

Healing of Critically Sized Femoral Defects, Using Genetically Modified Mesenchymal Stem Cells from Human Adipose Tissue

BRETT PETERSON, M.D.,¹ JEFFREY ZHANG, D.D.S., Ph.D.,¹ ROBERTO IGLESIAS, M.D.,¹
MICHAEL KABO, Ph.D.,¹ MARC HEDRICK, M.D.,² PROSPER BENHAIM, M.D.,^{1,2}
and JAY R. LIEBERMAN, M.D.¹

ABSTRACT

The FDA has approved the clinical use of recombinant bone morphogenetic proteins (BMPs). However, the use of recombinant BMPs in humans has required large doses of the proteins to be effective, which suggests that the delivery method of bone morphogenetic proteins needs to be optimized. Gene therapy is an alternative method to deliver such recombinant proteins, and gene transfer techniques have been tested on a variety of cell types including bone marrow cells, skin fibroblasts, peripheral blood monocytes, and muscle-derived cells. In this study, we sought to determine the ability of BMP-2-producing human adipose-derived mesenchymal stem cells to heal a critically sized femoral defect in a nude rat model. After approval by the human subjects protection committee, human adipose tissue was obtained from healthy donors. The lipoaspirate was processed as previously described (De Ugarte, D.A., *et al.* *Cells Tissues Organs* 174, 101, 2003). Cells were grown in culture and infected with a BMP-2-carrying adenovirus. Five million cells were applied to a collagen-ceramic carrier and implanted into femoral defects as previously described (Zuk, P.A., *et al.* *Mol. Biol.* 13, 4279, 2002). All animals were killed at 8 weeks. Femora were dissected out and underwent radiographic, histologic, and biomechanical analysis. Eleven of the 12 femora in the group treated with human processed lipoaspirate (HPLA) cells genetically modified to overexpress BMP-2 had healed at 8 weeks. This was assessed by radiographs, by mechanical testing, and by histology. The one femur that did not heal had a subacute infection. All eight of the femora treated with the rhBMP-2-impregnated collagen-ceramic carrier healed. No statistically significant difference was detected between these two groups. Evaluation of the control groups: group II (collagen-ceramic carrier with HPLA cells) and group III (collagen-ceramic carrier alone) showed that none of the femora had healed by 8 weeks. Our results indicate that HPLA cells genetically modified by adenoviral gene transfer to overexpress BMP-2 can induce bone formation *in vivo* and heal a critically sized femoral defect in an athymic rat. The HPLA cells alone did not induce significant bone formation. However, when combined with an osteoinductive factor these cells may be an effective method for enhancing bone healing and the tissue engineering of bone.

¹UCLA Department of Orthopedic Surgery, and ²UCLA Department of Plastic Surgery, David Geffen School of Medicine at UCLA, Los Angeles, California.

INTRODUCTION

MESENCHYMAL STEM CELLS have been detected in human adipose tissue.¹ These adipose-derived mesenchymal stem cells have the potential to differentiate into bone, cartilage, or muscle when grown in the appropriate tissue culture medium.^{2,3} Interestingly, these cells are relatively easy to expand in culture and exhibit low rates of senescence even after 15 or more passages.^{4,5} The potential to harvest stem cells from adipose tissue is particularly promising because adipose tissue is in abundant supply and can be harvested with minimal morbidity via liposuction. Here we report that human adipose tissue-derived mesenchymal stem cells can be genetically modified to overexpress bone morphogenetic protein 2 (BMP-2) and to induce healing of critically sized femoral defects in athymic rats.

The tissue engineering of bone requires a combination of osteoinductive factors, osteoprogenitor cells, a matrix to serve as a scaffold for cells, and an adequate blood supply. Seminal experiments by Urist and co-workers demonstrated that bone contained osteoinductive molecules, which Urist termed bone morphogenetic proteins (BMPs).^{6,7} The osteoinductive potential of recombinant BMPs has been demonstrated in animal models⁸⁻¹² as well as in clinical studies.¹³⁻¹⁵ The Food and Drug Administration (FDA) approved the use of recombinant human BMP-2 for spine fusion and granted a humanitarian device exemption for the use of BMP-7 (OP-1) to treat recalcitrant nonunions. However, the results of clinical trials have been somewhat disappointing, especially because the use of recombinant BMPs in humans requires large doses of the protein to be effective.¹⁵⁻¹⁷ These results suggest that the collagen carriers presently being used are not the most efficient protein delivery vehicles. Furthermore, although the high doses of recombinant BMPs induce bone formation, these recombinant proteins are expensive, and there are concerns about potential oncogenic effects, especially if redosing is necessary. Clearly, the delivery of these recombinant proteins needs to be optimized.

Concurrent with the work on osteoinductive molecules, some investigators have pursued what are termed "cell-based" strategies to induce bone formation. This work has focused on the delivery of osteoprogenitor cells rather than osteoinductive molecules. In a variety of animal models, bone marrow-derived mesenchymal stem cells, administered with an appropriate carrier, have demonstrated the ability to heal critically sized bone defects.¹⁸⁻²¹ Even in large animal models such as a canine femoral defect model, Bruder and Fox were able to demonstrate that autologous mesenchymal stem cells could induce healing of defects that otherwise progressed to nonunions.²¹ The use of autologous mesenchymal stem cells to treat bone defects is appealing from a safety

standpoint and also because bone defects frequently occur in locations where there is a paucity of osteoprogenitor cells. However, these autologous bone marrow-derived mesenchymal stem cells have not yet been demonstrated to be effective in patients, and many clinicians and investigators theorize that both an osteoinductive signal and osteoprogenitor cells may be needed to heal large bone defects or bone defects associated with a compromised vascular supply.

Genetic enhancement of mesenchymal stem cells could potentially increase their osteoinductive ability. *Ex vivo* gene therapy is a strategy to deliver both osteoprogenitor cells and an osteoinductive factor to a specific anatomic site. In *ex vivo* gene therapy, cells are harvested, processed, and expanded in culture. In culture, the cells can be genetically modified to overexpress an osteoinductive signal such as one of the BMP molecules. The modified cells can be loaded onto an osteoconductive scaffold and then implanted at a bone defect site.

One potential advantage of *ex vivo* gene therapy strategies over *in vivo* strategies is that the cell type to be used as the delivery vehicle can be selected and expanded in culture. Most work with mesenchymal stem cells has been done with bone marrow-derived cells,^{20,22,23} but investigators have demonstrated that muscle, skin, and even fat may have potential.²⁴⁻²⁷ In our laboratory, we have successfully used BMP-2-producing bone marrow-derived cells via adenoviral gene transfer to heal critical-sized bone defects and to induce spinal fusion in rodent models.^{28,29} Other investigators have demonstrated that genetically modified muscle-derived cells can induce bone formation and also become incorporated in the new bone that is formed.^{24,25,30,31}

Zuk *et al.* have demonstrated that adipose tissue contains a population of cells that show multimesenchymal lineage potential *in vitro*.² Given the appropriate medium, these cells have the ability to differentiate into an osteoblastic lineage. In addition, these cells can be easily expanded in culture and infected with adenoviral vectors, and in contrast to bone marrow-derived mesenchymal stem cells, the number of mesenchymal stem cells in fat does not seem to decline with age.³ This makes adipose tissue an attractive source of mesenchymal stem cells. Another advantage of using mesenchymal stem cells such as those derived from adipose tissue is that both paracrine and autocrine responses can be elicited.³²⁻³⁴ The transduced adipose-derived stem cells not only secrete BMP but also can respond to the BMP themselves, thus amplifying the body's biologic response to this therapy. In addition, the endogenously secreted BMP-2 from genetically modified cells may have greater biological activity because of appropriate posttranslational modifications.³⁵ Finally, depending on the vector chosen, *ex vivo* gene therapy may allow either brief or more sustained production of the osteoinductive protein.

We have previously demonstrated that adipose-derived mesenchymal stem cells transduced with a BMP-2-carrying adenovirus can induce bone formation when implanted in a hindlimb muscle pouch in severely compromised immunodeficient (SCID) mice.²⁶ The purpose of this study was to assess the efficacy of BMP-2-producing human adipose-derived stem cells to heal critically sized femoral defects in nude rats. This study would provide the proof of concept that these cells could be used to enhance bone repair in humans.

MATERIALS AND METHODS

Adenoviral vector

The complementary DNA (cDNA) for hBMP-2 (Genetics Institute, Cambridge, MA) was introduced into an adenoviral vector as previously described.²⁹ This adenoviral vector, AdBMP-2, was used to transduce human adipose tissue-derived mesenchymal stem cells.

Culture of human adipose tissue-derived mesenchymal stem cells

By an internal review board-approved protocol, human adipose tissue was obtained from healthy donors who had undergone liposuction. All of the donors were between 30 and 50 years of age. The lipoaspirate was washed with phosphate-buffered saline, and digested with collagenase as previously described.^{1,2} Enzyme activity was neutralized with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine cells (FBS). Cells were further processed, filtered, and plated. All cells were grown in DMEM plus 10% FBS and maintained at 37°C and 5% CO₂. Cells were expanded in culture through three to five passages before infection with the BMP-2-carrying adenovirus.

Adenoviral infection

When the adherent cells were nearly confluent, 5 million human-derived processed lipoaspirate (HPLA) cells were infected with the BMP-2-carrying adenovirus at a multiplicity of infection (MOI) of 100. Cells were kept in culture overnight after infection. The next day, the supernatant of the infected cells was removed and assayed for BMP-2 production by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.³⁶ The cells were then trypsinized, washed, centrifuged, and counted. Five million infected cells were then concentrated into a volume of approximately 100 μ l and applied to the collagen-ceramic carrier in an Eppendorf tube.

Collagen-ceramic carrier

A collagen-ceramic carrier (compression-resistant matrix, calcium phosphate ceramic plus type I bovine colla-

gen; Medtronic Sofamor Danek, Memphis, TN) was used to deliver cells to the femoral defect. The carrier was cut with a scalpel into a 5 \times 5 \times 7 mm block and placed into a sterile Eppendorf tube. Transduced, adipose-derived stem cells were then loaded onto the carrier and allowed to soak in for approximately 5 min. All of the femoral defects received the collagen-ceramic carrier.

In vivo bone formation at an orthotopic site

Approval was obtained from the Institutional Animal Review Committee before beginning any animal studies. Rats were anesthetized with isoflurane inhalational anesthetic and monitored by an assistant during surgery. At surgery, a polyethylene plate was secured to the femur of an anesthetized rat, using Kirschner wires and cerclage wires as previously described.²⁹ A 6-mm critically sized femoral defect was created in the rat hindlimb, using a high-speed burr. Once the defects were created, a collagen-ceramic carrier impregnated with either normal saline, 20 μ g of recombinant human BMP-2 (rhBMP-2), 5 million human-derived processed lipoaspirate (HPLA) cells, or 5 million AdBMP-2-transduced HPLA cells were implanted in the defect site. The defects were then covered with a muscle pouch and the skin was closed with Vicryl. All rats were able to bear their full weight after surgery.

Study groups

Thirty-two nude rats were placed into one of four treatment groups. In group I, 12 rat femoral defects were implanted with collagen-ceramic carrier containing 5 million AdBMP-2-infected HPLA cells. In group II, six rat femoral defects were implanted with collagen-ceramic carrier containing 5 million uninfected HPLA cells. In group III, six rat femoral defects were implanted with collagen-ceramic carrier alone. In group IV, eight rat femoral defects were implanted with collagen-ceramic carrier containing 20 μ g of rhBMP-2. Radiographs were taken 4 and 8 weeks after surgery. All animals were killed at 8 weeks. All femora were explanted, palpated to assess healing, and underwent histologic or biomechanical analysis (Table 1).

Radiographic review

Animals were anesthetized and radiographs were made at 4 weeks and at 8 weeks. The collagen-ceramic carrier was radioopaque, making it difficult to distinguish or quantify new bone formation at 4 weeks. At the 8-week time point, each radiograph was examined by three blinded independent observers and given a score depending on the amount of bone that had formed. Femora were evaluated for radiographic evidence of healing, using a graded scoring system (0, minimal to no evidence of new bone formation; 1, evidence of bone formation, complete healing questionable; 2, solid-appearing bone, complete healing). Radiographic scores were summed to

TABLE 1. SUMMARY OF STUDY GROUPS

Group	Total no. of defects	No. studied radiographically	No. studied histologically	No. of sections studied histomorphometrically
I: AdBMP-2-infected human-derived processed lipoaspirate (HPLA) cells	12	12	5	10
II: Uninfected HPLA cells	6	6	6	10
III: Collagen-ceramic carrier alone	6	6	6	10
IV: rhBMP-2 with carrier	8	8	3	10

give a maximum score of 6. Femora with a cumulative score of 5 or greater were considered healed. Results of the radiographic evaluation were subjected to statistical analysis. The Fisher exact test was used to compare the average score of each group, and the κ statistic was calculated to quantify interrater reliability.

Histologic techniques

Twenty femora were analyzed histologically (Table 1). These included five femora in group I (AdBMP-2-infected HPLA cells), six femora in group II (uninfected HPLA cells), six femora in group III (collagen-ceramic carrier alone), and three femora in group IV (20 μ g of rhBMP-2-treated limbs).

After sacrifice, femurs were dissected out and fixed in 40% ethanol. Specimens were dehydrated, and embedded in polymethylmethacrylate. Serial cross-sections, 150 μ m thick, were cut from the central portion of each defect, mounted on plastic slides, milled, and polished, and the surface was stained with toluidine blue. Sagittal sections of the proximal and distal host-defect interfaces were also prepared.

Histomorphometric analysis

Cross-sections of the femora were also analyzed histomorphometrically. After transverse sectioning with a diamond band saw and toluidine blue staining, 10 cross-sections of femora from each of the groups were scanned with a charge-coupled device (CCD) camera. For each stained cross-section that was scanned, the total cross-sectional area of bone was measured, using the Bioquant Osteo system (Bioquant Image Analysis, Nashville, TN). In addition, the total area of bone formation and bone density was calculated. For each parameter, a mean was calculated for the specimens in each group. The mean values were subjected to statistical analysis.

Mechanical testing

At 8 weeks, all rats were killed and femora were explanted. The surrounding soft tissue, pins, and polyacetylene plates were removed from the femora. The explanted femora were then assessed manually for healing. Only femora from group I (AdBMP-2-infected HPLA cells) and

from group IV (rhBMP-2) were found to have healed sufficiently to undergo biomechanical testing. Biomechanical testing could not be done on femora from either group II (uninfected HPLA cells) or from group III (collagen-ceramic carrier alone) because none of the femora had sufficient bone formation to be adequately tested. Six femora from group I (AdBMP-2-infected HPLA cells) and five femora from group IV (rhBMP-2) underwent biomechanical testing. In addition, six femora from age-matched rats in which no defect was created were tested biomechanically and served as controls. The ends of the bones were mounted in polymethylmethacrylate. Each specimen was suspended in a Burstein-Frankel torsion tester and the loading of the specimens was initiated by pendulum impact. The data were recorded in real time on a storage-and-oscilloscope photographer. Specimens were tested until failure. Rotational displacement and torque to failure were recorded. From these data torsional stiffness and energy to failure were calculated. For each of the groups, mean values and confidence intervals for maximum torque, energy to failure, and torsional stiffness were calculated and were subjected to statistical analysis.

Statistical methods

The radiographic findings on rats at 8 weeks were assessed with a Fisher exact test. The radiographs were reviewed by three observers, and the κ statistic was calculated as a measure of interobserver reliability. The mean values from the results of the mechanical testing were compared among all groups (when possible) by one-way analysis of variance and the post hoc Student-Newman-Keuls test. For the histomorphometric analysis, mean total bone area and mean bone density were calculated. Confidence intervals were calculated as well and differences were assessed by Student *t* test.

RESULTS

Radiographic analysis

Eleven of the 12 femora in group I (HPLA cells infected with the BMP-2-carrying adenovirus) had healed

radiographically at 8 weeks (Fig. 1 and Table 2). The one femur that did not heal had a subacute infection. All eight of the femora in group IV (rhBMP-2 impregnated collagen–ceramic carrier) healed (Fig. 1 and Table 2) No statistically significant difference in radiographic evidence of healing was detected between femora treated with AdBMP-2-infected HPLA cells or rhBMP-2 was detected. Evaluation of the femora in group II (uninfected HPLA cells) and in control group III (collagen–ceramic carrier alone) indicated little to no evidence of healing based on analysis of plain radiographs. No radiographs of femora from rats in these groups appeared to have healed defects although this was occasionally more difficult to determine because of the radioopaque collagen–ceramic carrier (Fig. 1 and Table 2). The κ statistic (0.82) revealed excellent agreement among the three observers who reviewed the 8-week radiographs.

Histologic analysis

Histologic analysis of femora from group I (AdBMP-2-infected HPLA cells) showed new bone formation extending the length of the defect (Fig. 2). The ceramic portion of the carrier was still identifiable at the 8-week time point. New bone formed both around the collagen–ceramic carrier and within the interstices of the carrier. The ceramic was incorporated within the new bone that formed in femoral defects treated with AdBMP-2-infected HPLA cells. Examination of the host–defect in-

terface demonstrated solid bone formation at both ends of the femoral defects (Fig. 3A).

Analysis of group II (HPLA cells) and group III (collagen–ceramic carrier alone) demonstrated sparse periosteal and endosteal new bone formation at the proximal and distal ends of the defect. The ceramic portion of the carrier was still present in the middle of the femoral defect, but there was little to no new bone formation across the defect or in the interstices of the osteoconductive carrier even at 8 weeks (Fig. 3B and C).

Femora of rats in group IV (collagen–ceramic carrier with rhBMP-2) showed histologic evidence of healing similar to that of femora of group I rats. No qualitative differences were noted between bone induced by BMP-2-expressing HPLA cells and bone induced by recombinant BMP-2.

Histomorphometric analysis

Histomorphometric analysis was done on toluidine blue-stained cross-sections of femora from each of the groups. Ten cross-sections from each of the groups were scanned with the CCD camera and the total cross-sectional area of bone was measured, using the Bioquant Osteo system (Bioquant Image Analysis). The mean cross-sectional area of bone in the healed defects of femora treated with BMP-2-expressing HPLA cells was $4.7 \pm 0.4 \text{ mm}^2$. This was significantly higher than the mean cross-sectional area of bone induced by HPLA cells alone

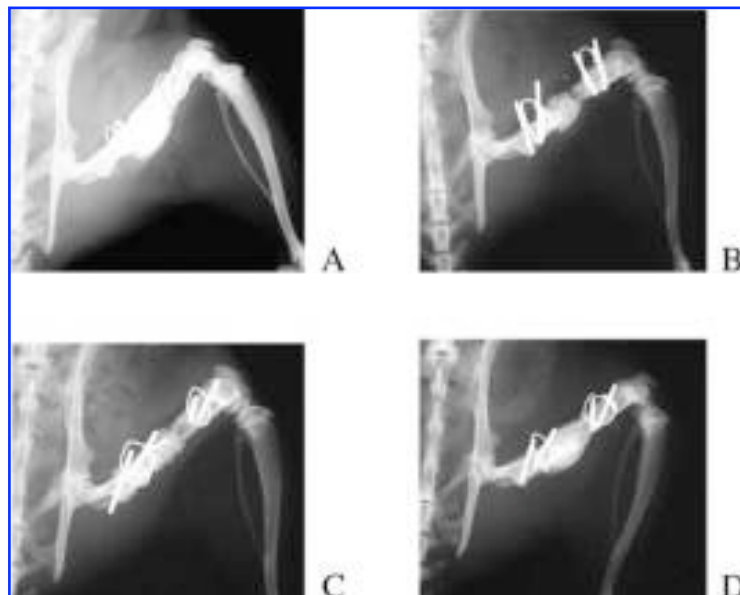


FIG. 1. (A–D) Radiographs of specimens at the 8-week time point. The collagen–ceramic carrier was implanted in all femoral defects. (A) Group I, BMP-2-producing human processed lipoaspirate (HPLA) cells. Bone formation is seen around and incorporating the collagen–ceramic carrier. (B) Group II, HPLA cells alone. Little to no bone formation is noted. (C) Group III, carrier alone. (D) Group IV, rhBMP-2-impregnated collagen ceramic carrier resulted in bone formation that was distinguishable on plain radiographs.

TABLE 2. RADIOGRAPHIC SCORES AT 8 WEEKS

Group	Total no. of defects	No. studied radiographically	Radiographic score at 8 weeks	p Value (vs. group III)
I: AdBMP-2-infected human-derived processed lipoaspirate (HPLA) cells	12	12	5.4 ± 0.5	<0.02
II: Uninfected HPLA cells	6	6	0.9 ± 0.2	NS
III: Collagen–ceramic carrier alone	6	6	0.8 ± 0.2	—
IV: rhBMP-2 with carrier	8	8	5.7 ± 0.2	<0.02

(group II, $0.4 \pm 0.2 \text{ mm}^2$) or by carrier alone (group III, $0.3 \pm 0.2 \text{ mm}^2$). The mean cross-sectional area of bone in femora treated with rhBMP-2 (group IV) was $5.2 \pm 0.6 \text{ mm}^2$. Mean bone area and mean bone density were similar between group I (BMP-2-expressing HPLA cells) and group IV (rhBMP-2) (Fig. 4).

Biomechanical testing

Biomechanical testing was performed to quantitatively assess the structural properties of the healed femora. Biomechanical testing could not be done on any femur from group II (uninfected HPLA cells) or group III (carrier alone) because none had sufficiently healed. After removal of the polyacetylene plate, femora from these treatment groups were not rigid enough to be tested in the Burstein–Frankel torsion device. Specimens that were rigid enough were tested to failure in torsion. Femora that were tested failed just proximal or distal to the healed defect site (Fig. 5). At this location, the diameter of the healed femora was smaller than at the healed defect site. Six femora from group I (AdBMP-2-infected HPLA cells), five femora from group IV (rhBMP-2), and six age-matched control femora without defects were tested. Specimens were tested in torsion, and energy to failure and torsional stiffness were calculated. Mean values and confidence intervals were calculated for peak torque, energy to failure, and torsional stiffness. No statistically significant differences were detected between group I, group IV, and the control nonoperated femora with re-

spect to average torque to failure, torsional stiffness, or energy to failure (Table 3). There was a relatively high standard deviation for each of the group averages. There was a trend toward higher stiffness in the nonoperated femora, whereas the healed femora from either group I (AdBMP-2-infected HPLA cells) or group IV (rhBMP-2) tended to have lower torsional stiffness.

DISCUSSION

This study demonstrates that BMP-2-producing human adipose-derived mesenchymal stem cells in a collagen–ceramic carrier can heal critically sized femoral defects in athymic rats. In contrast, when human adipose tissue-derived mesenchymal stem cells were placed in a femoral defect with just collagen–ceramic carrier, critically sized defects did not heal. Bone defects treated with BMP-2-producing adipose-derived mesenchymal stem cells were healed in 11 of 12 animals by 8 weeks after cell implantation. The one femur that did not heal was found to have a subacute infection. Biomechanical testing revealed no statistically significant difference in energy to failure, torque to failure, or torsional stiffness between femora treated with BMP-2-producing bone marrow cells or recombinant BMP-2 and nonoperated age-matched control femora. We purposely chose a high dose ($20 \mu\text{g}$) of BMP-2 because it is a more stringent comparison group with which to assess the quality of

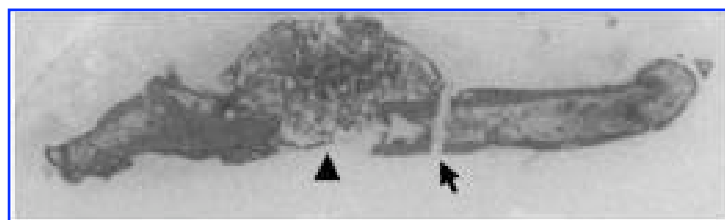


FIG. 2. Toluidine blue-stained sagittal section of full-length femur. This femoral defect had been treated with 5 million AdBMP-2-infected HPLA cells. Some of the collagen–ceramic carrier shifted to an eccentric position in the defect, but osseous union still occurred. Arrowhead points to the 6-mm defect site, which is filled with new bone and residual ceramic carrier. Arrow points to previous Kirschner wire site that had been used to hold the plate to the bone during healing.

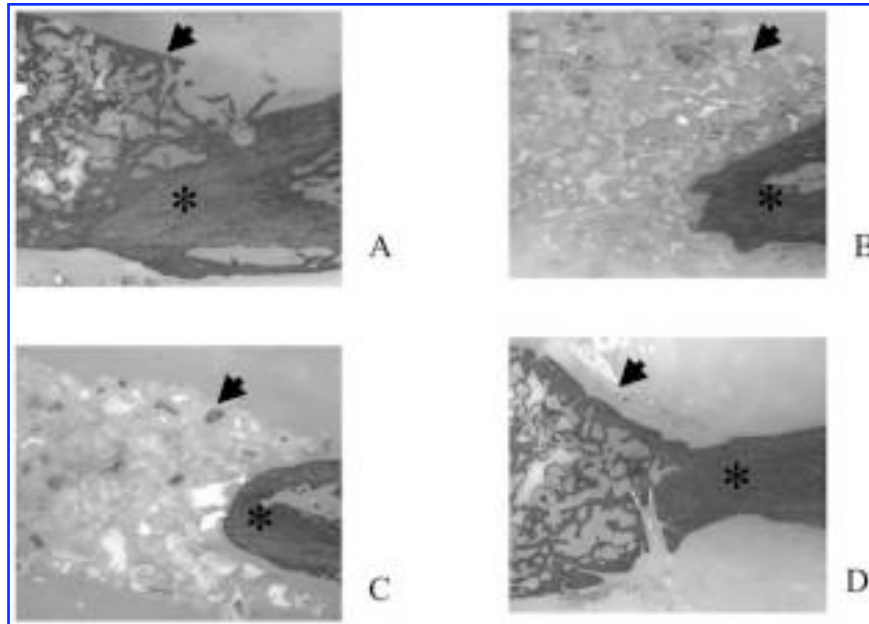


FIG. 3. Higher power view of toluidine blue-stained sagittal sections of host-defect interface. (A) Group I, BMP-2-producing human processed lipoaspirate (HPLA) cells. New bone is seen bridging between the cut ends of the rat femoral cortices. Bone is also seen interdigitating within the collagen-ceramic carrier. (B) Group II, HPLA cells alone. Little new bone formation is seen within or around the collagen-ceramic carrier. Some endosteal new bone did form. (C) Group III, carrier alone. Results are similar in appearance to group II, with sparse periosteal new bone formation and some endosteal bone formation, but no evidence of potential for healing across the defect site. (D) Group IV, rhBMP-2 along with ceramic carrier resulted in bone formation, which formed a continuous cortex with the host cortex. This was similar in appearance to group I. Asterisk marks the cut end of rat femur. Arrow points to bone or carrier present in the defect site.

bone formed by transformed mesenchymal stem cells; 5 μg of recombinant BMP-2 is able to heal this defect (our unpublished data). These results are promising regarding a future strategy in which AdBMP-2-infected adipose-derived mesenchymal stem cells could be adapted to treat bone repair problems such as delayed unions or nonunions in patients.

The clinical problems of delayed unions and nonunion

represent failures in bone repair.³⁷ The biology of bone repair requires an osteoinductive signal, host responding cells, a matrix, and an adequate vascular supply. In the past, autogenous bone has been the “gold standard” bone graft material for the treatment of atrophic nonunions. However, the significant morbidity associated with the harvest of autogenous graft and the limited amount of autologous bone available make other options attractive.^{38,39}

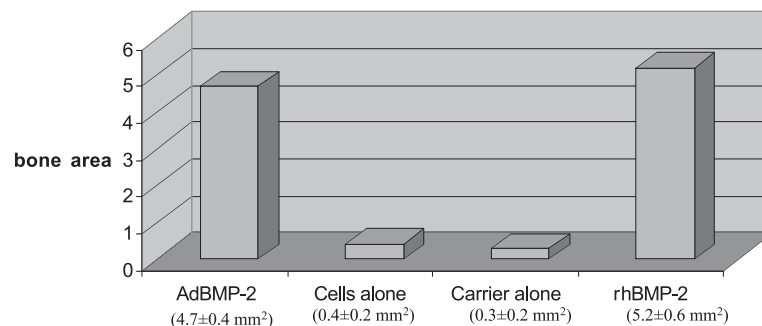


FIG. 4. Histomorphometric analysis of toluidine blue-stained cross-sections of femora from each of the four groups. The bar graphs demonstrate mean bone area for each of the groups. Both AdBMP-2-infected HPLA cells (group I) and rhBMP-2 (group IV) induced significantly more bone than the other two groups, but there was no significant difference between either total bone area or bone density measurements for femora treated with rhBMP-2 or AdBMP-2-infected HPLA cells.

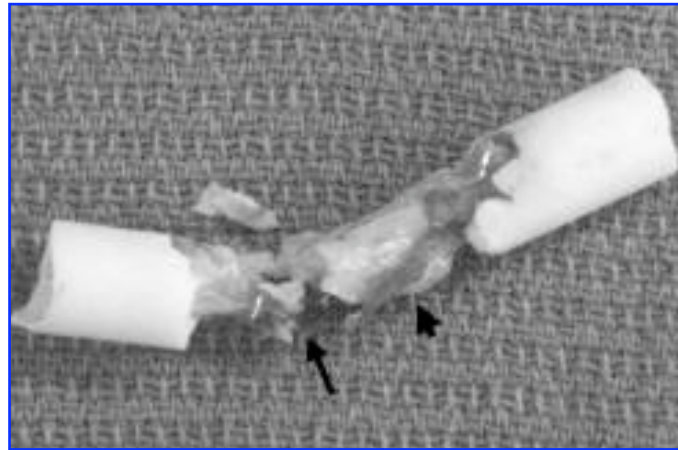


FIG. 5. Digital photograph of healed femur that had been treated with AdBMP-2-infected HPLA cells. This femur was explanted and cleaned of soft tissue. The ends of the bone were embedded in polymethylmethacrylate, and the specimen was then tested to failure in the Burstein–Frankel torsion device. The healed femoral defect can be seen (arrowhead). In addition, the site at which failure occurred (arrow) and the PMMA-embedded ends of the bone are visible. This result was typical for femora from either group I (AdBMP-2-infected HPLA cells) or group IV (rhBMP-2).

Recombinant bone morphogenetic proteins are now clinically available, but extremely high doses are necessary to induce bone in humans,^{13,14,40} suggesting that the delivery of recombinant bone-inducing proteins needs to be optimized. There are concerns that a single dose of exogenous protein will not induce an adequate osteoinductive response in patients with poor vascularity, a damaged soft tissue envelope, or a history of nicotine or steroid use. In these cases, alternative methods of bone growth factor delivery, such as regional gene therapy with genetically manipulated stem cells, may be attractive. In addition, alternative viral genetic delivery systems are being developed. One of the most promising of these viral delivery systems is based on human parvovirus adeno-associated virus (AAV) type 2 because of its high transduction efficiencies and biosafety ratings. Using Sprague-

Dawley rats and a replication-deficient AAV carrying the BMP-2 transgene, Chen *et al.* demonstrated bone formation after directly injecting the recombinant AAV into rat hindlimbs.⁴¹ Other investigators have had success in regulating BMP-2 expression by recombinant AAV by incorporating a tetracycline-sensitive promoter.⁴² Although no studies have yet shown healing of a critically sized bone defect, AAV strategies are promising. Clearly gene therapy will not be necessary to treat all bone repair problems, but once safer and more effective vectors can be developed, gene therapy may become one aspect of a comprehensive tissue-engineering strategy.

We have previously demonstrated that an *ex vivo* gene therapy strategy using bone marrow cells transduced with a BMP-2-carrying adenovirus could be used to heal critically sized defects in syngeneic rats.²⁹ In our own lab-

TABLE 3. BIOMECHANICAL TESTING

Group	Energy to failure		Torsional stiffness		Peak torque	
	Result ^a	p Value ^b	Result ^a	p Value ^b	Result ^a	p Value ^b
I: AdBMP-2-infected human-derived processed lipoaspirate (HPLA) cells	5.7 ± 3	NS	0.26 ± 0.2	NS	1.7 ± 1.1	NS
II: Uninfected HPLA cells	—	—	—	—	—	—
III: Collagen–ceramic carrier alone	—	—	—	—	—	—
IV: rhBMP-2 with carrier	8.6 ± 2.0	NS	0.21 ± 0.1	NS	1.9 ± 0.9	NS
Nonoperated femora	5.7 ± 2.2	—	0.5 ± 0.3	—	2.4 ± 1	—

^aData represent mean values and 95% confidence intervals.

^bVersus nonoperated femora.

oratory, rat fibroblasts transduced with the same BMP-2-carrying adenovirus were unable to heal these defects (our unpublished data), suggesting that not only the osteoinductive protein but also the cellular delivery vehicle was critical. Other investigators have used muscle-derived stem cells to heal critically sized defects and have shown that these transplanted cells directly participate in bone formation.^{30,31,43,44} Lee *et al.* infected muscle-derived mesenchymal stem cells with a BMP-2-carrying adenovirus and healed calvarial defects in SCID mice.⁴⁴ Using fluorescence *in situ* hybridization techniques, Lee *et al.* showed that a small percentage of the muscle-derived cells that were implanted into the calvarial defects had differentiated into osteoblasts *in vivo*.

Compared with other tissue sources for mesenchymal stem cells, human adipose tissue is an attractive source of autologous stem cells. In most patients, there is a more than adequate supply of adipose tissue, and large amounts of it may be safely available by liposuction. Once harvested, large numbers of mesenchymal stem cells can be obtained from a relatively small amount of fat.⁴ These cells are a plentiful, expandable, and attractive source of stem cells for tissue engineering. In addition, the number of mesenchymal stem cells derived from adipose tissue does not appear to decrease with age.³ Theoretically, these stem cells could be harvested from elderly patients.

It is also important to note that adipose-derived stem cells were not able to heal a defect if they were not genetically manipulated to overexpress BMP-2. It has been reported that muscle-derived mesenchymal stem cells alone cannot heal critically sized defects, either.³⁰ In contrast, it has been shown that mesenchymal stem cells derived from bone marrow can heal critically sized bone defects.¹⁸ These findings suggest that these cells have varying degrees of native osteoinductive potential, which may have an impact on cell-based therapies. Comparative studies are necessary to determine which cellular delivery vehicles—fat-derived, muscle-derived, or bone marrow-derived cells—are the most effective for *ex vivo* gene transfer strategies. In addition, in this study, the adipose tissue was harvested from relatively young and healthy patients undergoing liposuction procedures. Further studies need to be performed with stem cells from elderly patients to determine whether osteoinductive potential is maintained with age.

For a gene therapy strategy to be used clinically, it will need to be efficacious and have a high safety index. If these difficulties can be solved, it will greatly add to the ability of clinicians to treat bone loss problems. In simple cases allograft or autograft bone will be the most effective treatment, in others a recombinant protein may be necessary, and finally gene therapy-based strategies may be needed to treat the most difficult problems. Each of these therapies will represent one aspect of an overall strategy for the tissue engineering of bone.

ACKNOWLEDGMENT

This work was supported by a grant from the NIH to J.R.L. (RO1 AR46789).

REFERENCES

- Zuk, P.A., *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell.* **13**, 4279, 2002.
- Zuk, P.A., *et al.* Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng.* **7**, 211, 2001.
- Morizono, K., *et al.* Multilineage cells from adipose tissue as gene delivery vehicles. *Hum. Gene Ther.* **14**, 59, 2003.
- De Ugarte, D.A., Ashjian, P.H., Elbarbary, A., and Hedrick, M.H. Future of fat as raw material for tissue regeneration. *Ann. Plast. Surg.* **50**, 215, 2003.
- De Ugarte, D.A., *et al.* Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* **174**, 101, 2003.
- Urist, M.R., Nogami, H., and Mikulski, A. A bone morphogenetic polypeptide. *Calcif. Tissue Res.* **21**(Suppl.), 81, 1976.
- Urist, M.R. Bone: Formation by autoinduction. *Science* **150**, 893, 1965.
- Salkeld, S.L., Patron, L.P., Barrack, R.L., and Cook, S.D. The effect of osteogenic protein-1 on the healing of segmental bone defects treated with autograft or allograft bone. *J. Bone Joint Surg. Am.* **83**, 803, 2001.
- Kirker-Head, C.A., Gerhart, T.N., Armstrong, R., Schelling, S.H., and Carmel, L.A. Healing bone using recombinant human bone morphogenetic protein 2 and copolymer. *Clin. Orthop.* **349**, 205, 1998.
- Gerhart, T.N., *et al.* Healing segmental femoral defects in sheep using recombinant human bone morphogenetic protein. *Clin. Orthop.* **293**, 317, 1993.
- Cook, S.D., Baffes, G.C., Wolfe, M.W., Sampath, T.K., and Rueger, D.C. Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. *Clin. Orthop.* **301**, 302, 1994.
- Cook, S.D., Salkeld, S.L., Patron, L.P., Sargent, M.C., and Rueger, D.C. Healing course of primate ulna segmental defects treated with osteogenic protein-1. *J. Invest. Surg.* **15**, 69, 2002.
- Friedlaender, G.E., *et al.* Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. *J. Bone Joint Surg. Am.* **83**(Suppl. 1), S151, 2001.
- Boden, S.D., Zdeblick, T.A., Sandhu, H.S., and Heim, S.E. The use of rhBMP-2 in interbody fusion cages: Definitive evidence of osteoinduction in humans. Preliminary report. *Spine* **25**, 376, 2000.
- Boden, S.D., Kang, J., Sandhu, H., and Heller, J.G. Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans: A prospective, randomized clinical pilot trial. 2002 Volvo Award in Clinical Studies. *Spine* **27**, 2662, 2002.
- Govender, S., *et al.* Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: A

- prospective, controlled, randomized study of four hundred and fifty patients. *J. Bone Joint Surg. Am.* **84**, 2123, 2002.
17. Sakou, T. Bone morphogenetic proteins: From basic studies to clinical approaches. *Bone* **22**, 591, 1998.
 18. Bruder, S.P., *et al.* Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. *J. Orthop. Res.* **16**, 155, 1998.
 19. Bruder, S.P., Kraus, K.H., Goldberg, V.M., and Kadiyala, S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J. Bone Joint Surg. Am.* **80**, 985, 1998.
 20. Caplan, A.I., and Bruder, S.P. Mesenchymal stem cells: Building blocks for molecular medicine in the 21st century. *Trends Mol. Med.* **7**, 259, 2001.
 21. Bruder, S.P., and Fox, B.S. Tissue engineering of bone: Cell based strategies. *Clin. Orthop.* **367**(Suppl.), S68, 1999.
 22. Boden, S.D., *et al.* Lumbar spine fusion by local gene therapy with a cDNA encoding a novel osteoinductive protein (LMP-1). *Spine* **23**, 2486, 1998.
 23. Viggswarapu, M., *et al.* Adenoviral delivery of LIM mineralization protein-1 induces new-bone formation *in vitro* and *in vivo*. *J. Bone Joint Surg. Am.* **83**, 364, 2001.
 24. Bosch, P., *et al.* Osteoprogenitor cells within skeletal muscle. *J. Orthop. Res.* **18**, 933, 2000.
 25. Musgrave, D.S., *et al.* Human skeletal muscle cells *in vivo* gene therapy to deliver bone morphogenetic protein-2. *J. Bone Joint Surg. Br.* **84**, 120, 2002.
 26. Dragoo, J.L., *et al.* Bone induction by BMP-2 transduced stem cells derived from human fat. *J. Orthop. Res.* **21**, 622, 2003.
 27. Rutherford, R.B., *et al.* Bone morphogenetic protein-transduced human fibroblasts convert to osteoblasts and form bone *in vivo*. *Tissue Eng.* **8**, 441, 2002.
 28. Wang, J.C., *et al.* Effect of regional gene therapy with bone morphogenetic protein-2-producing bone marrow cells on spinal fusion in rats. *J. Bone Joint Surg. Am.* **85**, 905, 2003.
 29. Lieberman, J.R., *et al.* The effect of regional gene therapy with bone morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. *J. Bone Joint Surg. Am.* **81**, 905, 1999.
 30. Wright, V., *et al.* BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. *Mol. Ther.* **6**, 169, 2002.
 31. Musgrave, D.S., *et al.* *Ex vivo* gene therapy to produce bone using different cell types. *Clin. Orthop.* **378**, 290, 2000.
 32. Moutsatsos, I.K., *et al.* Exogenously regulated stem cell-mediated gene therapy for bone regeneration. *Mol. Ther.* **3**, 449, 2001.
 33. Turgeman, G., *et al.* Engineered human mesenchymal stem cells: A novel platform for skeletal cell mediated gene therapy. *J. Gene Med.* **3**, 240, 2001.
 34. Turgeman, G., Aslan, H., Gazit, Z., and Gazit, D. Cell-mediated gene therapy for bone formation and regeneration. *Curr. Opin. Mol. Ther.* **4**, 390, 2002.
 35. Niyibizi, C., *et al.* Potential role for gene therapy in the enhancement of fracture healing. *Clin. Orthop.* **355**(Suppl.), S148, 1998.
 36. Lieberman, J.R., *et al.* Regional gene therapy with a BMP-2-producing murine stromal cell line induces heterotopic and orthotopic bone formation in rodents. *J. Orthop. Res.* **16**, 330, 1998.
 37. Wu, C.C. Bone grafting techniques in treating fracture nonunion. *Changeng Yi Xue Za Zhi* **23**, 319, 2000.
 38. Younger, E.M., and Chapman, M.W. Morbidity at bone graft donor sites. *J. Orthop. Trauma* **3**, 192, 1989.
 39. Colterjohn, N.R., and Bednar, D.A. Procurement of bone graft from the iliac crest: An operative approach with decreased morbidity. *J. Bone Joint Surg. Am.* **79**, 756, 1997.
 40. Friedlaender, G.E. OP-1 clinical studies. *J. Bone Joint Surg. Am.* **83**(Suppl. 1), S160, 2001.
 41. Chen, Y., *et al.* Gene therapy for new bone formation using adeno-associated viral bone morphogenetic protein-2 vectors. *Gene Ther.* **10**, 1345, 2003.
 42. Gafni, Y., *et al.* Gene therapy platform for bone regeneration using an exogenously regulated, AAV-2-based gene expression system. *Mol. Ther.* **9**, 587, 2004.
 43. Young, B.H., Peng, H., and Huard, J. Muscle-based gene therapy and tissue engineering to improve bone healing. *Clin. Orthop.* **403**(Suppl.), S243, 2002.
 44. Lee, J.Y., *et al.* Effect of bone morphogenetic protein-2-expressing muscle-derived cells on healing of critical-sized bone defects in mice. *J. Bone Joint Surg. Am.* **83**, 1032, 2001.

Address reprint requests to:

Jay R. Lieberman, M.D.

UCLA Department of Orthopedic Surgery

David Geffen School of Medicine at UCLA

Center for Health Sciences 76-134

10833 Le Conte Avenue

Los Angeles, CA 90095-3075

E-mail: jlieberman@mednet.ucla.edu

This article has been cited by:

1. Nadav Kimelman , Gadi Pelled , Gregory A. Helm , J. Huard , Edward M. Schwarz , Dan Gazit . 2007. Review: Gene- and Stem Cell-Based Therapeutics for Bone Regeneration and Repair. *Tissue Engineering* **13**:6, 1135-1150. [[Abstract](#)] [[PDF](#)] [[PDF Plus](#)]
2. Marcus Jünger, Uzer Degistirici, Andreas Knipper, Johannes Fischer, Martin Sager, Rüdiger Krauspe. 2007. Bone Healing and Migration of Cord Blood-Derived Stem Cells Into a Critical Size Femoral Defect After Xenotransplantation. *Journal of Bone and Mineral Research* **22**:8, 1224. [[CrossRef](#)]
3. Kazunori Shimizu, Akira Ito, Tatsuro Yoshida, Yoichi Yamada, Minoru Ueda, Hiroyuki Honda. 2007. Bone tissue engineering with human mesenchymal stem cell sheets constructed using magnetite nanoparticles and magnetic force. *Journal of Biomedical Materials Research Part B Applied Biomaterials* **82b**:2, 471. [[CrossRef](#)]
4. Charles A. Gersbach, Jennifer E. Phillips, Andrés J. García. 2007. Genetic Engineering for Skeletal Regenerative Medicine. *Annual Review of Biomedical Engineering* **9**:1, 87. [[CrossRef](#)]
5. W. K. Hsu, B. T. Feeley, L. Krenek, D. B. Stout, A. F. Chatziioannou, J. R. Lieberman. 2007. The use of 18F-fluoride and 18F-FDG PET scans to assess fracture healing in a rat femur model. *European Journal of Nuclear Medicine and Molecular Imaging* **34**:8, 1291. [[CrossRef](#)]
6. Daisuke Matsumoto , Katsujiro Sato , Koichi Gonda , Yasuyuki Takaki , Tomokuni Shigeura , Takahiro Sato , Emiko Aiba-Kojima , Fumiko Iizuka , Keita Inoue , Hirotaka Suga , Kotaro Yoshimura . 2006. Cell-Assisted Lipotransfer: Supportive Use of Human Adipose-Derived Cells for Soft Tissue Augmentation with Lipoinjection. *Tissue Engineering* **12**:12, 3375-3382. [[Abstract](#)] [[PDF](#)] [[PDF Plus](#)]
7. J.M. McMahon , S. Conroy , M. Lyons , U. Greiser , C. O'shea , P. Strappe , L. Howard , M. Murphy , F. Barry , Dr. T. O'brien . 2006. Gene Transfer into Rat Mesenchymal Stem Cells: A Comparative Study of Viral and Nonviral Vectors. *Stem Cells and Development* **15**:1, 87-96. [[Abstract](#)] [[PDF](#)] [[PDF Plus](#)]
8. Jason R. Dudas, Kacey G. Marra, Gregory M. Cooper, Virginia M. Penascino, Mark P. Mooney, Shao Jiang, J Peter Rubin, Joseph E. Losee. 2006. The Osteogenic Potential of Adipose-Derived Stem Cells for the Repair of Rabbit Calvarial Defects. *Annals of Plastic Surgery* **56**:5, 543. [[CrossRef](#)]
9. Eileen Gentleman, Julia M. Polak. 2006. Historic and current strategies in bone tissue engineering: Do we have a hope in Hench?. *Journal of Materials Science Materials in Medicine* **17**:11, 1029. [[CrossRef](#)]
10. Jose Becerra, Enrique Guerado, Silvia Claros, Monica Alonso, Maria L Bertrand, Carlos Gonzalez, Jose A Andrades. 2006. Autologous human-derived bone marrow cells exposed to a novel TGF-1 fusion protein for the treatment of critically sized tibial defect. *Regenerative Medicine* **1**:2, 267. [[CrossRef](#)]
11. Kotaro Yoshimura, Tomokuni Shigeura, Daisuke Matsumoto, Takahiro Sato, Yasuyuki Takaki, Emiko Aiba-Kojima, Katsujiro Sato, Keita Inoue, Takashi Nagase, Isao Koshima. 2006. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *Journal of Cellular Physiology* **208**:1, 64. [[CrossRef](#)]
12. Jean-Thomas Vilquin, Philippe Rosset. 2006. Mesenchymal stem cells in bone and cartilage repair: current status. *Regenerative Medicine* **1**:4, 589. [[CrossRef](#)]
13. N Kimelman, G Pelled, Zul Gazit, D Gazit. 2006. Applications of gene therapy and adult stem cells in bone bioengineering . *Regenerative Medicine* **1**:4, 549. [[CrossRef](#)]