Controlled Release of Growth Factors on Allograft Bone *in vitro*

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Abstract Allografts are important alternatives to autografts for treating defects after major bone loss. Bone growth factors have both local autocrine and paracrine effects and regulate the growth, proliferation, and differentiation of osteoprogenitor cells. To study the effects of prolonged, continuous, local delivery of growth factors on bone growth, we developed a new microelectromechanical system (MEMS) drug delivery device. Bone marrow cells from mice were seeded on mouse allograft discs and cultured in osteogenic media with osteogenic protein 1 (OP-1) and/or basic fibroblast growth factor (FGF-2) delivered from MEMS devices for 6 weeks. We monitored bone formation by changes of bone volume using micro-CT scanning and release of osteocalcin using ELISA. The data suggest the MEMS devices delivered constant concentrations of OP-1 and FGF-2 to the media. Bone marrow cells grew on the allografts and increased bone volume. Addition of OP-1 increased bone formation whereas FGF-2 decreased bone formation. Local delivery of growth factors over a prolonged period modulated the differentiation of osteoprogenitor cells on allograft bone.

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Introduction

Over 1,000,000 bone graft procedures are performed each year in the United States. Autograft, the gold standard, serves as an osteoconductive scaffold and contains both osteoinductive growth factors and osteoprogenitor cells. However, geometrically and structurally appropriate autografts are limited to a few sites and the amount of cancellous autograft is limited; further the required surgical procedure at the donor site increases the risk of blood loss, infection, hematoma formation, and other complications such as chronic pain [1, 24, 26]. One alternative to autograft is allograft, which accounts for over 200,000 bone graft procedures in the United States every year [27]. However, allograft bone lacks viable osteoprogenitor cells and has low levels of growth factors. As a result, the incorporation of allograft bone proceeds more slowly than autograft bone. Synthetic scaffolds could potentially be used to deliver both osteogenic cells and growth factors to large bone defects, although an appropriate completely biodegradable scaffold has not yet found its way into clinical use.

In vitro and *in vivo* studies have confirmed bone-specific growth factors exhibit autocrine and paracrine effects on the proliferation, differentiation, and maturation of osteoprogenitor cells [12]. Fibroblast growth factor basic (FGF-2) is expressed in mesenchymal cells, as well as mature osteoblasts, and is stored in the extracellular matrix [11]. Mouse models and human genetic mutations demonstrate FGF-2/FGFR2 is important for bone formation [8, 25]. Mice with the recessive phenotype of FGF-2IIIc (–/–) have retarded long bone ossification and decreased expression of the Runx-related transcription factor 2 (RUNX2) [8]. Schedlich et al. [23] reported FGF-2 stimulates osteocalcin (OC) with cAMP synergistically.

RUNX2 was also up-regulated by FGF-2 or FGFR2 mutation [34]. This suggested the FGF-2 may directly stimulate the differentiation of osteoprogenitor cells and mainly involves MAP kinases [16]. Osteogenic protein 1 (OP-1, also known as BMP-7) is also osteoinductive [5] and is expressed in the kidney [18] and cartilage [6]. OP-1 stimulates the differentiation of osteoprogenitor cells [4, 7] and has been approved for the treatment of long bone defects in humans since 2002.

Micro- and nanosystems for drug delivery offer the possibility of precise dosage and time control for highly potent drugs [13, 20]. There have been great advances in drug delivery devices made of biodegradable polymers. However, most of these rely on polymer erosion for control of drug delivery [9, 10]. Since this erosion-based control is limited by the properties of the polymers and surrounding tissue, precise control of drug release may not be attainable. Microelectromechanical system (MEMS) technology enables the shaping of microstructures on silicon or polymer chips using etching, deposition, and molding processes adapted from semiconductor technology. MEMS-based drug delivery devices are typically comprised of arrays of microreservoirs loaded with drugs and covered with thin metal or polymer membranes. Selective opening of the membranes by electrochemical reaction or biodegradation delivers defined amount of drugs under precise time schedules. However, to date, these devices have been limited to only pulsatile or short-period release profiles based on the degradation of the polymer used [21].

We have developed biodegradable MEMS devices for delivery of OP-1 and FGF-2 for a prolonged time period in a controlled fashion [22]. The devices deliver a relatively constant concentration of OP-1 and FGF-2 in culture media. An *in vitro* allograft model using murine bone discs was also developed to test the effects of growth factors on osteoprogenitor cells.

We hypothesized continuous delivery of OP-1 and FGF-2 could modulate the growth and differentiation of osteoprogenitor cells using a murine allograft bone model *in vitro*.

Materials and Methods

Micromolds were fabricated by a combination of wet and reactive ion etching processes. 85/15 poly(L-lactide-co-glycolide) (85/15PLGA, Inherent Viscosity = 2.3) was obtained from Purac (Lincolnshire, IL, USA). The polymer was compression molded into 25- and 75- μ m-thick films at 150°C under 1.9 MPa for 2 minutes in a molding press (Carver Auto C press; Carver, Inc, Wabash, IN, USA). A reservoir and microchannels of the micromold were pattern-transferred to the 75- μ m-thick films at 160°C under

1.9 MPa for 2 minutes in the Carver press. The microstructured layer and the other plain layer (25- μ m-thick film) were stacked together with a thin Teflon[®] sheet (McMaster-Carr, Los Angeles, CA) inserted in the reservoir area between the two layers. This assembly was thermally bonded at 70°C under 345 kPa for 5 minutes in a pneumatic press (Model AP7; Air-mite, Chicago, IL) with heating pads (Model EFH SH 4×5-10-115, McMaster-Carr). Later, the Teflon[®] sheet was removed from the assembled device, leaving the reservoir area open for loading of the growth factors. The pastelike mixture of the growth factors was loaded into the reservoir by a syringe. Each device had 20 μ L PEG6000 (0.4 g/mL) only, PEG6000 (0.4 g/mL) plus FGF-2 (85 μ g/mL) or OP-1 solution (170 μ g/mL), or PEG6000 (0.4 g/mL) plus FGF-2 (85 μ g/mL) and OP-1 solution (170 μ g/mL). Devices with PEG only were used as controls. Then, the device was sealed by a heat sealer (Model AN90; Andersen Products, Inc, Haw River, NC, USA). Assembled devices had a reservoir filled with a mixture of growth factors and PEG and four distinct microchannels for the release of growth factors. The microchannels were well preserved without deformation after thermal assembly. The devices were rolled so they could be fit into the wells of the microplates (Fig. 1).

To determine the release profiles of the growth factor-loaded MEMS devices, we incubated the devices in basal medium (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μ g/mL streptomycin [Invitrogen Inc, Carlsbad, CA, USA]) up to 28 days. The media were changed every 4 days and the amount of FGF-2 and OP-1 in the released medium was determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, samples, together with assay diluents, were added into 96-well plates that were precoated with antibodies and incubated for 2 hours at room temperature. After three times washes, conjugates were added into the same plates and incubated for 2 hours at room temperature. Colorific substrates were applied into the plates after three times washes. The optical densities of the protein-antibody complexes were determined using a Spectra M2 microplate reader (Molecular Devices, Downingtown, PA, USA) set at 450 nm and 570 nm.

Institutional guidelines for the care and use of laboratory animals were observed in all aspects of this project. Ten-week-old C57 male mice were euthanized with CO₂ gas and the femora and tibiae were excised and cleaned of soft tissue. Each bone was cut using a Buehler Isomet Plus Precision Saw (Lake Bluff, IL, USA) into 1.5-mm-thick slices made from the metaphyses of the distal femora and proximal tibiae. Slices were taken from the metaphysis to obtain bone discs with trabecular bone in the marrow

Fig. 1A–B (A) Diagrams illustrate the device configuration and principle of operation. The device has a microfabricated reservoir and microchannels in a biodegradable polymer layer. This layer is sealed by another biodegradable polymer layer. Drugs and osmotic agents are loaded in the reservoir. Water flows into the reservoir by osmosis and a hydrostatic pressure builds up in the reservoir leading to inflation and increased pressure within the device. This pressure drives drug molecules through release channels of the device. (B) A photograph shows a sample device and zoomed-in views show a microchannel. Channels are 50 μm wide and 50 μm deep.

space. Each bone disc slice was then quantitatively evaluated at a resolution of 10.5 μm using a Scanco Medical micro-CT (Southeastern, PA, USA). The micro-CT scans allow characterization of the trabecular bone within the medullary space of the bone disc before and after various treatments. The data collected included the total bone volume, the total trabecular number (to determine formation of new trabeculae), and trabecular thickness (indicating new bone deposition on existing trabeculae). All data from each allograft bone disc were saved for later comparisons with postharvest scans after various treatments. After scanning, each graft was placed in a labeled 0.5-mL centrifuge tube with sterile saline and stored at -80°C . Before seeding with cells, the grafts were defatted and sterilized by placing the bone discs in a 1:1 solution of methanol:chloroform for 12 hours and then washed three times in 100% methanol, three times in sterile water, and three times in sterile saline.

To harvest the osteoprogenitor cells, bone marrow was collected from the femora of C57BL male mice aged 10 to 12 weeks old. The mice were sacrificed with CO_2 gas, shaved, and sterilized in 70% ethanol for 5 minutes and then 90% ethanol for 5 minutes before surgery. While maintaining sterile technique, the femora were surgically removed. Using a syringe and 25-gauge needle, the bone marrow was flushed by injecting 4 mL basal medium (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin [Invitrogen Inc, Carlsbad, CA, USA]) through the marrow cavity into a 50-mL centrifuge tube. The cells were spun down and washed three times with basal medium. The cells were then resuspended in the same medium and counted with a hemocytometer and replated in T-75 culture flasks at a concentration of 120×10^6 cells per flask. Cells were allowed to expand in 37°C with 5% CO_2 incubator until confluence reached 75% (about 1 week), with a medium change every 3 days to remove nonadherent cells. The cells were lifted with 0.25% trypsin/EDTA (Invitrogen Inc), washed three times with basal medium, and resuspended in augmented osteogenic medium (basal medium with 50 $\mu\text{g}/\text{mL}$ L-ascorbic acid, 0.01 mol/L β -glycerophosphate, and 10 nmol/L dexamethasone [Sigma Aldrich, St Louis, MO, USA]).

Each allograft bone disc was placed into one well of a 96-well plate. One $\times 10^6$ cells in 300 μL osteogenic medium were added directly onto each bone disc in a dropwise fashion. The plate was placed in the incubator for 72 hours to allow cell attachment to the disc.

After the 72-hour seeding period, each graft was removed and placed into one well of 24-well ultralow cluster plates (Corning Inc, Corning, NY, USA) with the MEMS devices in 2 mL fresh osteogenic medium. Each plate was placed in the incubator and the medium changed every 5 days, and the spent media were collected and stored at -80°C .

After harvest, the seeded bone discs were fixed for 30 minutes in 4% paraformaldehyde. The grafts were then placed in PBS and rescanned by micro-CT. The bone volume, trabecular number, and trabecular thickness were determined again. Data from each graft were then compared to the data from the first scan of that particular graft. This allowed an accurate and quantitative method of evaluating any changes in bone volume and trabecular characteristics of the allograft bone discs.

For quantification of OC in the medium, we used an OC ELISA kit from Biomedical Technologies Inc, Stoughton, MA. We followed the manufacture's protocol carefully. The optical densities were determined using a Spectra M2 microplate reader set at 450 nm.

Data were reported as mean $\pm$ standard error. We used a one-way analysis of variance (post hoc multicomparisons

with Tukey test) to compare differences in osteocalcin releases and changes of bone volume between control and treatment groups. To evaluate the release profiles of the OP-1 and control groups, the slopes of the curves and areas under the curves (AUC) were used to describe the rate and amount of OC released. We used SPSS 14.0 (SPSS Inc, Chicago, IL, USA) for all analyses.

Results

The MEMS devices delivered constant concentrations of OP-1 and FGF-2. The concentration of FGF-2 in the media was maintained at approximately 15 ng/mL while the OP-1 had a relatively constant release of approximately 4 ng/mL. We noted minor ($p > 0.05$) variation between samples at different time points after Day 4 (Fig. 2.).

Bone marrow cells differentiated on allografts. After 6 weeks of culture with bone marrow cells, there was an increase ($p < 0.01$) of approximately 55% in bone volume

of the allografts (Figs. 3, 4), accompanied by increases of both trabecular number ($p < 0.01$) and thickness ($p < 0.01$). The release profile of OC demonstrated a detectable level at Day 15. OC levels remained high and increased continuously after Day 20, which agrees with previous experiments using similar conditions on culture plates alone [12].

OP-1 increased bone formation on allografts. At the end of the 6-week culture period, both bone volume ($65\% \pm 7.5\%$) and released OC level (60.1 ± 8.2 ng/mL) in the OP-1 group were higher ($p < 0.01$ and $p = 0.51$, respectively) than in the control group ($55\% \pm 3.7\%$ and 50.3 ± 8.5 ng/mL, respectively) (Figs. 3–5). The average trabecular thickness of the OP-1 group was slightly higher than the control group. The OC levels at Day 20 of the OP-1 group were lower ($p < 0.05$) compared to the control group, suggesting a delay in differentiation. The slope of

Fig. 2 A graph shows the release profiles of FGF-2 and OP-1 from the devices. The released FGF-2 and OP-1 from MEMS devices were measured by an ELISA kit that complexed the growth factor with antibody. The profiles demonstrated relatively constant release of both growth factors ($n = 4$).

Fig. 4 A graph of the changes in bone volume (BV), trabecular thickness (TbTh), and trabecular number (TbN) of allografts using micro-CT after 6 weeks of cell culture. Bone volumes in the control, OP-1, and FGF-2 groups were increased 55%, 65%, and 14% respectively. ($n = 4$; * $p < 0.05$ versus the FGF-2 and FGF-2/OP-1 groups, # $p < 0.05$ versus FGF-2 and FGF-2/OP-1 groups, & $p < 0.05$ versus the FGF-2 group; one-way analysis of variance).

Fig. 3 Visualization of the changes in bone volume by micro-CT is shown. The allografts seeded with murine osteoprogenitor cells were scanned by micro-CT before and after the 6-week cell culture period. The results demonstrated bone increase in the control group, with further bone increase with OP-1 and less increase with FGF-2.

Fig. 5 A graph shows the release profiles of OC from bone marrow cells during cell culture. In the control group, OC demonstrated a detectable level at Day 15 and increased continuously after Day 20. OC in the OP-1 group began appearing from Day 20 and increased quickly thereafter. There was no OC detected in the FGF-2 group. (n = 4; *p < 0.05 versus control group; one-way analysis of variance).

control group (0.44 ± 0.03) was lower ($p < 0.05$) than the OP-1 group (2.79 ± 0.37), while the AUC of both groups demonstrated no substantial differences, which indicated cells in the OP-1 group differentiated faster, although later, than cells in the control group.

FGF-2 decreased bone formation on allografts. Both micro-CT data and released OC demonstrated large decreases in bone formation on allografts in the FGF-2 and OP-1/FGF-2 groups, compared with the control and OP-1 groups (Figs. 3–5). The bone volume of the FGF-2 group increased 14%, while the OP-1/FGF-2 group increased about 17%. The bone volume of both FGF-2 and OP-1/FGF-2 groups was significantly lower than that of both OP-1 and control group ($p < 0.05$) (Fig. 4). No released OC could be detected in either the FGF-2 group or the OP-1/FGF-2 group (Fig. 5).

Discussion

The devices used in the current experiments are different from conventional biodegradable drug delivery systems. While the release of growth factors from conventional systems is controlled by degradation of polymers, our devices are controlled by the microgeometries of the devices and the permeability of the polymer used. Degradation of the devices occurs after drug release is completed. The potential advantages of this decoupling between drug release and device degradation include improved control and accuracy of drug release that can be preprogrammed, avoidance of the acidic environment from polymer degradation, and avoidance of a reaction between the drugs and the degraded polymers [3, 15].

Although only a single release profile (continuous) was used in this experiment, the delivery device can be

modified so multiple different drug release profiles, including different start times, durations, and dosages, can be examined. For example, a single device containing two drug reservoirs with individual release channels of different lengths can release two different drugs at different time points and concentrations. The stiffness of the devices employed in these experiments limits their usage for in vitro studies. New, more flexible materials will be needed for in vivo use. Our in vitro allograft model also has the same limitations as other in vitro models: the lack of stimuli from surrounding tissues/organs such as paracrine, endocrine and other influences that may affect chemotaxis, proliferation and differentiation of the cells on the allograft scaffold. However, the model and drug delivery device provide a “proof of principal” in which the local delivery of growth factors may be accomplished in a more rational, cost-effective way with potentially reduced adverse effects.

The released OC and micro-CT data demonstrated the seeded bone marrow cells differentiated into bone cells and generated new bone on the allografts. Compared with the control group without growth factor, addition of OP-1 stimulated an additional 10% increase in bone formation, which agrees with our previous experiment, in which OP-1 was pipetted at 330 ng/ml daily (data not shown), and studies from other laboratories demonstrating OP-1 stimulates the differentiation of osteoprogenitor cells [2, 28, 29, 31–33]. Differentiation of the osteoprogenitor cells without addition of growth factors was synchronous with an increase of OC between Days 15 and 20 followed by a slower increase later on (slope of 0.44) (Fig. 5). With the addition of OP-1, cellular differentiation and OC expression became asynchronous, demonstrating a slower increase of OC release from Day 15 despite continued cellular differentiation.

Interestingly, in this short-term in vitro experiment, FGF-2 inhibited new bone formation markedly. Previous studies by our group [17] with the same allograft model with daily addition of FGF-2 manually (165 ng/ml) (data not shown) reported high cellularity histologically with little new bone formation. The addition of FGF-2 in this model appears to maintain the osteoprogenitor cells in a proliferative state, delaying differentiation. This is confirmed by a recent study demonstrating FGF-2 inhibited the differentiation of adipose tissue-derived stromal cells into osteoblasts [19]. While different experimental models and drug release schedules may explain some of the findings, the timing of adding FGF-2 may be one of the key factors related to the outcome. FGF-2 from bone marrow cells cultured in osteogenic media is expressed only in the stage of differentiation [12]. Therefore, the continuous addition of FGF-2 in this experiment appears to prevent or delay the osteoprogenitor cells from entering the differentiation stage.

Various growth factors, such as IGF-1 [30], IL-6 [32], BMP-2 [35, 36], and CDMP-1 [31], were reported to enhance the effects of OP-1. Two recent studies reported FGF-2 also inhibited the stimulatory effects of OP-1 [4, 14]; however, we found neither synergic nor antagonistic effects between OP-1 and FGF-2 in the present experiment, which is consistent with a previous *in vivo* study [12].

Our drug delivery device, combined with an *in vitro* allograft model, provided a novel tool to study growth factor regulation of differentiation of osteoprogenitor cells. Our approach is a first step to address drug delivery in allografts. The difference between this model and the clinical situation is in our model we simulate continuous release of growth factors (similar to what occurs physiologically) whereas in the clinical situation growth factors were added once at surgery but in much higher concentrations and therefore the growth factors are degraded quickly. In future experiments we will modify the devices to release different growth factors at their specific physiologic profiles that will decrease the dosages and cost of exogenous growth factors, alleviate the adverse effects of high concentration of growth factors and hopefully be more potent stimulating bone formation. This research has the potential to improve allograft incorporation into host bone in clinical situations of bone loss.

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