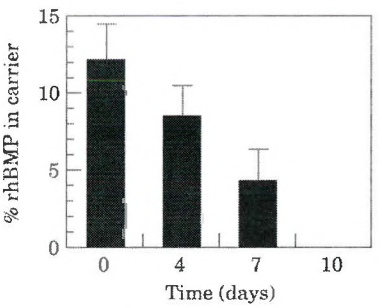


Fig. 2. In vitro release of rhBMP from foamspheres. Loading and release of rhBMP-2 averaged from three independent foamsphere preparations was determined over time. % rhBMP in carrier refers to the amount of rhBMP used initially for the loading of the foamspheres. All values represent the means ± standard deviations. 50 mg of lyophilized foamspheres were added to 1 ml of PBS-6M urea and the appearance of rhBMP in the supernatant was determined to calculate the amount of rhBMP retained in the foamspheres. After 10 days, the sum of released rhBMP and the amount of rhBMP initially entrapped in the foamspheres was identical within the error of the measurements.

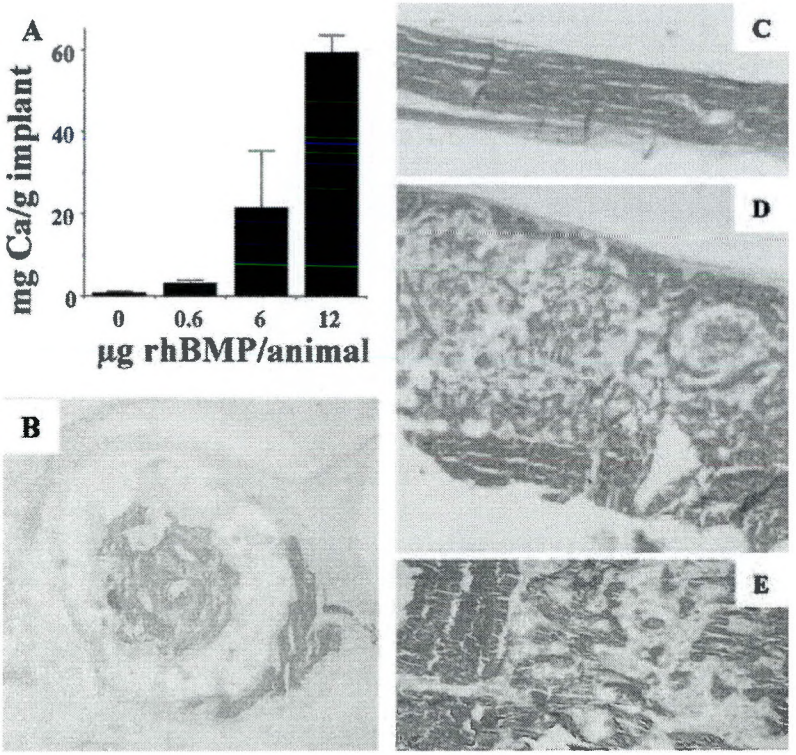


Fig. 3. Calcium content of rhBMP-2-loaded foamspheres and associated histological sections. An increase of the calcium content of foamspheres placed on top of the calvarial bone of rats correlated with the amount of applied rhBMP (A). The calcium content was determined 33 days after implantation from a probe taken from implanted (foamsphere) material located at least 1 mm above the original calvarial bone. Each value represents the mean ± standard deviation from 4–8 probes. Histological section of a foamsphere where 0.6 µg rhBMP was applied to the animal (B). On the right, a histological section of an untreated calvarial bone (no carrier, no rhBMP) (C) is compared to a calvarial bone of an animal treated with foamspheres containing 12 µg rhBMP (D), which is also shown in a 5 × magnification (E). All the implants were Goldner-Trichrome stained where calcified bone appears green and osteoid red.

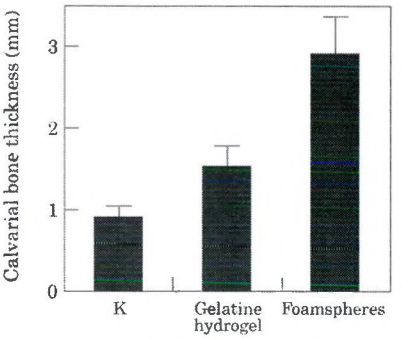


Fig. 4. Augmentation of the calvarial bone with rhBMP-2-loaded carriers. 12 µg rhBMP-2 was applied via foamspheres or via gelatine-based hydrogels. The animals were sacrificed 33 days after application and the thickness of the calvarial bone was determined from the Goldner-Trichrome-stained histosections. Results from untreated (control) bones, where no rhBMP and no carrier was applied, is shown along with gelatine- and foamspheres-treated calvarial bones with 12 µg rhBMP-2 applied. The foamspheres that had a higher retention of rhBMP-2 clearly led to greater augmentation of the calvarial bone. The columns represent the means ± standard deviation.

In vivo test of the rhBMP-loaded foamspheres

33 days after implantation, the foamspheres placed on top of the calvarial bone of rats covered an area between 1.0–1.5 cm^2. As shown in Fig. 3A, the mean calcium content of foamspheres determined from 4–8 animals applied with 0, 0.6 or 12 µg rhBMP-2 depended on the amount of rhBMP-2 applied ($P<0.004$). For eight animals tested with 6 µg rhBMP-2, the value for P equalled 0.029. Histology revealed that no ossification occurred in the absence of rhBMP-2. If the amount of applied rhBMP-2 was below 1 µg, bone formation was induced in and around foamspheres (Fig. 3B) without leading to a complete ossification of the implant. In contrast, when foamspheres were loaded with 12 µg rhBMP-2 (Fig. 3D), extra bone covered the entire foamsphere implant. Although the shapes of dissolved foamspheres were still recognizable, the entire carrier material had disappeared without leaving any fibrous tissue behind (Fig. 3E). In order to demonstrate the efficacy of our foamspheres as rhBMP-2 carrier for bone augmentation, we compared them to gelatine-based hydrogels. As shown in Fig. 4, 12 µg rhBMP-2 applied via hydrogels increased the maximal calvarial bone thickness from 0.9 ± 0.1 mm ($n=6$) without rhBMP to 1.5 ± 0.8 mm ($n=6$; $P=0.04$) with rhBMP. If the same amount of rhBMP was applied via foamspheres, the maximal thickness of the calvarial bone reached 2.9 ± 0.8 mm ($n=6$; $P=0.002$).

Discussion

Numerous delivery systems for rhBMPs have been tested, but none have demonstrated sustained delivery of rhBMP-2 for local application. In order to develop a system by which bioactive rhBMP is released over days rather seconds or minutes, we encapsulated pure rhBMPs into PLGA. The first trials with encapsulation of BMP in PLGA were performed with partially purified bovine BMP preparations[7]. However, the determination of the retention of impure, bovine BMP proved to be difficult because only the total protein released was measured. Since the BMP only represented a small fraction of the total protein, extrapolating the release data for BMP only from these numbers was not possible. Interestingly, the numbers for the release of crude bovine BMP and our results with pure rhBMP-2 were identical. This implies that BMP release is governed by the degradation of the encapsulation material, which was identical in both studies. In terms of loading efficiency, however, the results were very different. Only 5–15% of pure rhBMP but 91% of impure native BMP[7] was encapsulated in the foamspheres, which points to the possibility of modulating BMP loading efficiency by the addition of proteins, which are present in native BMP preparations, to pure rhBMP preparations.

rhBMP release kinetic from diverse hydroxyapatites and calcium phosphates was shown to be biphasic. $t_{1/2}$ of the

second phase of rhBMP release determined *in vivo* ranged from 2–17 days[19]. Gelatine based-hydrogels, which we used as reference material in the bone augmentation model, showed $t_{1/2}$ values determined *in vivo* of 15 days[23]. *In vitro* with no diffusion barrier in place, the apparent $t_{1/2}$ of gelatine-based hydrogels was below 25 minutes[23] and much shorter than the 5 days determined for the foamspheres in a similar setting. An additional benefit of the foamspheres is that the rhBMP release could be further modulated by the choice of the PLGA. Besides the extended *in vitro* $t_{1/2}$ for rhBMP release achieved by foamspheres, the quality of unreleased rhBMP was also different. rhBMP was added to the outside of all the other carriers[3–5,13]. Therefore, upon implantation, even the unreleased rhBMP is subject to bodily fluids and degradation as it is located on the surface of the diverse materials. In contrast, rhBMP delivered by foamspheres is encapsulated by a shell of PLGA that prevents rhBMP degradation and increases the chance that rhBMP released from foamspheres has retained its bioactivity. The efficiency of rhBMP delivery systems cannot be evaluated from *in vitro* or *in vivo* $t_{1/2}$ values exclusively. It also needs *in vivo* studies, where bone formation is compared in either a bone defect or augmentation model.

The advantage of slower release of rhBMP-2, especially at ectopic sites, has been demonstrated with gelatine-based hydrogels[23]. Interestingly, the same rhBMP-2-loaded hydrogels evaluated in an orthotopic bone regeneration assay were less effective than rhBMP-2 delivered in solution[6], most likely due to the slow degradation of the carrier material. In our bone augmentation assay, where bone is generated orthotopically as well, slow release of rhBMP by foamspheres led to larger amounts of bone than by the use of hydrogels, which gave faster release of rhBMP. Therefore, slower release at orthotopic sites can also be advantageous. If rhBMP-loaded foamspheres were used in calvarial defects in rabbits, they performed worse than the faster degradable gelatine hydrogels. It may be that the rate of degradation of the foamspheres was much slower than the local healing rate in the rabbit, leading to a physical blocking of the healing process within the defect.

For clinical application of rhBMP, the perfect application would involve a synchronized release of rhBMP and degradation of the delivery system that was matched to the generation of new bone in the defect. One major variable in this system is the number of mesenchymal stem cells, which not only decreases with age but also varies in individuals of the same age[15]. Especially for this aspect, a slow-release rhBMP delivery system is an advantage as was shown in the rabbit skull model[6]. In the defects where rhBMP-2 was applied with gelatine-based hydrogels, the number of osteoblasts in the defect and at the dura mater side was significantly elevated compared to defects where rhBMP-2 was applied in liquid form. This result indicates that a slow release of rhBMP supports the chemotactic attraction of bone-forming cells, which is needed, even at orthotopic sites, if the number of mesenchymal stem cells is limited. In conclusion, the apparent differences in the efficiencies of rhBMP carrier combinations for diverse applications could mean that in future, not a single but an array of rhBMP carriers with diverse rhBMP release and carrier degradation kinetics should be available to guarantee optimal rhBMP applications for all clinical situations.

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