Use of Genetically Engineered Bone-Marrow Stem Cells to Treat Femoral Defects: An Experimental Study

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Background: Treatment of osteonecrosis continues to be a challenging problem. The replacement of necrotic bone with graft materials that promote osteogenesis and angiogenesis may provide better outcomes for early stage disease. In this study, genetically engineered bone-marrow stem cells were used to enhance repair of a defect in the distal aspect of the femur.

Methods: Cloned bone-marrow stem cells were transfected with traceable genes. Osteoblastic and angiogenic properties of the cells were analyzed. A defect was created bilaterally in the distal portion of the femur of twenty-four mice to mimic a core decompression procedure. The cloned cells were transplanted into each defect of the right femur while the left femur served as control. Bone formation was evaluated radiographically and histomorphometrically. In addition, in twenty-four additional mice, the cells were injected into subcutaneous sites, muscles, and into the renal capsule (eight mice in each group) to evaluate ectopic osteogenesis.

Results: Radiopaque tissue appeared two weeks after the cells were transplanted into bone defects and at ectopic sites. Histologic analysis demonstrated that these tissues consisted of newly formed bone from transplanted cells that expressed traceable genes. Four of six bone defects that received cell transplantation were filled with new bone at four weeks, and all of the defects (n = 6) demonstrated complete healing at six weeks. On the control side, complete repair was seen in only two of six bone defects at four weeks and in three of six defects at six weeks. Histomorphometric analysis showed that transplantation of marrow stem cells into bone defects produced more bone at an earlier time-point than occurred in the controls.

Conclusions: This study demonstrated that cloned bone-marrow stem cells can directly form bone after transplantation into bone defects and at ectopic sites, indicating that the in vitro expanded bone-marrow stem cells can serve as a graft material to enhance bone repair and to treat osteonecrosis.

Clinical Relevance: As an alternative graft material, bone-marrow stem cells may provide new and as yet technologically unachievable solutions to many clinical problems in the areas of musculoskeletal reconstruction and tissue regeneration.

Treatment of osteonecrosis of the femoral head continues to be a challenging problem. Hip arthroplasty is generally successful, but long-term results may be less than optimal, especially in active young adults. Alternative treatments such as core decompression and bone-grafting procedures provide some benefits, but long-term success is not always reliable. Replacement of necrotic bone at an early stage of the disease to promote osteogenesis and angiogenesis as well as to heal subchondral bone lesions may provide better outcomes for patients with the disease. Autogenous cancellous bone is currently the gold-standard graft material, but its supply is limited and, in addition, donor-site complications can occur. Allografts are useful but not as desirable as autografts because of the problem of immunogenicity and the potential to transmit disease. Growth and differentiation factors, delivered in the form of proteins and genes, have been widely tested both in animal studies and clinically for their potential to enhance bone repair, including the treatment of osteonecrosis, but no long-term results are available. Autologous marrow transplantation has been used as a bone-graft substitute in the treatment of fracture nonunions and has been shown to be effective. Successful use of bone-marrow transplantation in the treatment of osseous defects is based on the osteogenic property of these cells. However, the prevalence of stem cells in a marrow preparation is low (approximately one in 100,000 cells) which means that cell expansion will be needed if these cells are to be used for the treatment of bone defects. Since few ideal substitutes for or alternatives to
bone grafts have been found25,30, we have used a cloned, genetically engineered bone-marrow stem cell expanded in culture to attempt to enhance defect repair in a mouse model.

Materials and Methods

A bone-marrow stem cell, D1-BAG, cloned from Balb/c mice and transduced with LacZ and neomycin resistance genes, was tested for its osteogenic properties and cryobiologically preserved in a cell bank31-36. Since the vector deoxyribonucleic acid (DNA) used for transduction encodes for β-galactosidase and neomycin resistance, these cells can be selected with use of neomycin in culture and identified with use of X-gal (a chromogenic substrate for β-galactosidase) stain31,35. The cells were cultured for seven to ten days before they were tested in vitro and in vivo32-34. Both the osteoblastic and angiogenic properties of the cells were examined with use of Northern blot analysis to detect osteocalcin and vascular endothelial growth factor (VEGF) gene expression. The cells were also stained for alkaline phosphatase with use of the von Kossa technique to establish their osteogenic properties. Both the in vitro and in vivo experiments were carried out with use of the same source of cells. A suspension containing 2 × 10^7 cells per mL of phosphate-buffered saline solution (PBS) was prepared for cell transplantation32,33.

For messenger ribonucleic (RNA) analysis, the Northern blot analysis was hybridized with osteocalcin and VEGF complementary DNA (cDNA). The DNA isolation was carried out according to the protocol described by Gross-Bellard et al.37 The neomycin resistance gene (neo) was detected with a polymerase chain reaction. The following primer pairs were used for amplification of the neo sequences: 3’ primer-5’ GATGTTTCGCTTGGTGG, 5’ primer-5’ CTTTTGCAAGACCGACC yielding a 280-base-pair product33,34.

The in vivo study with animals was approved by the Institutional Animal Care and Use Committee. Forty-eight eight-week-old Balb/c mice were used in this study (twenty-four mice [forty-eight femora] in the defect model group and twenty-four mice in the injection model group). Animals received general anesthesia with ketamine (80 mg/kg) and xylazine (7 mg/kg) and were positioned prone. In the defect model group, a midline anterior longitudinal incision was made to expose the distal aspect of the femur. A 1.2-mm defect was created through the lateral femoral condyle bilaterally with an electric drill to mimic a core decompression. The wound was then irrigated with normal saline solution to remove osseous debris, and the core defect was dried with a sponge. Two million cells were transplanted into the defect of the right femur. In the left femur, which served as a control, the core defect was injected with PBS only (without cells). The defects were sealed with gel foam. No postoperative immobilization devices were used. An equal number of cells were injected either at subcutaneous sites, in the hindquarter muscles, or into the renal capsule of the twenty-four mice in the injection model group (eight mice for each site) to evaluate ossification at ectopic sites. For sub-renal capsular transplantation, a lateral abdominal incision was used to
expose the kidney. A needle was carefully used to prepare a pocket under the renal capsule, and the cells were then injected into the pocket. Animals were killed at two, four, six, and eight weeks (six animals from the bone-defect group and two animals from each of the three injection model groups [subcutaneous, intramuscular, and renal] at each time-point). Defect repair was evaluated radiographically, and the contribution to osteogenesis by transplanted cells was studied histomorphometrically on tissue sections stained with X-gal as well as biochemically on DNA extracts with use of nucleotide primers for the neomycin resistance gene.

With use of an HP43805-N radiographic system (Hewlett-Packard, McMinnville, Oregon), both anteroposterior and lateral radiographs of the distal aspect of the femur were made immediately after the operation and immediately after the animals were killed.

For histologic analysis, the specimens were prepared as described previously. Five to 7-µm sections were cut and counterstained with hematoxylin and eosin. To quantitate bone formation, five sections from each specimen were scanned with use of a Nikon LS-3510 AF Scanner (Nikon, Melville, New York) and Adobe Photoshop (Adobe Systems, San Jose, California). The areas of new bone were determined quantitatively with use of the Image-Pro software package (Media Cybernetics, Silver Springs, Maryland). For each specimen, the area of new bone in five sections was measured and the average of those measurements was determined.

Statistical analysis was performed by the Division of Biostatistics and Epidemiology, Department of Public Health Sciences, University of Virginia. Bone-formation data were analyzed with two-way analysis of variance. Hypotheses related to comparisons of bone formation were assessed with use of a Bonferroni multiple-comparison criterion with an experimental Type-I error rate of 0.05. Statistical computations were performed with use of version 6.12 of the SAS statistical software package and PROC MIXED mixed-model software package (SAS/STAT; SAS Institute, Cary, North Carolina).

Results

D1-BAG cells are multipotent and primarily osteogenic. Ten days after they were cultured in Dulbecco modified Eagle medium (DMEM), D1-BAG cells stained positively for alkaline phosphatase and with the von Kossa stain. Both osteocalcin messenger RNA (mRNA) and VEGF mRNA were detected with use of Northern blot analysis. D1-BAG cells stained positively with X-gal, were readily identifiable (Fig. 1), and expressed the neomycin resistance gene.

There were no surgical or postoperative complications, and no animals were excluded from the study. Two weeks after surgery, radiopaque tissue was seen at transplantation sites with D1-BAG cells in the right femur but not in the control left femur. Two weeks after the cells were transplanted into the bone defects, new bone formation became evident (Fig. 2) in bone defects, in muscle, at subcutaneous sites, and in the renal capsule.

Histologic analysis demonstrated that these tissues consisted of newly formed bone from transplanted cells that stained positively with X-gal (Fig. 2) and contained neo DNA. The repair tissue did not contain cartilaginous areas, which indicated that ossification surrounding the D1-BAG cells had not yet completed the endochondral process. At four weeks, four of six femora showed a defect that was filled with new bone. At six weeks, all of the defects (n = 6) contained fully re-
stored bone. However, on the control side that was injected with PBS (no cells), two of six defects at four weeks, three of six defects at six weeks, and five of six defects at eight weeks showed complete repair. All histologic sections of bone defects (n = 24) were examined histomorphometrically; the data showed that transplantation of marrow stem cells into bone defects produced more bone at an earlier time-point than that which was seen in the control group, and the process of enhanced ossification continued throughout the observation period (p < 0.05) (Fig. 3).

Discussion

The repair processes in bone defects and in fractures represent a complex cascade of cellular recruitment and differentiation directed by growth factors and cytokines that ultimately regenerate the osseous structure. The process is more complicated in the repair of necrotic bone because of the lack of a blood supply. Bone-grafting is one of the most effective methods to enhance bone repair but has associated problems and limitations. Bone-graft substitutes have been extensively studied, and some of the results are promising. Successful use of bone-graft substitutes is based on three elements: specific cells designated to make bone, a biodegradable polymeric scaffold on which the cells can attach and grow, and bioactive factors (such as cytokines) that induce either rapid cell proliferation or specific cell differentiation. Early results from the treatment of osteonecrotic lesions with autologous bone graft that included bone-marrow mononuclear cells. After twenty-four months, the group treated with the bone-marrow graft had a significant reduction in pain (p = 0.021) and joint symptoms compared with the other group. Five of the eight hips treated with only core decompression had radiographic evidence of deterioration, whereas only one of the ten hips treated with the bone-marrow graft had such deterioration (p = 0.016). Unfortunately, little is known of the fate of these grafts at the sites of tissue regeneration. Clonal stem-cell lines can be pivotal tools to define the characteristics of marrow stem cells in fracture-healing and bone repair, but information on this topic is limited. With use of gene-labeling techniques, the present study demonstrated that the cloned bone-marrow stem cell can directly form bone after transplantation into bone defects or into ectopic sites, indicating that the in vitro expanded bone-marrow stem cells can serve as a grafting material to enhance healing of femoral defects.

Bone-marrow stem cells offer promise for necrotic bone repair. Unfortunately, the isolation of marrow stem cells at sufficient purity and quantity remains a challenge. Connolly et al. have successfully used aspirated marrow, which contains mesenchymal stem cells (MSCs), to treat fractures and skeletal defects. Bruder et al. have used ex vivo culture-expanded MSCs to treat critical-size bone defects. This approach provides the cellular machinery directly to the fracture and/or defect sites and may be particularly useful for patients with fracture nonunions and delayed unions. Moreover, studies have demonstrated that MSCs can serve as a vehicle to deliver therapeutic genes and factors that enhance fracture repair. The advantages of using MSCs as a vehicle for ex vivo gene delivery are that the transfected cells would not only provide...
growth factors but also could enhance healing by providing osteoprogenitor cells. By successfully transfecting a cloned bone-marrow stem cell with LacZ and neomycin resistance genes, the present study demonstrated that cloned bone-marrow stem cells were useful as a carrier of genetically engineered factors for the treatment of skeletal disease.

Angiogenesis is a critical process in necrotic bone repair as well as in fracture-healing35-39. Invading vessels bring not only nutrients to the fracture site but also cells that participate in the repair process. Deficiencies in vascularity and angiogenesis will lead to a delayed union or nonunion35-36. Angiogenesis involves several steps, including degradation of the existing basement membrane and endothelial cell migration. These processes are likely to be controlled by angiogenic factors, including VEGF54-56,58,59. One study has found that VEGF can bind to osteoblasts and increase their migration and differentiation capabilities60. VEGF also plays an important role in bone growth via the endochondral ossification pathway and enhances bone formation in vivo61-63. Interestingly, the cloned bone-marrow stem cell used in this study expressed VEGF, which might have contributed to enhanced core defect repair by potentiating osteogenesis and angiogenesis through the local delivery mechanism. From this point of view, bone-marrow stem cells are ideal graft materials for the treatment of early stage osteonecrosis by providing osteogenic cellular elements as well as angiogenic growth factors. Numerous osteoinductive growth factors, such as bone morphogenetic proteins, are expressed at the fracture site and act in autocrine and paracrine fashion to attract and stimulate differentiation of stem cells into bone cells64. Some of the growth and differentiation factors have been used in patients13,40,49. However, the molecular form, dose, timing, and delivery system need further investigation. The results of the present study demonstrate the feasibility of using such a cloned marrow stem cell to investigate the complex mechanisms of bone repair.

One limitation of this study was that defects were created in normal bone, which may not reproduce the condition of osteonecrosis. This model was used because there is no suitable animal model in which to study osteonecrosis45,47. This defect and/or decompression procedure mimicked the clinical situation of osteonecrosis as a model in which all of the necrotic tissue was removed, so that the blood supply to the area that is newly filled with graft material can be reestablished45,51,52,68. The model seems reasonable considering that recent clinical studies on the treatment of osteonecrosis, which made use of core decompression together with bone graft and growth factors, did not reveal graft survival problems53,54,62.

In summary, the current study demonstrates that the cloned bone-marrow stem cell can directly form bone after transplantation into bone defects and at ectopic sites, indicating that the in vitro expanded bone-marrow stem cells can serve as a graft material that may enhance bone repair and therefore may be useful in the treatment of osteonecrosis. In addition, this study demonstrates that genetic labeling can be used as a tool in studies of cell differentiation in vivo and that bone-marrow stem cells can serve as a carrier of genetically engineered factors in the treatment of musculoskeletal diseases.

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