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Effect of Autologous Bone Marrow Stromal Cell Seeding and Bone Morphogenetic Protein-2 Delivery on Ectopic Bone Formation in a Microsphere/ Poly(Propylene Fumarate) Composite

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A biodegradable microsphere/scaffold composite based on the synthetic polymer poly(propylene fumarate) (PPF) holds promise as a scaffold for cell growth and sustained delivery vehicle for growth factors for bone regeneration. The objective of the current work was to investigate the *in vitro* release and *in vivo* bone forming capacity of this microsphere/scaffold composite containing bone morphogenetic protein-2 (BMP-2) in combination with autologous bone marrow stromal cells (BMSCs) in a goat ectopic implantation model. Three composites consisting of 0, 0.08, or 8 μg BMP-2 per mg of poly(lactic-co-glycolic acid) microspheres, embedded in a porous PPF scaffold, were combined with either plasma (no cells) or culture-expanded BMSCs. PPF scaffolds impregnated with a BMP-2 solution and combined with BMSCs as well as empty PPF scaffolds were also tested. The eight different composites were implanted subcutaneously in the dorsal thoracolumbar area of goats. Incorporation of BMP-2–loaded microspheres in the PPF scaffold resulted in a more sustained *in vitro* release with a lower burst phase, as compared to BMP-2–impregnated scaffolds. Histological analysis after 9 weeks of implantation showed bone formation in the pores of 11/16 composites containing 8 μg/mg BMP-2–loaded microspheres with no significant difference between composites with or without BMSCs (6/8 and 5/8, respectively). Bone formation was also observed in 1/8 of the BMP-2–impregnated scaffolds. No bone formation was observed in the other conditions. Overall, this study shows the feasibility of bone induction by BMP-2 release from microspheres/scaffold composites.

Introduction

Bone tissue engineering is a challenging field that strives to create alternative methods for current autograft and allograft treatments to restore bone defects or reinforce existing malfunctioning bone. Current strategies are mainly based on three components: (1) scaffolds, (2) (progenitor) cells, and (3) growth and differentiation factors. A three-dimensional biodegradable scaffold is the starting point for most regenerative strategies, providing initial mechanical strength and a framework for attachment and proliferation of cells. The cells are responsible for the matrix deposition that precedes ossification and can be locally recruited after implantation or seeded before implantation. Growth factors can be added to the scaffold to induce cell differentiation toward the osteogenic lineage. These tissue-engineering strategies have proven to be successful in many studies; however, upscaling bone tissue engineering toward clinical applications remains challenging.

The ideal biomaterial for bone regeneration should have good mechanical properties, support cell attachment and differentiation, and allow controlled release of bioactive factors for the modulation of cellular function. Further, the scaffold must biodegrade into nontoxic products to permit natural bone formation and remodeling. Poly(propylene fumarate) (PPF)–based materials are promising as biodegradable scaffolds for filling skeletal defects.[1,2] Crosslinking of the linear polyester PPF through double bonds along its polymer backbone results in a biocompatible, biodegradable scaffold with mechanical properties similar to human trabecular bone.[3–5] The injectable nature of the material makes it easy to shape the scaffold into a desired interconnected porous structure.[6,7] Crosslinked PPF scaffolds have shown to be suitable substrates for the *in vitro* proliferation and

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Table 1. Experimental Groups and Implant Compositions

	PPF scaffold	Cells	Initial mps loading (μg BMP/mg PLGA)	Composite (Mps/PPF/porogen) (%)	BMP/ scaffold (μg)	Number of scaffolds
1	Blank	No cells	No mps	0/30/70	0	4
2	$BMP^{impregnated}$	BMSCs	No mps	0/30/70	50	8
3	Mps^{empty}	No cells	0	6/24/70	0	8
4	Mps^{empty}	BMSCs	0	6/24/70	0	8
5	Mps-BMP^{low}	No cells	0.08	6/24/70	0.04–0.38	8
6	Mps-BMP^{low}	BMSCs	0.08	6/24/70	0.04–0.38	8
7	Mps-BMP^{high}	No cells	8.0	6/24/70	39	8
8	Mps-BMP^{high}	BMSCs	8.0	624/70	39	8

Mps, microparticles; PLGA, poly(lactic-co-glycolic acid); BMP, bone morphogenetic protein-2; PPF, poly(propylene fumarate); BMSCs, bone marrow stromal cells.

osteoblastic differentiation of bone marrow stromal cells (BMSCs).[8,9] Although these material characteristics are favorable for bone regeneration, PPF scaffolds are not osteoinductive. Making PPF scaffolds osteoinductive by adding the appropriate growth factors would be an improvement.

Bone morphogenetic protein-2 (BMP-2) is a very potent growth factor capable of inducing bone formation in both orthotopic and ectopic implantation sites.[10–13] However, due to its short *in vivo* half-life and localized actions, BMP-2 requires a delivery vehicle to sustain its release at the implantation site. Biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres are ideal candidates for such a sustained release of BMP-2.[1] Previous studies have shown that BMP-2 can be successfully encapsulated into PLGA microspheres with retention of its bioactivity.[14] Further, the PLGA microspheres could be incorporated into PPF scaffolds to form microsphere/scaffold composites with a decreased burst release of loaded molecules.[1]

The purpose of this study was to investigate the effect of the incorporation of BMP-2–loaded microspheres in PPF scaffolds in a goat ectopic implantation model. To test whether the addition of progenitor cells would influence the bone regeneration capacity of the composite, some of the scaffolds were combined with autologous BMSCs before implantation.

Materials and Methods

Experimental design

Five different porous PPF scaffolds were prepared consisting of scaffolds alone (blank), scaffolds impregnated with a solution containing 50 μg BMP-2 ($BMP^{impregnated}$), and scaffolds with incorporated PLGA microspheres containing 0, 0.08, or 8.0 μg BMP-2/mg PLGA (Mps^{empty}, Mps-BMP^{low}, and Mps-BMP^{high}, respectively; Table 1). A 50 μg dose of BMP-2 was used for the $BMP^{impregnated}$ scaffolds since this was approximately the initial amount used for the fabrication of the Mps-BMP^{high} scaffolds. The *in vitro* BMP-2 release profile from the scaffolds was determined in phosphate-buffered saline (PBS). The composites were combined with autologous plasma containing either no cells or autologous BMSCs, and implanted ectopically in goats. After 9 weeks, the composites were harvested and characterized for tissue ingrowth, bone formation, and porosity by histology and histomorphometry.

Materials

PLGA (75:25 lactic-to-glycolic ratio, Mw = 62 kDa, Medisorb®; Lakeshore Biomaterials, Birmingham, AL), poly(vinyl alcohol) (PVA, 87–89% mole hydrolyzed, M_w = 13–23 kDa; Sigma-Aldrich, St. Louis, MO), and isopropanol (IPA; Sigma-Aldrich) were used for the microsphere preparation. PPF with a molecular weight of 3100 and a polydispersity index of 2.7 was synthesized by a two-step reaction process as previously described.[2] *N*-vinylpyrrolidinone (NVP; Acros, Pittsburgh, PA) and bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO; Ciba Specialty Chemicals, Tarrytown, NY) were used for the scaffold fabrication. BMP-2 (kindly provided by Wyeth Pharmaceuticals, Madison, NJ) was concentrated by centrifuging at 5000 *g* in a Centricon-10 filter unit (Amicon, Beverly, MA) and reconstituted to the appropriate concentrations in an aqueous buffer (pH 4.5) consisting of 5 mM glutamate, 5 mM NaCl, 0.5% sucrose, 2.5% glycine, and 0.01% polysorbate 80 (all from Sigma-Aldrich). An enzyme-linked immunosorbent assay (ELISA, Quantikine BMP-2 Immunoassay®; R&D Systems, Minneapolis, MN) was used for the entrapment efficiency and release assay.

Microsphere fabrication

A water-in-oil-in-water (W1-O-W2) double-emulsion-solvent-extraction technique was used for microsphere preparation.[14] Briefly, 50 μL of a 0, 0.40, or 40 mg/mL BMP-2 solution was emulsified in a solution of 250 mg PLGA in 1 mL dichloromethane using a vortexer at 3050 rpm. The entire mixture was re-emulsified for 30 s in 2 mL of 1% w/v aqueous PVA solution to create the double emulsion. The content was then added to 100 mL of 0.3% w/v aqueous PVA solution and 100 mL of 2% w/v aqueous IPA solution with stirring for 1 h. The extraction of the dichloromethane to the external alcohol phase resulted in precipitation of the dissolved polymers and subsequently the formation of microspheres. The microspheres were collected by centrifugation, washed twice with ddH_2O, and finally vacuum dried. The resulting powder was stored at −20°C prior to use.

Scaffold fabrication

Porous PPF scaffolds were fabricated using a salt leaching technique. Sodium chloride particles that were sieved to a size range of 300–500 μm were used as porogen. Briefly, 60 μL of an initiator solution (100 mg/mL of BAPO in

dichloromethane) was added to a solution of 0.9 g of PPF in 0.27 mL NVP and mixed well with a spatula. The PPF/NVP/BAPO paste was then combined with the appropriate microsphere formulation (0 or 6% w/w) and 70% w/w NaCl particles as indicated in Table 1. The mixture was forced into glass cylindrical vials with a diameter of 6 mm and placed under UV light (PS135; Matcon, Middenbeemster, The Netherlands) for 30 min for photocrosslinking. The cylindrical scaffolds were then cut into 3-mm-thick disks and sterilized by ethanol evaporation. Finally, the disks were placed in PBS for 12 h to leach out the NaCl particles. Eight blank PPF scaffolds were impregnated with 19 μL of a solution containing 50 μg BMP-2. All implants were cryopreserved at −20°C until use.

Microsphere and scaffold characterization

The entrapment efficiency of BMP-2 in the PLGA microspheres was determined by normalizing the actual amount of entrapped BMP-2 to the starting amount. Approximately 10 mg of microspheres was dissolved in 0.75 mL of dichloromethane and 0.75 mL of a strong desorption buffer consisting of 0.5 M arginine, 0.5 M NaCl, and 50 mM K_2HPO_4. The BMP-2 was extracted over a period of 48 h with a buffer change after 24 h. The concentration of the extracted BMP-2 was analyzed by ELISA following the manufacturer's instruction. To determine the *in vitro* BMP-2 release profile, the scaffolds were placed in microcentrifuge tubes containing 1.0 mL of pH 7.4 PBS and maintained at 37°C on an orbital shaker set at 100 rpm to ensure continuous mixing. At days 0.5, 1, 2, 3, 5, 7, 9, 13, 17, 21, and 24, the supernatant was collected, stored at −20°C, and replaced with fresh PBS. The samples were assayed for BMP-2 concentration using the BMP-2 ELISA.

BMSC culture and seeding conditions

The animal experiments were approved by the Institutional Animal Care and Use Committee. Ten adult Dutch milk goats were obtained from a professional stockbreeder at least 4 weeks prior to surgery. Autologous BMSCs were derived and expanded 3 weeks preoperatively. Briefly, bone marrow aspirates were taken from both iliac wings under general anesthesia. The BMSCs in the aspirates were culture expanded in standard culture medium containing 15% fetal bovine serum (FBS; Gibco, Paisly, Scotland) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Gibco) with media changes every 3 days. The proportion of BMSCs in the bone marrow aspirates was determined by a colony-forming efficiency (CFU) assay in which nucleated cells from the aspirate were plated in two 25 cm^2 flasks at a density of 1×10^5 cells/cm^2. After 8 or 9 days, the colonies were washed with PBS, fixed in 8% formalin, stained with methylene blue, and counted under an inverted microscope. The rest of the expanded cells were cryopreserved after passage 2 in medium containing 30% FBS and 10% dimethylsulfoxide (DMSO; Sigma-Aldrich) in aliquots of 1×10^7 cells/mL until use.

On the day of surgery, aliquots of cryopreserved cells were thawed on ice, washed with autologous serum, and resuspended in serum at a concentration of 1×10^6 cells/mL. The cell viability was determined by trypan blue exclusion. The BMSCs were transported to the operating room on ice in aliquots of 5.5 mL. During surgery, plasma was obtained by centrifuging 10 mL of venous blood at 1200 *g* in a plastic tube. The BMSCs aliquots were centrifuged at 300 *g*, the medium was decanted, and the cells were resuspended in autologous plasma. Plasma with no cells or with BMSCs (0.5×10^6 cells/scaffold) was combined with the prewetted scaffolds and allowed to clot before implantation.

Surgical procedure

Each goat received six implants in the thoracolumbar area according to a randomized scheme (Table 2). Prior to surgery, the goats were sedated by an intravenous injection of detomidine, and general inhalation anesthesia was provided by a halothane gas mixture. Six subcutaneous pockets were created in the dorsal thoracolumbar area by blunt dissection and filled with one of the implants according to a randomized scheme. To increase the statistical power to determine an effect of BMSCs, groups 5 and 6, and groups 7 and 8 (Tables 1 and 2) were implanted pairwise. The pockets were closed with a nonresorbable suture to indicate their location postmortem. In addition to the subcutaneous implantations, in the course of separate studies, the goats also received ceramic implants intramuscularly and in osteoconduction chambers on the transverse processes of L3 and L5.[15,16]

Table 2. Implantation Scheme and Location of the Experimental Groups

	Pocket contents					
Goat	*1*	*2*	*3*	*4*	*5*	*6*
1	$M^{em}+C$	M^{em}	$B^{lo}+C$	B^{lo}	$B^{hi}+C$	B^{hi}
2	$B^{im}+C$	Bl	$M^{em}+C$	M^{em}	$B^{lo}+C$	B^{lo}
3	$B^{hi}+C$	B^{hi}	$B^{im}+C$	Bl	$M^{em}+C$	M^{em}
4	$B^{lo}+C$	B^{lo}	$B^{hi}+C$	B^{hi}	$B^{im}+C$	M^{em}
5	M^{em}	$M^{em}+C$	B^{lo}	$B^{lo}+C$	B^{hi}	$B^{hi}+C$
6	Bl	$B^{im}+C$	M^{em}	$M^{em}+C$	B^{lo}	$B^{lo}+C$
7	$B^{hi}+C$	B^{hi}	M^{em}	$B^{im}+C$	Bl	$M^{em}+C$
8	$B^{lo}+C$	B^{lo}	$B^{hi}+C$	B^{hi}	M^{em}	$B^{im}+C$
9	$M^{em}+C$	$B^{im}+C$	$B^{lo}+C$	B^{lo}	$B^{hi}+C$	B^{hi}
10	B^{hi}	$B^{hi}+C$	$M^{em}+C$	$B^{im}+C$	$B^{lo}+C$	B^{lo}

Pocket location		
Cranial		
1	S	4
2	p i	5
3	n e	6
Caudal		

B^{im}, $BMP^{impregnated}$; M^{em}, Mps^{empty}; M^{lo}, Mps-BMP^{low}; M^{hi}, Mps-BMP^{high}; Bl, blank; C, autologous bone marrow stromal cells.

Mps, microparticles; BMP, bone morphogenetic protein-2.

Postoperative pain relief was achieved by buprenorphine. To monitor the dynamics of calcification, the fluorochrome markers calcein green (10 mg/kg intravenously; Sigma-Aldrich), oxytetracyclin (32 mg/kg intramuscularly according to the manufacturer's instructions; Mycofarm, Boxmeer, The Netherlands), and xylenol orange (80 mg/kg, intravenously; Sigma-Aldrich) were administered after 3, 5, and 7 weeks, respectively. At 9 weeks postimplantation, euthanasia was performed by an overdose of pentobarbital (Organon, Oss, The Netherlands).

Histology and histomorphometry

After explantation, the implants were fixed in a 4% phosphate-buffered formaldehyde solution (pH 7.4). The implants were dehydrated in graded series of alcohol and embedded in methylmethacrylate. After polymerization, sections were cut using a sawing microtome (Leica SP1600; Leica Microsystems, Nussloch, Germany) and stained with methylene blue/basic fuchsin or with hematoxylin and eosin for routine histology and histomorphometry. An additional unstained section was sawn for fluorescence microscopy. Using light and fluorescence microscopy, the general tissue response, bone formation, and fluorochrome labels were evaluated. For histomorphometry, high-resolution (300 dpi), low-magnification (40×) digital micrographs covering the complete implant were made of blinded sections. The areas of interest were pseudocolored, and the colorized pixels were measured to calculate the percentage of scaffold porosity and bone area relative to the available pore space.

Statistical analysis

Both *in vitro* ($n=4$) and *in vivo* ($n=4$ or 8) results are reported as means ± standard deviations. Analysis of variance (ANOVA) with Bonferroni-corrected *post hoc* tests were used to analyze differences in porosity between the experimental groups. A two-tailed paired Student's *t*-test was used to determine the effect of cells on the amount of newly formed bone in the Mps-BMP^{high} scaffolds (groups 7 and 8). All tests were performed by SPSS (version 13.0; SPSS, Chicago, IL), and the level of significance was set at $p=0.05$.

Results

Scaffold characterization

The entrapment efficiency of BMP-2 in the microspheres loaded at 8.0 μg/mg PLGA was 82 ± 3.6% ($n=4$), which resulted in a scaffold loading of approximately 39 μg BMP-2 per Mps-BMP^{high} implant. Despite the use of the strong desorption buffer and repetition of the extraction procedure, it was difficult to estimate the entrapment efficiency of the 0.08 μg/mg loaded microspheres. The entrapment efficiency of these microspheres varied between 8.1% and 78% ($n=9$). Based on these encapsulation yields, the amount of BMP-2 in the Mps-BMP^{low} scaffolds was estimated between 0.04 and 0.38 μg BMP-2 per implant. Since the BMP-2 loss during the impregnation process of the blank scaffolds was limited, the BMP^{impregnated} scaffolds were loaded with 50 μg of BMP-2. This was also approximately the starting amount for the fabrication of the Mps-BMP^{high} scaffolds.

The BMP-2 release profiles from the microsphere/scaffold composites and scaffolds impregnated with a BMP-2 solution are shown in Figure 1. The BMP^{impregnated} scaffolds showed a considerable initial burst release of 34 ± 2.9 μg in the first day, followed by a rapid decline of the released amounts. The Mps-BMP^{high} scaffolds showed a much lower burst of 0.49 ± 0.07 μg in the first day, followed by a prolonged sustained release. The Mps-BMP^{low} scaffolds showed no measurable *in vitro* BMP-2 release during the first 2 days with a low sustained release for the rest of the experiment.

BMSC characterization

The bone marrow aspirates contained $5.6 \pm 1.5 \times 10^6$ nucleated cells/mL, which showed a CFU of 1.9 ± 0.6 colonies per 1.0×10^5 cells. The doubling time of the proliferating BMSCs was 1.2 ± 0.6 days, resulting in cryopreservation of 60–100 million cells within 3 weeks after obtaining the aspirates. Trypan blue exclusion indicated 95% cell viability after thawing.

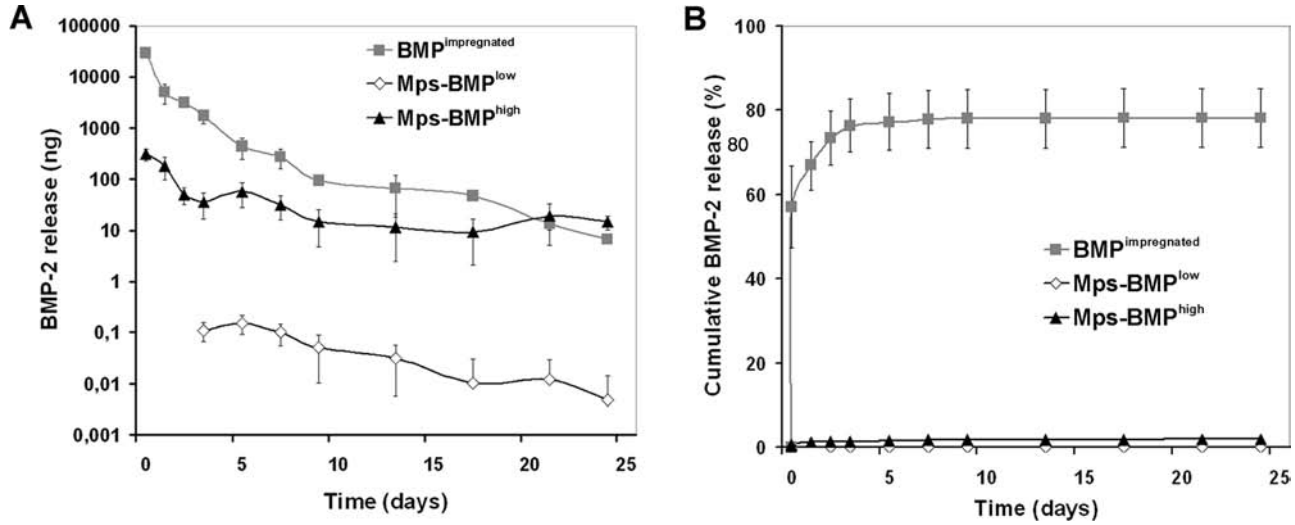

FIG. 1. BMP-2 release profiles expressed as (**A**) amount of released protein (in ng) and (**B**) normalized release (as % of initial loading) from different composites in PBS at 37°C.

Animals

During the experiments, all goats remained in good health and did not show any complications. After 9 weeks of implantation, all implants were easily identified and retrieved.

Histology

Microscopic evaluation of transverse sections showed that all composites maintained their original shape. All scaffolds were surrounded by fibrous tissue from which well-vascularized connective tissue grew into the pores of the scaffold (Fig. 2A, B). Microfragmentation of PLGA was seen in the intact PPF network, indicating degradation of the microspheres (Fig. 2B). No signs of PPF degradation could be observed. The tissue response to the PLGA-PPF materials was relatively uniform. There was a mild foreign body reaction to the implanted materials as indicated by some inflammatory cells inside the pores of the scaffolds, including macrophages and giant cells at the polymer–tissue interface.

Bone formation was observed in the pores of 11/16 of the Mps-BMP^{high} scaffolds and 1/8 of the BMP^{impregnated} scaffolds. No bone was formed in the blank, Mps^{empty}, or Mps-BMP^{low} scaffolds. The newly formed bone was found throughout the implants, mainly clustered in the center of the pores with little contact between the bone and scaffold (Fig. 2C, D). The bone had a woven or lamellar appearance, with osteocytes inside the matrix and osteoblasts lining the bone surfaces (Fig. 2D, E). One of the BMP^{impregnated} scaffolds loaded with BMSCs showed trabecular bone in the fibrous capsule of the implant. Unfortunately, the excision margin of the other BMP^{impregnated} scaffolds was not large enough to investigate the bone formation outside the scaffold borders. Despite little autofluorescence of the tissue, no fluorochromes could be detected in the bone that had formed in the polymer scaffolds (Fig. 2F). Fluorescence microscopy did show all fluorochromes in scaffolds implanted in the same animals in the course of another study. This indicates the onset of bone mineralization was between 7 and 9 weeks, after the administration of the final fluorochrome.

Histomorphometry

Table 3 summarizes the measurements of scaffold porosity and bone formation for the different implant conditions. Bone formation was observed inside the pores of 1/8 of the BMP^{impregnated} scaffolds, 5/8 of the Mps-BMP^{high} scaffolds without cells, and 6/8 of the Mps-BMP^{high} scaffolds with

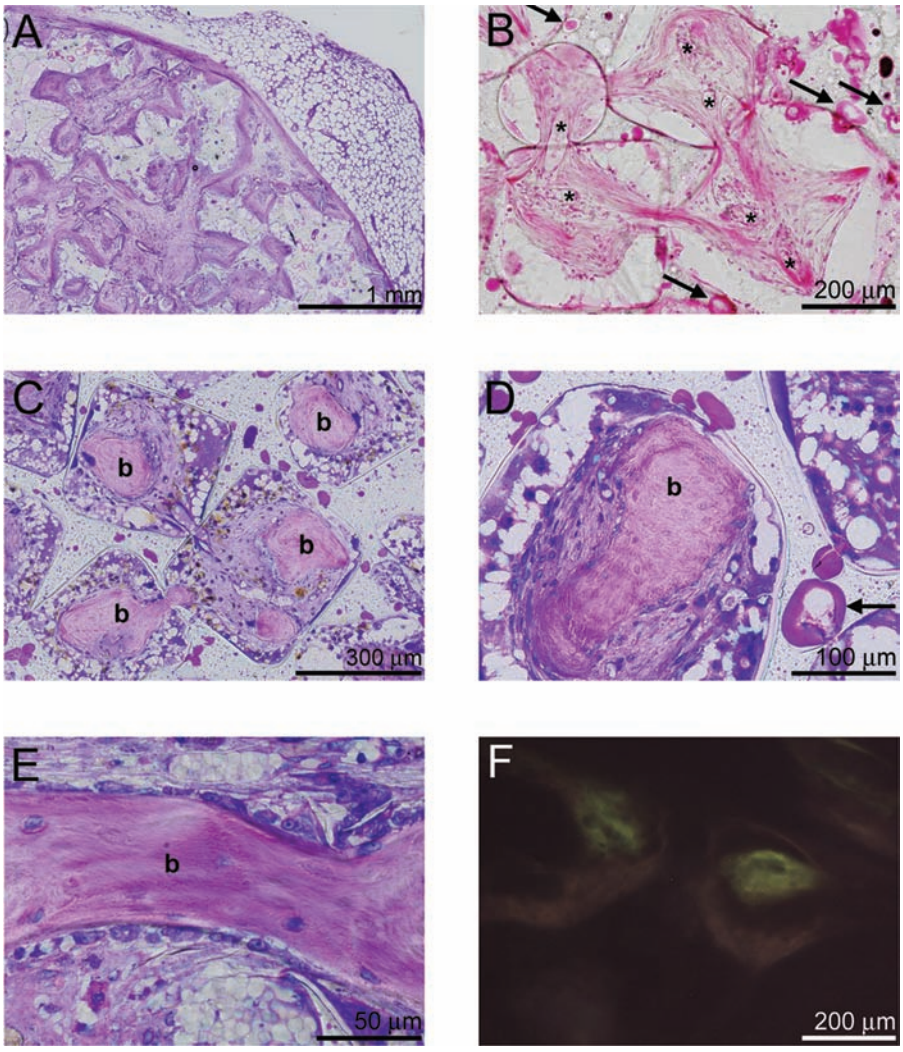

FIG. 2. Methylene blue-basic fuchsine (**A, C, D, E**) and hematoxylin and eosin (**B**) stained or unstained (**F**) histological sections of (**A, B**) Mps-BMP^{low} and (**C–F**) Mps-BMP^{high} composites after 9 weeks of subcutaneous implantation in goats. (**A**) Overview and (**B**) detailed view of an Mps-BMP^{low} implant showing ingrowth of well-vascularized fibrous tissue into the pores of the composite (vessels indicated by asterisks) and microfragmentation of PLGA in the intact PPF network (indicated by the central excavation and uneven staining of the microspheres at arrows). (**C**) Bone formation (indicated by letter b) was seen in the center of the pores of the Mps-BMP^{high} implants. The bone had a woven (**D**) or lamellar (**E**) appearance, with osteocytes inside the matrix and osteoblasts on the surface. (**F**) Despite the little autofluorescence of the tissue, no fluorochromes could be detected in the newly formed bone. Color images available online at www.liebertonline.com/ten.

Table 3. Histomorphometry Results of the Implants

	Scaffold	*Cells*	*Porosity (%)*	*Scaffolds (with bone/total)*	*Bone (% of pore space)*
1	Blank	No cells	77.4 ± 4.5[a]	0/4	0
2	$BMP^{impregnated}$	BMSCs		1/8	0.0 ± 0.1
3	Mps^{empty}	No cells	71.7 ± 1.7	0/8	0
4	Mps^{empty}	BMSCs		0/8	0
5	Mps-BMP^{low}	No cells	73.1 ± 2.4	0/8	0
6	Mps-BMP^{low}	BMSCs		0/8	0
7	Mps-BMP^{high}	No cells	75.3 ± 2.2[a]	5/8	2.9 ± 3.0
8	Mps-BMP^{high}	BMSCs		6/8	2.7 ± 2.4

Scaffold porosity (mean ± SD), incidence of bone formation, and the percentage of bone area (mean ± SD) of the available pore space.
[a]Significantly higher than Mps^{empty} and Mps-BMP^{low} ($p < 0.05$, ANOVA).
Mps, microparticles; BMP, bone morphogenetic protein-2; BMSCs, bone marrow stromal cells.

BMSCs. The porosity of the blank/$BMP^{impregnated}$ and Mps-BMP^{high} scaffolds was significantly higher compared to the Mps^{empty} and Mps-BMP^{low} scaffolds ($p < 0.05$). The bone formation in the Mps-BMP^{high} implants was $2.9 \pm 3.0\%$ (varying from 0% to 7.2%, $n = 8$) and $2.7 \pm 2.4\%$ (varying from 0% to 5.6%, $n = 8$) of the available pore space for the scaffolds without cells and with BMSCs, respectively. BMSC seeding had no effect on bone formation. In the one-PPF implant impregnated with BMP solution, 0.2% of the pore space was filled by newly formed bone.

Discussion

This study demonstrates that PPF scaffolds can be rendered osteoinductive by incorporation of BMP-2–loaded PLGA microspheres. Compared to BMP-2–impregnated scaffolds, microsphere incorporation resulted in a lower burst and a more sustained *in vitro* release over a prolonged period of time. In contrast to the high burst release of the BMP-2–impregnated scaffolds, the sustained BMP-2 release from the PLGA microspheres resulted in a higher amount of ectopic bone in the scaffold pores after 9 weeks of implantation in goats. In the current experimental setup, the addition of autologous BMSCs before implantation had no significant effect on the osteoinductive capacity of the construct.

Previous *in vitro* studies have shown that PLGA microspheres are effective vehicles for sustained delivery of BMP-2.[14,17–19] The BMP-2 retention is based on physical entrapment as well as protein–polymer interactions as a result of ionic, hydrophobic, and/or hydrogen bonding.[17,18] During the fabrication process, the encapsulation and interactions lead to a reasonable encapsulation efficiency of the Mps-BMP^{high} microspheres. Probably due to the strong protein–polymer interactions, only a fraction of BMP-2 could be extracted from the Mps-BMP^{low} microspheres resulting in an underestimation of their entrapment efficiency. An alternative to the extraction/ELISA method for the entrapment efficiency measurements could be radioactive labeling of the BMP-2 prior to incorporation its into the microspheres. This method would obviate protein extraction and could also be used for the *in vitro* and *in vivo* release measurements.[20–26]

The PLGA microspheres were incorporated into the PPF scaffold in an attempt to better maintain local *in vivo* concentrations at osteoinductive levels for sufficient time. The impregnation of PPF scaffolds with a high BMP-2 dose is inherent to minimal retention and failed to consistently produce bone inside the scaffolds. In contrast, BMP-2 incorporation into PLGA microspheres resulted in a gradual *in vitro* release from the PPF composites. This gradual BMP-2 release was probably responsible for bone induction in the higher-loaded microsphere/scaffold composites. Previous ectopic studies in rodents reported that BMP-2–induced bone formation was related to the dosage.[27–31] Apparently, the local BMP-2 concentrations in the composites with a lower loading were not sufficient to induce bone formation. Although the amounts released by the higher-loaded scaffolds were sufficient to induce bone formation, both microsphere formulations released < 2.5% of the incorporated BMP-2 in the first 24 days. Therefore, further optimization of the BMP-2 pharmacokinetics is required to obtain a release profile that coincides better with the normal rate of bone formation.

Surprisingly, the preoperative seeding of autologous cryopreserved BMSCs on the composite formulations in this study did not enhance ectopic bone formation. Although the mechanism of bone induction in ceramics is completely different, the osteogenic potential of autologous BMSCs in goats was shown in ceramic scaffolds where they clearly enhanced ectopic bone formation.[15,32,33] Preoperative seeding of cryopreserved BMSCs resulted in similar amounts of newly formed bone in ceramic scaffolds as in precultured constructs.[33] Therefore, this seeding method was also employed for this study to overcome complicated logistics for the preparation of preoperatively cultured constructs. Although the plasma polymerization during the preoperative seeding method resulted in a 100% cell loading efficiency and retention during implantation procedure, the behavior of the cells *in vivo* on the scaffolds applied in this study is unknown.

The absence of bone formation in the cell-seeded composites without BMP-2 was as expected, since these synthetic polymers do not possess osteoinductive characteristics in contrast to ceramics. Therefore, the initiation of the BMSC osteogenesis in the PPF scaffolds is likely to depend on BMP-2 signaling. However, the absence of an effect of autologous BMSCs on bone formation in BMP-2–loaded scaffolds is less clear. *In vitro* studies have shown that the osteogenic capacity of BMSCs in the presence of BMP-2 is dose dependent.[34,35] Since the histological results show large amounts of nonresorbed PLGA in the PPF matrix after 9 weeks of follow-up, the degradation of the PLGA microspheres and the sub-

sequent release of the BMP-2 from the microspheres might have been too slow. This could have resulted in insufficient BMP-2 concentrations in the early days of implantation to induce differentiation of the seeded cells, since BMSC-derived osteogenesis normally starts within 3 weeks after implantation.[15] In the absence of such an osteoinductive stimulus, the undifferentiated BMSCs could have differentiated to the fibrous tissue in the microsphere/scaffold composite. The bone formation and late ossification by the BMP-2 released at a later time point are probably derived from host cells that were locally recruited from for example the circulation.[36]

In conclusion, this study shows that sustained release of BMP-2 from microspheres contained in PPF scaffolds can induce ectopic bone formation in these scaffolds that are otherwise nonosteoinductive. In contrast to the enhanced ectopic bone formation on ceramic scaffolds by BMSCs, the addition of stem cells to the scaffolds in the current study did not further enhance bone formation, possibly due to a slow rise of the local BMP concentration and a critical delay before the osteoinductive threshold dose is reached. Future studies should be aimed at improving the timing and rate of BMP-2 release to further stimulate the extent and rate of bone formation, either alone or in combination with mesenchymal stem cells.

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