Successful repair of large bone defects in orthopedic trauma remains a clinical challenge. Although bone possesses repair capacity, it is often insufficient to overcome large bone loss. In most cases, surgical intervention is required to augment the bone healing process. Clinical approaches involve the use of vascularized and non-vascularized bone grafts or the application of growth factors such as recombinant human bone morphogenetic protein 2 (rhBMP2) and collagen. Clinical success has usually been limited by the necessity of harvesting autologous cells and/or vascularized bone grafts. Both cell-based therapies and non-viral (plasmids) gene delivery systems have been explored using a variety of systems including viral (retrovirus, adenovirus, and AAV) and non-viral (plasmids) gene delivery systems. The limited transduction efficacy in vivo and risk of chromosomal integration have limited the potential of these approaches.

In this study, we developed a cell-based molecular therapy that allows the use of allogeneic cells transduced with AdBMP2, thus circumventing problems associated with donor site morbidity. The use of osteoinductive proteins such as rhBMP2, released in vivo to augment bone regeneration, has been developed for clinical use. Unfortunately, large amounts (6–12 mg) of rhBMP2 protein are required to achieve a reliable bone induction and delivery of the large amounts of this protein has led to production of antibodies as well as osteolysis of the bone often before it has completely repaired as well as other adverse effects. To circumvent these problems much emphasis has been focused on linking the protein to a variety of matrices that would allow for a slower release over time. These systems are problematic due to the difficulty in cross-linking large amounts of the protein to the biomaterial, which would release the protein slowly over time in an active form. For these reasons, molecular therapies to provide long-term secretion of BMP2 at physiological levels from human cells has been explored using a variety of systems including viral (retrovirus, adenovirus, and AAV) and non-viral (plasmids) gene delivery systems. Previous research has also shown that injected PEGDA hydrogels also allow for sufficient release of therapeutic proteins, like insulin, for diabetic therapy and BMP2 for bone formation. In our system, the polymer mesh size is such that BMP2 can diffuse out of the microbeads but neither immune-activated host

---

Grant sponsor: Defense Advanced Research Agency (DARPA); Grant number: W911NF-09-1-0040; Grant sponsor: Department of Defense Orthopedic Extremities Program (DAMD); Grant number: W81XWH-07-1-0215.

Correspondence to: Elizabeth A. Olmsted-Davis
(T: 713-798-1253; F: 713-798-1230; E-mail: edavis@bcm.edu)
© 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.
cells nor immunoglobulin can diffuse in, therefore offering protection of the microencapsulated cells from the host immune system.

Here we extend these studies by demonstrating that delivery of low doses (96 ng/day) of endogenously produced BMP2 leads rapid and reliable bone formation and healing in a critical size femur defect. The rate of healing occurred as early as 3 weeks, or more rapid than similar experiments using high dose recombinant BMP2. In these studies injection of these microspheres into the void region led to rapid healing of a 5 mm femoral defect. This newly formed bone was well-integrated at the defect edges with the skeletal bone, and allowed the animals to ambulate normally without additional fixation as early as 2 weeks after delivery. These results indicate that these osteoinductive microspheres may be a safe and efficacious substitute for rhBMP2 and provide a clinically novel option to bone graft in the regeneration of traumatic bone defects.

MATERIALS AND METHODS

Cell Culture

Wistar skin fibroblasts (WSF) were generated from a skin biopsy from a Wistar rat. W20-17, a murine bone marrow stromal cell line, was obtained as a gift from Genetic Institute, Cambridge, MA and was propagated as previously described.23 All cells were propagated in a humidified incubator at 37°C/5% CO₂ in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100,000 U/L penicillin, 100 mg/L streptomycin, 0.25 mg/L amphotericin B (Invitrogen Life Technologies, Gaithersburg, MD), and 3 mg/L tetracycline (Sigma).

Adenovirus and Cell Transduction

A replication defective first generation human type 5 adenovirus (Ad5) deleted in regions E1 and E3 was constructed to contain the cDNA for human BMP2 in the E1 region of the viral genome.24 The control vector included the same adenovirus but lacked a transgene cassette (Ad5empty cassette). The virus particle (vp) to plaque-forming unit (pfu) ratios of Ad5BMP2 and Ad5empty cassette were 1:120 and 1:106, respectively, and all viruses were shown to be negative for virus but lacked a transgene cassette (Ad5empty cassette). The virus particle (vp) to plaque-forming unit (pfu) ratios of Ad5BMP2 and Ad5empty cassette were 1:120 and 1:106, respectively, and all viruses were shown to be negative for replication-competent adenovirus. WSF were transduced with Ad5BMP2 or Ad5empty cassette control virus at a concentration of 5×10⁵ cells per 1 ml of hydrogel precursor solution into the mineral oil, emulsifying by vortex for 2 s while exposing to white light for an additional 20 s. Microspheres were isolated through centrifugation at 330g.

Preparation of Cells for Injection in the Defect

Ad5BMP2 or Ad5empty cassette transduced cells were suspended in PBS and directly injected using a 22 gauge needle and syringe at a concentration of 5×10⁵ cells per 1 ml of PBS. Microencapsulated cells were prepared at a concentration of 2×10⁶ cells per 100 μL of hydrogel and a total volume of 1 ml of PBS containing microencapsulated cells were loaded into a syringe with an 18-gauge needle.

Quantification of BMP2 Protein and Activity After Transduction of Cells With Ad5BMP2

BMP2 protein was measured in culture supernatant 24 h after transduction of cells with Ad5BMP2 using a Quantikine BMP2 immunoassay ELISA kit (DBP200; R&D Systems, Minneapolis, MN). BMP2 biological activity was quantified in culture supernatant 24 h after transduction by alkaline phosphatase (AP) assay using W20-17 cells.26 Briefly, W20-17 cells were plated in 24-well plates at subconfluent density and 24 h later, the media was replaced with 200 μL of fresh media and 200 μL of conditioned culture media. W20-17 cells were then assayed 72 h later for AP activity using a chemiluminescence procedure as previously described.24

Rat Femur Critical Size Defect Model

Wistar rats between 9- and 11-week-old (350–440 g) were shaved, disinfected, and a linear incision made over the lateral aspect of the gluteal region of the rat from the palpable greater trochanter of the femur to just above the knee joint. The musculature was separated to expose the femur. Four 6” long 0.45” (1.1 mm) diameter end-threaded K wires were placed in the bone at a 90° angle: two in the proximal and two in the distal end of the femur 2 mm apart. All pins were securely anchored within a tubular external fixation device placed along the length of the femur. A 5 mm osteo-periosteal defect was made in the middle part of the bone using a high-speed burr. At the time of surgery, microencapsulated cells were then injected into the defect void by placement into a sutured muscle pocket. Animals were allowed to recover, and resume normal activity. Rats tolerated this device well with <5% loss of animals due to failure in the fixation. All treated rats were capable of walking and full weight bearing after surgery. Radiological, histological, and biomechanical analyses have been performed at various time points after surgery (2, 3, 6, 9, and 12 weeks). All animal studies were performed in accordance with the standards of Baylor College of Medicine, Department of Comparative Medicine, after review and approval of the protocol by the Institutional Animal Care and Use Committee (IACUC).

Radiographic Evaluation and Microcomputed Tomography

At various time point of the study, animals were radiographed while under anesthesia using an XPERT model faxitron (Kubtec, Fairfield, CT) in blipanar projections. Hind limb of the animal was set at an exposure of 15 s and acceleration voltage of 30 kV. Radiographic analyses were performed using microcomputed tomography (microCT) system. Specimens were scanned at 0.1 mm in plane resolution with 0.25 mm thick contiguous slices using a Stratec XCT.
RESEARCH SA PLUS pQCT SCANNER from Orthometrix, Inc. (White Plains, NY). This scanner provides data calibrated to bone equivalent density. Three dimensional reconstructions and multi planar orthogonal reconstructions were used to visualize new bone bridging the defect. Total volume of new bone within the defect as a function of time was calculated from 3D rendering using a mineral density threshold of 600 Hounsfield units.

Biomechanical Analysis
Hind limbs (defect and intact contralateral) were harvested at 3 and 12 weeks \((n = 5)\), soft tissues removed and the proximal and distal ends of the femurs embedded in poly(methyl methacrylate) (PMMA) perpendicular to their long axis and adjusted to a plumb line oriented to the center of rotation of the testing system (Universal MTS Bionix 3700). The specimens were mounted using clamps and torsion was applied in a displacement control mode at a constant rate of \(0.5 \, \text{s}^{-1}\) in direction replicating femur external rotation. Torsional load-to-failure was calculated and stiffness established from the most linear region of the load–deflection curve and ultimate strength was defined as the peak load causing angular displacement >30˚.

Histological Analysis
Animals were euthanized at 2, 3, 6, 9, or 12 weeks postsurgery, hind limbs isolated and external fixator removed leaving the pins in place. The whole limb was then formalin fixed and decalcified (Richard-Allan Scientific Decalcifying Solution, Thermo Scientific, Kalamazoo, MI) for 6 days. The remaining pins were removed, tissue was trimmed for mounting in the cassettes and then processed and paraffin embedded. Serial sections (4 \(\mu\)m) covering the whole femur area were prepared and every fifth section were stained with hematoxylin and eosin for histological analysis. The image reconstruction was done by assembling sequential images covering the whole area of interest (2× magnification each image).

Statistical Analysis
The results are reported as mean ± SD. For BMP2 ELISA and AP assay, \(n = 6\) and a standard \(t\)-test determined significance \(p < 0.005\), “\(p < 0.001\). For stiffness and strength data, \(n = 3\) and a standard \(t\)-test were performed. For bone volume data, \(n = 3\) and ANOVA test determined significance \(p < 0.05\).

RESULTS
Bone Formation Within the Femur Defect Requires PEGDA Hydrogel
Figure 1A shows a schematic of our approach. Briefly, allogeneic skin fibroblasts were transduced with Ad5BMP2 and 24 h later microencapsulated through addition of a hydrogel precursor solution (PEGDA) in a mineral oil emulsion, forming cross-linked microsphere structures upon exposure to white light. This injectable therapy is delivered into the target site of a critical size defect in the rat femur. The femur was chosen because it is the largest weight-bearing bone in the body and is one of the most challenging for clinical repair. In this model, threaded pins were placed across the ends of the femur allowing for a 5 mm gap to be created in the midshaft of the bone. The pins were held in place by an external fixation device so that the rat could ambulate without either compression of the defect or significant motion. Osteoinductive microspheres are then directly injected into the femur defect (Fig. 1A). Each microsphere measures between 50 and 350 \(\mu\)m as shown in Figure 1B.
We first performed dose escalation studies. No bone formation was observed in the defect in animals receiving $2 \times 10^6$ encapsulated cells (Fig. 2A) whereas complete healing was achieved with $2 \times 10^7$ microencapsulated cells (Fig. 2B). Quantitation of BMP2 levels released from osteoinductive microspheres in culture indicate that 48 ng of BMP2 was produced per $10^7$ encapsulated cells over a 24 h period (Fig. 2E). Upregulation of endogenous cellular AP activity is commonly used for the assessment of osteogenic differentiation of stem cells.26,29 AP assay showed that BMP2 produced by the microspheres possesses bioactivity similar to the recombinant protein (Fig. 2F). We next evaluated bone formation and healing in the defect using the osteoinductive microspheres, as compared to a similar number of Ad5BMP2 transduced cells, which were not encapsulated in PEGDA hydrogel. When encapsulated BMP2-producing cells were directly injected into the defect, there was no apparent bone formation and/or healing (Fig. 2C); while the same cells, when microencapsulated, were able to induce bone formation (Fig. 2D). As expected, there was no bone healing in control animals that received Adempty transduced cells microencapsulated in the PEGDA hydrogel and the defect formed a non-union fracture (data not shown).

New Bone Bridged the Defect and Integrated With Native Bone
Consistent bone formation was observed at the targeted site at 3 weeks after surgery by X-ray (Fig. 3A) and microCT (Fig. 3F). At this time there was bone bridging the defect in 95% of the animals compared to 76% at 2 weeks (Fig. 3K). This bridging bone did not appear to have a cortical exterior (Fig. 3F) but rather a more immature trabecular nature. However, by 6 weeks (Fig. 3B and G) the new bone appeared to have a cortical exterior and trabecular interior that bridged the defect without gaps. This bone remained consistent in samples isolated at later time points 6–12 weeks (Fig. 3B–D and G–I). The bone formation appeared finite rather than continually invasive and all rats continued to appear healthy, ambulate normally, and had no additional injuries. Neither bone formation nor spontaneous healing was observed in any of the seven control animals (Fig. 3E and J).

Bone healing was studied longitudinally, in a subset of animals, to evaluate any changes or adverse events occurring from injection of microspheres (Fig. 4). In these studies the external fixator was removed 2 weeks after surgery without disruption of the newly forming bone (Fig. 4A), and the rat was X-rayed, and then followed radiographically at 2, 4, 12, 22, 34, and 45 weeks (Fig. 4A–F). The shape of the new bone did not change significantly from 2 weeks to 11 months, as seen in Figure 4. However, the bone became more mineralized with time, suggesting that remodeling was occurring.

When analyzed by histology bone filled the defect at 3 weeks (Fig. 5A). Microspheres were observed at the periphery and newly formed bone is also observed around the microspheres. Interestingly, new bone appears to be integrated or fused with the intact femur suggesting complete bone healing and supporting the results seen by microCT. However, at 3 weeks, some areas of cartilage and immature bone were observed. Also at 3 weeks bone appeared to be less remodeled, although there was the appearance of early cortical bone at the defect edges, suggesting the callus is still undergoing remodeling. At 6 weeks, the bone appeared to be more remodeled, with a cortical exterior and internal marrow cavity. Although immature bone -mineralized

Figure 2. Osteoinductive microspheres induce bone formation in a model of femoral critical size defect in rat. (A and B) Radiographs 3 weeks after introduction of critical size defect in the rat femur and delivery of varying numbers of BMP2 expressing cells within microspheres; (A) $2 \times 10^6$ cells and (B) $2 \times 10^7$ cells. (C and D) Radiographs at 2 weeks after surgery and after injection of Ad5BMP2 transduced cells (C) or microencapsulated Ad5BMP2 transduced cells (D). (E) ELISA showing the amount of BMP2 protein required for response. Statistically significant changes, as denoted by an asterisk, were determined using a standard t-test; $p = 0.0001; n = 6$. (F) Alkaline phosphatase activity in W20-17 cells after addition of conditioned media from Ad5BMP2- or Ad5Empty cassette-transduced cells (7,500 vp/cell) in monolayer or microencapsulated (microspheres). Statistically significant changes, as denoted by an asterisk, were determined using a standard t-test; ***$p < 0.0001; NS$, $p = 0.0781; n = 6$. 

JOURNAL OF ORTHOPAEDIC RESEARCH MONTH 2013

SONNET ET AL.
cartilage was still observed (Fig. 5B). At 12 weeks, no more cartilage is seen in the area of newly formed bone (Fig. 5C), and there is now a well-developed cortical exterior and trabecular interior as noted in the microCT images. Although not shown in this photomicrograph there was a contiguous marrow cavity.

**Biomechanical Analysis of the New Bone, Suggests That It Rapidly Obtains Strength Comparable to the Intact Femur**

Biomechanical torsional testing was performed on the contralateral intact and defect femurs isolated from the rats 3 and 12 weeks after surgery. The average torsional stiffness at 3 and 12 weeks were $0.026 \pm 0.011$ and $0.061 \pm 0.015$ N m/degree and 93% and 121% of the contralateral intact femur, respectively (Fig. 6A). The average torsional strength at 3 weeks was $0.35 \pm 0.13$ N m yielding 79% of the contralateral intact femur (Fig. 6B). The defect femur strength persisted and at 12 weeks, the average torsional strength was $0.41 \pm 0.12$ N m or 75% of the contralateral femur, suggesting that bone was continuing to be maintained within the defect. There was no statisti-
cally significant difference in stiffness and strength between defect and intact femur at 3 versus 12 weeks ($p = 0.2781$ and $p = 0.3390$, respectively).

MicroCT demonstrated well-mineralized bone formed within the defect as early as 3 weeks post-surgery. The new bone bridged the defect and was seamlessly integrated with the native bone at the defect ends. The process of new bone formation was robust, consistent, and conformed to defect site. The new bone exhibited homogeneity in morphology and the mineral content. At 2, 3, 6, 9, and 12 weeks intervals post-surgery, a steady increase in the average volume of new bone formation within the defect was observed (Fig. 6C). There was statistically significant twofold increase in average volume of new bone in the defect throughout the course of the experiment (2 weeks vs. 12 weeks, $p = 0.0181$). Furthermore, this increase in new volume correlated well with the tortional strength of the defect treated with osteoinductive microspheres ($R^2 = 0.6084$, $p = 0.025$).

**DISCUSSION**

The data collectively demonstrates rapid bone healing of a critical size femur defect in wild type rats after injection of osteoinductive microspheres. Our approach shows considerable benefits compared to other studies using BMP2.

First, we employ cells that continually express lower levels of BMP2 (96 ng/day) which are approximately 100-fold lower than similar studies using recombinant protein. This level of BMP2 is similar to the amount of protein that is incorporated and potentially released from bone during fracture. We have previously shown that transgene expression from the cells was sustained and locally retained within the microspheres for up to 15 days. Therefore, the sustained delivery of BMP2 at these lower levels will potentially avoid adverse events associated with the recombinant BMP2 protein.

Secondly, these osteoinductive microspheres rely on transduction of allogeneic cells as “carriers” for the BMP2 transgene. Since these cells cannot contribute directly to the bone formation, due to the PEGDA hydrogel encapsulation, there is no requirement for including stem cell populations and thus allows for the use of a previously qualified allogeneic cell line. Therefore, there is no limitation in the cell source and the materials can be manufactured and cryopreserved so that they are ready at the desired time of surgery.
These studies are the first proof of concept studies that low sustained delivery of BMP2 can lead to reliable and rapid bone heals a critical size femur defect in rats. Delivery of the microspheres resulted in rapid new bone formation only in the targeted bone void region. The results of biomechanical testing suggested that the callus was well-integrated with the pre-existing skeletal bone with torsional strength and stiffness recovering to 79% and 93%, respectively, as compared with the intact contralateral femur. This reacquisition of strength and stiffness is dramatic and appears as early as 3 weeks after the injection of the osteoinductive microspheres. The new bone formation within the defect was also followed through microCT and found to be increasing even at latter time points where histology did not show significant cartilage. This may in part be due to continued conversion of cartilage and immature bone to more well-mineralized bone. However, significant variation within each animal group suggests that the rate of bone formation and remodeling may be somewhat animal dependent. Previous studies using high dose recombinant BMP2 did not obtain similar bone repair before 12 weeks. The data again, suggests that the sustained expression of BMP2 at physiological levels may be significantly more effective at bone healing than its recombinant counterpart.

Histological evaluation of the callus appeared to progress as expected in fracture repair, with only minor amounts of cartilage detectable after 6 weeks. The bone appeared to undergo some remodeling exhibiting a uniform trabecular nature at 2 weeks to a more organized structure with a cortical exterior and remodeled bone marrow cavity that becomes apparent in the 6-week samples. The integration of new bone with the previous skeletal bone was observed as early as 2 weeks and longitudinal X-rays of femur healing showed that after 6 weeks the bone appeared to have a stable shape and size. Interestingly, the bone did not return to the exact shape of the contralateral femur, which may be in part due to the presence of the nonresorbable microbeads that remain in the location which may be in part due to the presence of the nonresorbable microbeads that remain in the location for up to 2–3 years within the newly formed bone. However the bone was stable and we observed no other adverse events associated with the animals even as long as 11 months.

This system holds potential for clinical translation. We recently have found that this system is capable of inducing soft tissues regeneration, including the rapid generation of new vasculature and nerves. These studies confirmed that our system was capable of recapitulating the biological functions of endogenous BMP2. Thus, these microspheres may also provide substantial soft tissue benefits. Further, antibiotics such as vancomycin could easily be incorporated into the PEG to provide a mechanism for treatment of infection. Thus, delivery of these microspheres may provide benefit towards both bone and soft tissue regeneration which is essential for functional limb salvage.

Current clinical approaches for healing traumatic bone injuries involve a multi-step process by which the bone defect is maintained with a spacer, while the soft tissues are allowed stabilize and infection is treated. With the unique properties of the PEGDA hydrogel, the microspheres could be injected multiple times without launching a secondary immune response. Therefore, the microspheres could be delivered several times to augment current clinical approaches or alternatively, could be engineered to potentially provide a one-step process. In addition to the potential to incorporate antibiotics, these microspheres can be designed to be totally degradable, through inclusion of a cellular “safety switch” that will induce cellular apoptosis in the carrier cells before the hydrogel degradation. The biomaterial can then be selectively degraded through inclusion of specific protease sites, that allow for tunable degradation after the injury is healed. Finally, we have already demonstrated the ability for these microspheres to be cryopreserved, without change in efficacy, and thus could be easily manufactured and distributed clinically.

ACKNOWLEDGMENTS

This work was supported in part by grants from Defense Advanced Research Agency (DARPA) W911NF-09-1-0040 and Department of Defense Orthopedic Extremities Program (DAMD) W81XWH-07-1-0215. The authors thank Angie Puentes for performing the microcomputer tomography, Rita Nistal for the histology, and Francis Gannon for his advices on histology.

REFERENCES


