Published in final edited form as:
Clin Orthop Relat Res. 2005 March ; (432): 242–251.

Selective Retention of Bone Marrow-Derived Cells to Enhance Spinal Fusion

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Abstract

Connective tissue progenitors can be concentrated rapidly from fresh bone marrow aspirates using some porous matrices as a surface for cell attachment and selective retention, and for creating a cellular graft that is enriched with respect to the number of progenitor cells. We evaluated the potential value of this method using demineralized cortical bone powder as the matrix. Matrix alone, matrix plus marrow, and matrix enriched with marrow cells were compared in an established canine spinal fusion model. Fusions were compared based on union score, fusion mass, fusion volume, and by mechanical testing. Enriched matrix grafts delivered a mean of 2.3 times more cells and approximately 5.6 times more progenitors than matrix mixed with bone marrow. The union score with enriched matrix was superior to matrix alone and matrix plus marrow. Fusion volume and fusion area also were greater with the enriched matrix. These data suggest that the strategy of selective retention provides a rapid, simple, and effective method for concentration and delivery of marrow-derived cells and connective tissue progenitors that may improve the outcome of bone grafting procedures in various clinical settings.

Bone grafting is widely used in orthopaedic surgery to treat acute fractures, fracture nonunions, and bone defects, and to achieve therapeutic arthrodesis. Each year, approximately 500,000 bone grafting procedures are done on patients in the United States.[4] The efficacy of the grafts used in these procedures generally is considered to be derived from their osteoconductive and osteoinductive properties.[2,10,14,22]

Osteoconduction can be defined as a scaffold function provided by a graft material that facilitates the attachment and migration of cells that contribute to new tissue formation.[25]

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[a]One of the authors (GFM) has or may receive payments or benefits from a commercial entity (DePuy Acromed, Raynham, MA), under a license agreement with The Cleveland Clinic Foundation, related to this work.

Each author certifies that his or her institution has approved the animal protocol for this investigation and that all experimentation was done in conformity with ethical principles of research.

One or more of the authors have received funding from the Musculoskeletal Transplant Foundation (MTF, Edison, NJ); Grant R01 AR-42997 from the National Institutes of Health (Bethesda, MD); and from The Cleveland Clinic Foundation (Cleveland, OH).

Osteoinduction broadly refers to biologic stimuli from diffusible or matrix-bound peptide growth factors and cytokines that promote osteoblastic progenitors to migrate, proliferate, and differentiate. The prototypical stimuli are the family of bone morphogenetic proteins (BMPs), but many factors contribute. Osteogenic and non-osteogenic cells, including endothelial cells,[39] may elaborate inductive factors.[5,10,14,15]

Although osteoconduction and osteoinduction must be present in a successful graft site, the efficacy of any osteoconductive material or osteoinductive stimulus depends entirely on the presence of a sufficient number of osteogenic progenitors in the graft or graft site (the osteogenic potential). Were it not for the presence of osteogenic cells in most graft sites, the implantation of an osteoconductive material or the delivery of an osteoinductive stimulus alone would be ineffective. Fortunately, osteogenic cells are present in viable local bone and periosteum and also are found in adjacent soft tissues, muscle, or fat.[6,31,33] Osteogenic cells also may be transplanted into the graft site with autogenous bone graft or bone marrow.

Despite the presence of osteoblastic progenitors in virtually all graft sites, many clinical settings are likely to be deficient in osteoblastic progenitors. These settings include atrophic nonunited fractures; large or segmental bone defects; regions of scarring after infection, trauma, or previous surgery; regions of previous radiation therapy; regions of osteonecrosis; and patients who are immuno-compromised because of systemic illness or chemo-therapy. The concept that the number of osteoblastic progenitor cells in many graft sites is suboptimal is supported by the large volume of animal and clinical data[10,15,22–24,27] indicating that the addition of bone marrow-derived cells to almost any osteoconductive or osteoinductive material results in a considerable improvement in outcome.

Recognizing the potential biologic value and low risk of surgical complications,[41] many surgeons have used bone marrow aspirated from the iliac crest as an adjuvant to bone grafting procedures, even in the absence of practical means of concentrating cells in the operating room.[8,11–13,32,35,36,38] Efforts have provided information to assist surgeons in optimizing methods for the use of bone marrow-derived cells. For example, it has been shown that the concentration of bone marrow-derived cells in a bone marrow aspirate is diluted rapidly by peripheral blood as the volume of the aspirate is increased. However, this effect can be reduced by limiting the volume of aspiration from a given needle site to 2 cc or less.[19] Using established means for assay of connective tissue progenitors from human bone marrow, some investigators have begun to characterize the effects of clinical variables (age, gender, and disease) on the population of connective tissue progenitors.[16,27]

To provide surgeons with practical means of concentrating and delivering bone marrow-derived cells for optimization of bone grafting procedures, we observed that connective tissue progenitor cells and other bone marrow-derived cells can be concentrated rapidly from bone marrow aspirates using selected materials with surface properties that facilitate the rapid attachment of osteoblastic progenitors.[18] One such material is allograft bone chips. We also reported that a cellular graft using allograft chips that is enriched fourfold in the number of connective tissue progenitors performs significantly better than allograft chips combined with bone marrow alone.[28] However, this improvement was shown to require the presence of a fibrin clot environment provided by nonanticoagulated bone marrow or blood.[28]

The current experiment was designed to expand on these observations by testing the hypothesis that demineralized cortical bone powder provides an effective alternative substrate for selective retention and to confirm that concentration of connective tissue progenitors using the selective retention strategy significantly improves the radiographic and mechanical performance of demineralized cortical bone powder used with or without conventional bone marrow aspirate in spinal fusions.

MATERIALS AND METHODS

Demineralized canine allograft cortical bone powder was used as the matrix for attachment and delivery of cells. All allograft matrices used in these experiments were prepared under sterile conditions from allograft bone from Beagles (femurs and humeri) at Osteotech, Inc. (Eatontown, NJ), using methods identical to those used to process demineralized human cortical bone allograft matrix for clinical use. Graft preparation was in accordance with the standards of the American Association of Tissue Banks.[1] Cortical bone was frozen with liquid nitrogen, milled to a powder, and then washed in alcohol-based solvents and detergents to remove fats and cellular debris. The matrix then was demineralized to less than 5% wt/wt residual calcium in 0.6 mol/L HCl, adjusted to neutral pH, and lyophilized. A range of particle size from 425 μm to 850 μm in diameter was selected. To limit the potential for histocompatibility differences between donors that might compromise paired comparisons between matrices, the graft matrices used in each dog were derived from one donor.

Allograft-bone marrow composites were evaluated in an established canine posterior segmental spinal fusion model.[20,21,26] Twelve male Beagles (Marshall Farms, North Rose, NY) were used (age, 12–15 months; weight, 11.8–13.4 kg). Study animals were cared for in accordance with the Principles of Laboratory Care and The Guide for the Care and Use of Animals.[29]

Detailed methods for the surgical procedure, animal care, and specimen harvest have been reported.[21] Briefly, localized fusions were done at three spinal fusion sites in each animal (L1–L2, L3–L4, and L5–L6). Each fusion site was separated by one normal mobile segment. Graft site preparation was done under constant irrigation using a high-speed burr (TURQ-3, 3-mm fluted ball; Anspach, Palm Beach Gardens, FL). The fusion site included the facet joints at each level and the space between the lamina at each site. Each animal received grafts of all three materials under evaluation, one at each site. To limit the potential for surgical bias and to ensure equal distribution of materials at each of the three graft sites, the site assigned to each material was determined randomly after site preparation. Twelve cards (two sets of six possible combinations of three materials in three sites) were prepared at the beginning of the experiment. Site assignments then were made intraoperatively by randomly drawing a card from this set of possible combinations after site preparation was complete. Internal fixation was applied to each segment using stainless-steel plates (0.125 inches × 0.4 inches × 1.4 inches) placed on either side of the spinous processes. These plates were fixed using threaded bolts (2-56 thread, 0.5 inches long) passing through each spinous process with locking nuts. Each animal received preoperative prophylactic antibiotics of penicillin G (500,000 units) given intramuscularly, then 250 mg ampicillin given orally each day for 5 days. Acepromazine and acetaminophen were used for perioperative pain. No external immobilization was used.

All animals were euthanized 12 weeks postoperatively using an overdose of pentobarbital. The lumbar spine was harvested intact. Plate fixation was removed. Quantitative assessment of the bone formation in each fusion segment was done using helical radiography, CT scanning, and three-dimensional (3-D) image analysis. The spines were frozen at −20°C until preparation for mechanical testing. Each fusion segment then was tested mechanically to failure for assessment of mechanical properties. The cross-sectional area of the fusion mass bridged with firm mineralized bone tissue then was assessed as a union score for each site.

The following cell-matrix composites were evaluated: demineralized cortical bone powder (DCBP) alone − DCBP (1.5 cc) + saline (1.5 cc); DCBP + aspirated bone marrow (ABM) − DCBP (1.5 cc) + ABM (1.5 cc); and enriched DCBP + ABM − enriched DCBP composite (1.5 cc) + ABM (1.5 cc). This enrichment was designed to result in an approximately fivefold increase in the number of osteoblastic progenitors.

After induction of general anesthesia, and before beginning the approach to the spine, a 3-mm skin incision was made using a Number 11 blade over the anterior aspect of the proximal humerus. Previous experience has shown that the proximal humerus in a canine is a reliable source of hematopoietic bone marrow containing osteoblastic progenitors and that the yield of cells and progenitors from the humerus is more reliable than aspirates taken from the iliac crest in a canine. A Lee-Lok bone marrow aspiration needle (Lee Medical, Ltd., Minneapolis, MN) was inserted through one cortical site. Ten separate 2-cc bone marrow aspirates were harvested from the proximal humeri with each dog under general anesthesia (five aspirates were harvested from each side). Aspiration sites were separated by approximately 1 cm by changing the direction and depth of the needle placement. Samples were aspirated into 10-mL syringes (Becton Dickinson and Co, Franklin Lakes, NJ). One aspirate on each side was harvested without heparin to provide a marrow clot. Four additional aspirates on each side were harvested into syringes containing 1 cc sodium heparin solution (1000 units/cc). Each syringe was inverted several times to ensure complete mixing with the heparin solution. The eight heparinized aspirates were pooled (approximately 30 cc total volume) to provide a noncoagulated, single-cell suspension of bone marrow-derived cells diluted in blood. A 1-cc aliquot of the pooled sample was used to assay the concentration of nucleated cells using a hemacytometer.

For preparation of the enriched composite, a 1.5-cc volume of demineralized cortical bone powder was loaded into a 10-cc syringe using a nylon mesh at the tip of the syringe to prevent passage of the powder out of the syringes. The cellularity of marrow aspirates and the prevalence of connective tissue progenitors varies significantly between humans[19] and dogs.[28] Therefore, to control for variation between dogs with respect to the number of cells and connective tissue progenitors transplanted, a sample of heparinized bone marrow containing 600 million nucleated cells was prepared for each dog. This then was passed through the matrix at a flow rate of 0.5 cc per minute (a linear flow rate of approximately 25 cc/minute) to prepare the enriched cell-matrix composite. The number of cells and osteoblastic progenitors present in the effluent solution from each preparation was assayed to determine the number of cells and osteoblastic progenitors selectively retained in the matrix. Sample preparation took approximately 20 minutes.

Once the enriched composite was returned to the operating suite, the final composite preparation was completed. Demineralized cortical bone powder (1.5 cc) was moistened with 1.5 cc of saline to form the DCBP-alone graft material. Demineralized cortical bone powder (1.5 cc) was mixed physically with 1.5 cc of the clotted nonheparinized bone marrow aspirate to form a paste of the DCBP + ABM material. Similarly, the enriched composite (1.5 cc) was mixed physically with 1.5 cc of the nonheparinized bone marrow aspirate to form a paste of the enriched DCBP + ABM material.

The number of osteoblastic progenitors in each sample was assayed using an established colony-forming assay.[16,19,27,28] This assay was used to determine the number of colonies formed after culture of a defined number of nucleated cells. Colonies assayed in this way include cells that can differentiate into bone and phenotypes other than bone (cartilage, fat, muscle, and fibrous tissue). This population of tissue-derived cells has been defined broadly as connective tissue progenitors.[23] When cultured under conditions that promote proliferation and osteoblastic differentiation, approximately 90% of all connective tissue progenitor colonies express alkaline phosphatase (ALP), an early marker of bone differentiation. The number of ALP-positive connective tissue progenitors can be used as an assay of osteoblastic progenitors.

Briefly, heparinized marrow suspensions were centrifuged at 1500 rpm for 10 minutes. The buffy coat was pipetted and re-suspended in 5 cc of alpha minimal essential medium (Gibco BRL, Grand Island, NY). Nucleated cells were counted using a hemacytometer. Cells were

plated at a density of 125,000 cells/cm^2 on 4-cm^2 slides (Lab-tek chamber slides, Fisher Scientific, Pittsburgh, PA). The culture medium consisted of 90% alpha minimum essential medium, 10% fetal bovine serum (Lot no. 6MO109, BioWhittaker, Walkersville, MD), dexamethasone (10^{-8} mol/L), and ascorbic acid (50 μg/cc). Slides were incubated at 37°C in 5% CO_2. The medium was changed once at Day 7. On Day 9, the slides were stained in situ for determination of ALP activity. Cell clusters containing eight or more cells also expressing ALP were counted as connective tissue progenitors.

To assay the number of cells and connective tissue progenitors delivered in each graft site, the matrix loading process was done and controlled in four steps. A heparinized suspension of bone marrow-derived cells and blood obtained by aspiration was prepared. Then the heparinized suspension was passed through the matrix under controlled conditions to allow selective retention of cells by adhesion of cells to the matrix surface. Next, the effluent containing cells that did not adhere to the matrix was collected. Finally, the initial sample and the effluent sample were assayed for nucleated cells and connective tissue progenitors.

Using the cell count and connective tissue progenitor data, calculations were done that showed the retention and selection of cells and connective tissue progenitors in the graft. These parameters included the number of cells and connective tissue progenitors retained in the graft, binding efficiency for nucleated cells and connective tissue progenitors (fraction of cells and connective tissue progenitors retained), selection ratio for connective tissue progenitors versus nucleated cells (relative efficiency of retention for connective tissue progenitors versus other nucleated cells), and fold increase in concentration for cells and connective tissue progenitors (relative change in concentration from the initial marrow aspirate to the final graft).

The raw data for each implanted cell-matrix composite included the number of nucleated cells and connective tissue progenitors in the original sample (N_O, CTP_O) and in the load effluent (N_{LE}, CTP_{LE}). The following calculations then were done for each implanted cell–matrix composite graft.

The number of cells and connective tissue progenitors retained in the matrix (N_R and CTP_R) was determined by the following equations: $N_R = N_O - N_{LE}$ and $CTP_R = CTP_O - CTP_{LE}$. The binding efficiency (BE) for connective tissue progenitors and nucleated cells (CTP_{BE} and N_{BE}) was calculated as follows: $N_{BE} = N_R/N_O$ and $CTP_{BE} = CFU_R/CTP_O$. The selection ratio (SR) for connective tissue progenitors versus nucleated cells was calculated in terms of the binding efficiency ratio ($SR = CTP_{BE}/N_{BE}$). A selection ratio greater 1.0 implies positive selection or enrichment of connective tissue progenitors with respect to other marrow cells. The fold increase in concentration (ΔC) for cells and connective tissue progenitors was calculated as a ratio of the concentration in the final graft versus the concentration in the initial sample: $N_{\Delta C} = (N_R/\text{graft volume}) \div (N_O/\text{aspirate volume})$; $CTP_{\Delta C} = (CTP_R/\text{graft volume}) \div (CTP_O/\text{aspirate volume})$ = [CTPs] in the graft/[CTPs] in the marrow aspirate.

There were minor differences in the volume of graft implanted in these three groups. In preparing each sample, 1.5 cc of demineralized cortical bone powder first was hydrated with 1.5 cc saline. After hydration, the volume of the matrix was increased to approximately 1.7 cc as a result of swelling of the matrix particles and collection of a layer of fluid in the spaces between particles. The resulting consistency was like that of wet sand. There was no observable change in the volume of the matrix after the enrichment process. This was not surprising, because the volume contributed by the 148 ± 50 million cells retained in the enriched matrix after passing the heparinized bone marrow sample through the matrix was small (≤ 0.05 cc, if compressed as a pellet) and readily accommodated the space between bone matrix particles. When the matrices in the DCBP + ABM group and the enriched DCBP + ABM group were combined with clotted bone marrow, the formed clot fragments seemed to be retained in the

matrix, displacing some of the fluid that was retained between particles after the loading process. After mixing, the graft was allowed to set for 10 to 15 minutes. Any excess fluid was allowed to drain from the matrix before implantation. Therefore, the final volume of the graft was the same (approximately 2.0 cc) in the DCBP + ABM group and the enriched DCBP + ABM group, but slightly lower in the DCBP-alone group (approximately 1.7 cc). This preparation method achieved the goals of the study and ensured that any differences between the DCBP + ABM group and the enriched DCBP + ABM group in the final number of cells and progenitors that were delivered to the graft site was the direct result of increases in the concentration of cells and progenitors achieved during the process or selective attachment. These differences were not attributable to any differences in the volume of the graft material that was transplanted into the graft site.

Quantitative assessment of the fusion mass was done using helical radiography, CT scanning, and automated three-dimensional image-processing techniques to determine the volume of each fusion mass (bone volume), the cross-sectional area at the center of each fusion mass (fusion area at center slice), and the mean bone mineral density (BMD) of the fusion mass (bone density).

A three-dimensional data set was acquired of all segments from L1–L6 in each spine using a Somatome Plus 40 CT scanner (Siemens Medical System Inc., Iselin, NJ). Scanning was done for 30 seconds at 120 kV(p), 210 mA, 1 second helical mode, 2-mm collimation, and at a table speed of 2 mm/second with a Siemens BMD phantom. Images were reconstructed using a bone algorithm and an image-to-image overlap of 1 mm.

Original software (D–image Dog 2 for Unix system), developed in the Department of Biomedical Engineering of our institution, was used to manipulate and observe the three-dimensional CT data. The area of the fusion mass (center slice area) was calculated by adding the number of segmented pixels in the transaxial plane at the middle section with a value of more than 1400 (366 Hounsfield units), then multiplying this sum by the appropriate pixel area (1 mm^2). The volume of the fusion mass (bone volume) was calculated by adding the segmented voxels with a value of more than 1400 units in the specified region of interest in 11 image slices (the center slice plus five slices above and five slices below the middle disc cross section) and multiplying this sum by the appropriate voxel volume (1 mm^3). The mean mineralization density (bone density) was calculated for the entire fusion mass and referenced to the density of a phantom. As previously described,[21] before testing, each specimen was thawed for 24 hours at room temperature. Testing was done on an MTS Bionix 858 Materials Testing System (MTS, Minneapolis, MN) using a custom four-point bending device. After three sinusoidal conditioning cycles, load displacement data were collected nondestructively with the specimens in flexion and extension and left and right bending. Failure testing then was done in right bending using the ramp function at 8 mm/second. Lateral bending was selected as the mode of failure because lateral bending stiffness was correlated most closely with union status in previous studies.[20,26] Load displacement curves were used to determine stiffness, maximum load, displacement to failure, and total energy to failure. Failure in all segments occurred in the transaxial plane through the midportion of the fusion mass, at the level of the disc space.

Immediately after mechanical testing, the surface of the fractured specimen was examined using a metal probe. By comparing both sides of the fracture surface, the degree of union was scored from 0 to 4 based on a regional grid system described previously.[20,21,26,28] A score of 4 represents complete fusion of both facet joints and the entire lamina. Scores of 0, 1, 2, 3, and 4 represent union across approximately 0%, 25%, 50%, 75%, and 100% of the cross-sectional area of the grafted volume, respectively. Scoring was done by two observers (YM and GFM), who were blinded with respect to the material grafted at each site.

An ordered logistic regression using the proportional odds model,[17] which adjusted for the correlation of observations in the same animal and potential differences between graft sites, was used to compare union scores for the three cell-matrix composites. Pairwise comparisons were made between cell-matrix composites. Repeated measures analyses of CT data (fusion volume, fusion area, and bone density) and mechanical stiffness data were done by SAS Proc Mixed (SAS, Inc., Cary, NC), which provided estimates of the means for each cell-matrix composite. The reported p values were two-sided, and a p value of 0.05 or less was considered statistically significant.

RESULTS

One animal had a deep wound infection involving the region of plate fixation overlying the enriched DCBP + ABM site; this dog was euthanized 3 weeks after surgery and was replaced, maintaining data from 12 animals and 36 graft sites for analysis.

Concentration and selection of connective tissue progenitors did improve graft performance, confirming the initial hypothesis that a stepwise improvement in union score would be seen when marrow cells were added to the matrix (DCBP versus DCBP + ABM) and when marrow cells and connective tissue progenitors were concentrated additionally using the selective retention strategy (DCBP + ABM versus enriched DCBP + ABM). The total union score was greatest for the enriched DCBP + ABM group (mean, 2.3) and was greater than the DCBP-alone group (mean, 0.5) ($p = 0.009$) and the DCBP + ABM group (mean, 1.3) ($p = 0.04$) (Table 1). The union scores in the DCBP + ABM group also were superior ($p = 0.05$) to those in the DCBP-alone group.

The union score data also showed an effect of graft site. In this experiment, grafts at the most proximal site (L1–L2) scored lower ($p = 0.03$) than grafts at the more distal sites. This finding has been observed in some previous experiments using this model, but does not impair the statistical comparison between materials, because the materials were distributed equally by site.[20,21,26,28]

Improved performance with the enriched DCBP +ABM graft also was reflected in the overall data for union rate (achievement of a union score greater than 0). The union rate was highest ($p = 0.018$, Fisher's exact test) in the enriched DCBP + ABM group, in which eight of 12 grafts healed (67%). The union rate for the DCBP + ABM group was six of 12 grafts (50%), compared with two of 12 grafts (17%) in the DCBP-alone group. Unions with higher scores also were most common in the enriched DCBP + ABM group, in which six of 12 sites (50%) achieved a score of 3.0 or higher. In contrast, only two of 12 (17%) sites in the DCBP + ABM group and only one of 12 (8.3%) sites in the DCBP-alone group achieved a score in this range ($p = 0.097$ and 0.034, respectively; Fisher's exact test).

Concentration and selection of connective tissue progenitors also was associated with improved mechanical performance. The enriched DCBP + ABM group showed greatest mechanical stiffness (mean, 13.8 ± 7.7 N/mm). This was greater ($p = 0.006$) than the mechanical stiffness in the DCBP-alone group (9.4 ± 3.3 N/mm). In contrast, the stiffness in the DCBP + ABM group (11.0 ± 4.5 N/mm) also was not greater ($p = 0.25$) than that in the DCBP-alone group (Table 2).

The data on maximum load, displacement to failure, and total energy to failure only could be calculated from specimens with bony fusion in which failure was associated with a defined yield point. Data for these parameters, therefore, were limited to only those graft sites that successfully fused. The mechanical performance of these fused segments did not differ among the three composites (with adjustment for union score) and was consistent with previous reports using this model (data not shown).[20,26,28]

Fusion volume and fusion area data also support the initial hypothesis of stepwise improvement when marrow cells and connective tissue progenitors were concentrated additionally using the selective retention strategy (DCBP + ABM versus enriched DCBP + ABM). Fusion volume and fusion area in the enriched + ABM group were greater than those in the DCBP + ABM group ($p = 0.02$ and $p = 0.05$, respectively) (Table 3). Similarly, the fusion volume and fusion area in the enriched + ABM group were superior to the DCBP-alone group ($p = 0.001$ and $p = 0.005$, respectively). The DCBP + ABM group also was superior to the DCBP group for fusion volume and fusion area ($p = 0.001$ and $p = 0.04$, respectively). There were no differences between groups in the mean density of the bone formed.

Data measuring cell and connective tissue progenitor retention in the enriched graft also support the initial hypothesis that demineralized cortical bone powder provides an effective substrate for selective retention and concentration of connective tissue progenitors. The enriched DCBP + ABM group delivered more cells ($p < 0.001$) (Table 4). The mean concentration of marrow-derived cells implanted in the enriched DCBP + ABM group was increased by a factor of 2.3 ± 0.5 by the loading process. The total number of cells implanted with the graft was 269 ± 41 million cells, in contrast to an estimated 122 ± 20 million cells in the DCBP + ABM group.

The enriched DCBP + ABM group also delivered more connective tissue progenitors (Table 5). The mean number of connective tissue progenitors implanted in the enriched DCBP + ABM group was increased by a factor of 5.6 ± 3.9 by the loading process. The mean number of connective tissue progenitors implanted with the graft was 39,400 ± 24,500, in contrast to an estimated 7400 ± 4080 connective tissue progenitors in the DCBP + ABM group ($p < 0.001$).

Connective tissue progenitors adhered selectively to the cortical bone powder. Overall, 61% (± 14%) of connective tissue progenitors that were exposed to the matrix were retained. In contrast, only 23% (± 8%) of all nucleated cells in bone marrow were retained. This represents a positive selection of connective tissue progenitors over other marrow-derived cells of 3.0 (± 1.5)-fold.

DISCUSSION

This experiment was designed to test the hypothesis that demineralized cortical bone powder provides an effective alternative substrate for selective retention and to recon-firm our previous observation[28] that the concentration of connective tissue progenitors using the selective retention strategy significantly improves the performance in spinal fusions of an already effective osteoconductive matrix material, either with or without the addition of a conventional bone marrow aspirate.

These data show that demineralized cortical bone powder can be used as a substrate for rapid intraoperative concentration of bone marrow-derived cells. The addition of a simple aspirate of bone marrow to demineralized cortical bone powder resulted in an improvement in union score, fusion area, and fusion volume. This finding is consistent with results of numerous reports[3,9,11,13,30,34,36,38] suggesting the benefit of adding cells harvested by aspirated bone marrow to a graft site. Moreover, these data also support the hypothesis, and our previous observation, that the efficacy of a graft can be increased through preparation of an enriched composite of marrow cells, using the surface of the matrix as a means of selectively attaching and retaining marrow-derived cells and connective tissue progenitors for transplantation into a graft site. Enrichment of the matrix in this way resulted in increases in union score, fusion area, and fusion volume beyond those achieved by using a bone marrow aspirate alone. This enriched matrix was far superior to the allograft matrix alone.

We did not directly compare the enriched matrix preparation with the contemporary gold standard of autogenous cancellous bone. However, the mean union score of 2.3 achieved in

this study by an enriched DCBP + ABM graft is within the range of union scores (2.0–3.0) reported using autogenous cancellous bone in this model.[20,21,26] In contrast, the union score of 1.3 achieved by addition of a simple bone marrow aspirate to the allograft matrix was not in this range.

Connolly et al[7] were the first to suggest that concentration of bone marrow-derived cells could be used to enhance the performance of a cellular bone graft. They showed that increasing the concentration of cells (approximately fourfold) from rabbit bone marrow using density gradient separation resulted in increased bone formation in diffusion chambers. Although this method seemed to be effective, it required the use of a centrifuge in the operating room. In contrast, the selective retention method described in the current study does not require a centrifuge, nor are cells exposed to other potentially toxic agents (Percoll or Ficol) that must be removed later in processing. The selective retention method proposed by us also has the opportunity to exclude cells that do not attach, reducing the population of cells that compete with the retained cells and connective tissue progenitors after implantation.

Using selective retention, cell-matrix composites enriched in the concentration and prevalence of osteogenic connective tissue progenitors can be prepared rapidly in the operating room. However, this and a previous study[28] should be viewed only as initial feasibility studies that show the value of selective retention. Many variables in the processing require improvement before this method is practical for clinical use, as discussed later. Despite promising performance in this established canine spinal fusion model, direct clinical evaluation in selected treatments is necessary to show clinical value. Several clinical assessments are being done.

Optimal use of this method in the operating room requires selection of matrices with an appropriate porous structure, surface area, surface chemistry, and handling properties for processing and for implantation. Although only one structure was assessed in the current study, it is interesting to compare the selective retention characteristics of the demineralized cortical bone powder used in this study with that of the allograft cancellous chips used in a previous study.[28] Using similar loading techniques, demineralized cortical bone powder, with greater available surface area than cancellous chips, showed a greater increase in connective tissue progenitor concentration, greater relative selection of connective tissue progenitors versus other cells in marrow, and higher overall union scores than those achieved using cancellous chips alone as the matrix substrate.

Optimization of a selective retention strategy for clinical use also requires practical methods for control of the rate and pattern of fluid flow through the matrix in the operating room. In this study, preparation of the cell matrix composites was done in a separate laboratory under a laminar flow hood, using a customized nylon mesh to retain the matrix and a variable vacuum manifold to control flow rates. However, controlled fluid flow conditions must be made practical and convenient for use in an operating room with a limited set of specialized materials and equipment.

Optimization also may involve improved methods to harvest bone marrow before processing. Clinical aspiration of bone marrow has been shown to be a simple, safe, and effective means of harvesting connective tissue progenitors in considerable numbers.[16,19,27] Appropriate techniques for harvesting bone marrow by aspiration have been defined and can be taught and learned readily.[19] No patient has reported pain, swelling, infection, or hematoma at the aspiration site that required a change in pain medication, limited progress in rehabilitation, or prolonged hospitalization. However, future techniques should limit the time that is required for marrow harvest and the dilution of bone marrow cells with peripheral blood that has been shown to occur when aspiration volume from any given site is not limited to 2 cc or less using a static single-hole needle.[19]

Optimization of these methods also may include improvements in our understanding of the individual biologic features of patients and patient selection. However, current data suggest that few patients would be excluded. In three previous reports of clinical bone marrow harvest and subsequent experience, it was shown that connective tissue progenitors can be harvested from the iliac crest of almost all patients,[16,19,27] regardless of age or gender. The only exceptions, in our experience, arise in sclerotic regions of bone in patients with osteopetrosis and in patients with marrow-packing disorders such as myelofibrosis. We also showed that there is considerable variation in the concentration and prevalence of connective tissue progenitors in the bone marrow of patients. This variation is partly a function of age and gender.[27] There is a slow decline in marrow cellularity with age in men and women. In women, this decline in marrow cellularity is compounded by a decline in the prevalence of connective tissue progenitors, a finding that may be related to the pathophysiology of bone loss and estrogen deficiency after meno-pause.[27] However, only a small part of the variation in connective tissue progenitor concentration and prevalence in the tissues of patients is attributable to identified clinical factors. Other uncharacterized factors of genetics, systemic health (or disease), medications, tobacco and alcohol use, and local tissue conditions must account for this variation.[27]

Given the wide variation in connective tissue progenitor concentration among patients, it seems likely that, in some individuals, a deficiency in the number of cells and connective tissue progenitors may result in a clinically important reduction in the osteogenic potential, systemically or in specific tissue regions. In the context of a bone grafting procedure, a deficiency of connective tissue progenitors could limit the value of implanted osteoconductive materials and osteoinductive materials (BMPs), by reducing the number of available target cells. If so, the strategy of selective retention, or other means of enriching the local population of connective tissue progenitors, might offer a means of reversing or limiting this effect, and improve clinical outcomes.

Regardless of the variation that may exist among patients in concentration of connective tissue progenitors in local tissues, the available data suggest that increasing connective tissue progenitor concentration in a graft site will improve local osteogenesis, even in healthy individuals. One of the most striking findings of our study is that relatively modest enrichment of the connective tissue progenitor population results in significantly enhanced bone graft performance, even in young, healthy Beagles. This is a biologic setting in which the graft site is comprised entirely of normal bone and healthy minimally traumatized soft tissues. One would not expect this setting to be deficient in osteoblastic progenitors. Therefore, the finding of improved results with only a modest increase in the number of cells and connective tissue progenitors suggests that the number of osteogenic progenitors may be suboptimal in the majority of graft sites, even in small graft sites in otherwise healthy patients. It also may imply that augmentation of the cell and progenitor pool may be desirable and even necessary in a wide range of clinical settings to achieve an optimal bone healing response, including settings in which effective osteoconductive materials or osteoinductive growth factors or both may be used. This possibility is supported by the findings of Yasko et al,[40] who showed in a small femoral defect model in young, healthy rats that the combination of whole bone marrow and a highly osteoinductive preparation of BMP-2 was significantly more effective than the BMP-2 preparation alone.

The optimal number of cells or progenitors required in a graft site is not known. Our study evaluated only one level of enrichment, which delivered a mean of 5.6-fold more connective tissue progenitors and a mean of 2.3-fold more marrow-derived cells than would be delivered by a simple bone marrow aspirate alone. Whether delivery of a larger number of cells or connective tissue progenitors will result in greater improvements in efficacy is speculation.

There is reason to predict, however, that there will be an upper limit of cell density that is desirable in a wound site. In a graft site, the capacity for diffusion of oxygen, carbon dioxide, and other nutrients is limited. As greater numbers of cells are delivered, these demands continue to increase and may exceed the capacity of diffusion into the graft site to support cell survival. As a result, beyond some threshold concentration, additional increases in the number of cells in a graft site might reduce the efficacy of the graft by inducing more profound hypoxia in the graft site and greater cell death.[24] The optimal number of connective tissue progenitors for transplantation and the optimal composition of the population of other marrow-derived cells needs to be determined experimentally. This is a topic of ongoing investigation.

We think the methods presented here for preparation of cellular grafts containing an enriched population of bone marrow-derived cells in an implantable matrix are valuable for optimizing the performance of bone-grafting materials using an integrated tissue engineering strategy. The elements of this conceptual framework are: efficient collection of tissue-derived cells containing stem cells and progenitor cells; rapid concentration and selection of a population of bone marrow-derived cells that includes cells that are capable of osteoblastic differentiation using the surface of an implantable matrix as the means of cell selection; delivery of these cells at a concentration sufficient to repopulate the graft site with bone-forming cells; delivery of cells in an environment in which their survival will not be precluded or threatened by the metabolic environment present in the graft site after implantation; choice of a matrix with surface and three-dimensional properties (pore size and architecture) that facilitate proliferation and migration of the desired cell population throughout the region in which the tissue is required; and creation of a biologic environment that contains an appropriate repertoire of cells and inductive stimuli to initiate all critical processes of local bone healing, including revascularization of the graft site and proliferation and differentiation of transplanted osteogenic cells. We think that this strategy, beginning with rapid, simple, safe, and inexpensive methods for concentration and delivery of bone marrow-derived cells and connective tissue progenitors, may improve the outcome of bone grafting in a wide range of clinical settings. Similar methods using bone marrow-derived cells also might be applied to various tissue engineering applications.

Acknowledgment

We thank Chizu Nakamoto, MD, PhD, for assistance in preparing this manuscript.

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TABLE 1

Union Scores by Animal, Material, and Site

Animal	DCBP-Alone			DCBP + ABM			Enriched + ABM		
	L1–L2	*L3–L4*	*L5–L6*	*L1–L2*	*L3–L4*	*L5–L6*	*L1–L2*	*L3–L4*	*L5–L6*
1		0				2.0	0		
2			2.5	0				3.0	
3		3.5		2.5					4.0
4	0				4.0				4.0
5	0					0		0	
6	0				2.5				1.5
7	0					4.0		3.0	
8		0				0	0		
9		0		0					3.0
10			0		0		2.5		
11			0	0				4.0	
12			0		0.5		0		
Site subtotal	0	3.5	2.5	2.5	7.0	6.0	2.5	10.0	12.5
Total score		6.0			15.5			25.0	
Mean		0.5			1.3*			2.3†	
Median		0			0.3			2.1	

* = Significantly greater than DCBP-alone (p = 0.05)

† = Significantly greater than DCBP + ABM (p = 0.04) and DCBP-alone (p = 0.009).

TABLE 2

Mechanical Stiffness Data

Graft Material	Stiffness (N/mm)	
	Mean* ± *SD	**95% CI**
DCBP-alone (n = 12)	9.4 ± 3.3	6.1, 12.7
DCBP + ABM (n = 12)	11.0 ± 4.5	7.7, 14.3
Enriched + ABM (n = 12)	13.8 ± 7.7*	10.5, 17.1

* = Greater than the DCBP-alone group (p = 0.006) but not greater than the DCBP + ABM group (p = 0.06)

TABLE 3

Computed Tomography Data

Outcome	DCBP-Alone (n = 12)	DCBP + ABM	Enriched + ABM (n = 12)
Fusion volume (mm^3)	2260 ± 459	2624 ± 533*	2953 ± 616†
Fusion area (mm^2)	102 ± 28	121 ± 31‡	140 ± 38§
Bone density	1876 ± 54	1849 ± 42	1854 ± 47

* = Greater than the DCBP-alone group ($p < 0.01$)

† = Greater than the DCBP + ABM ($p = 0.02$) and DCBP-alone group ($p < 0.0001$)

‡ = Greater than the DCBP-alone group ($p < 0.04$)

§ = Greater than the DCBP + ABM ($p = 0.05$) and the DCBP-alone groups ($p = 0.005$)

TABLE 4

Concentration of Cells in Cortical Bone Powder

Dog Number	Cells (Pooled Heparinized Sample) (× 10^6/cc)	Cells (Nonheparinized Sample) (× 10^6)	Cells Loaded in Matrix (× 10^6)	Cell Retained in Matrix (× 10^6)	Efficiency of Cell Attachment (%)	Cells Retained in Matrix (× 10^6/ cc)	Total Cells Implanted (× 10^6)	Fold Increase in Cells
1	72	108	725	125	17	83	233	2.2
2	117	176	648	58	9	39	234	1.3
3	88	132	600	131	22	87	263	2.0
4	80	120	661	185	28	123	305	2.5
5	74	111	630	156	25	104	267	2.4
6	76	115	642	92	14	61	207	1.8
7	78	117	632	218	34	145	335	2.9
8	84	126	627	98	16	65	224	1.8
9	88	132	600	139	23	93	271	2.1
10	68	102	704	164	23	109	266	2.6
11	64	96	677	212	31	141	308	3.2
12	82	123	600	195	33	130	318	2.6
Mean	81	122	646	148	23	99	269	2.3
SD	14	20	40	50	8	33	41	0.5

TABLE 5

Concentration of CTPs in Cortical Bone Powder

Dog Number	CTP (Pooled Heparinized Sample) CTPs/cc	CTP (Nonheparinized Sample) CTPs/cc	CTPs Loaded in Matrix CTPs/cc	CTPs Retained in Matrix CTPs/cc	Efficiency of CTP Attachment (%)	CTPs Retained in the Matrix CTPs/cc	Total CTPs Implanted CTPs/cc	Fold Increase in CTP	Selection Ratio CTPs vs Cells
1	2749	4123	87000	63990	74	42660	68113	16.5	4.3
2	5154	7731	28512	17302	61	11535	25033	3.2	6.8
3	3866	5799	72000	50426	70	33618	56225	9.7	3.2
4	9389	14084	77337	54489	70	36326	68573	4.9	2.5
5	3034	4551	25830	15402	60	10268	19953	4.4	2.4
6	2064	3096	17334	5234	30	3489	8330	2.7	2.1
7	9828	14742	79632	63072	79	42048	77814	5.3	2.3
8	8356	12534	62700	47882	76	31921	60416	4.8	4.9
9	4403	6605	30000	18014	60	12009	24619	3.7	2.6
10	3696	5544	38016	16416	43	10944	21960	4.0	1.9
11	2644	3966	27757	15667	56	10445	19633	5.0	1.8
12	4027	6041	29400	16035	55	10690	22076	3.7	1.7
Mean	4934	7401	47960	31994	61	21329	39395	5.6	3.0
SD	2717	4075	25523	21856	14	14570	24568	3.9	1.5