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Spine Fusion Using Cell Matrix Composites Enriched in Bone Marrow-Derived Cells

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Abstract

Bone marrow-derived cells including osteoblastic progenitors can be concentrated rapidly from bone marrow aspirates using the surface of selected implantable matrices for selective cell attachment. Concentration of cells in this way to produce an enriched cellular composite graft improves graft efficacy. The current study was designed to test the hypothesis that the biologic milieu of a bone marrow clot will significantly improve the efficacy of such a graft. An established posterior spinal fusion model and cancellous bone matrix was used to compare an enriched cellular composite bone graft alone, bone matrix plus bone marrow clot, and an enriched bone matrix composite graft plus bone marrow clot. Union score, quantitative computed tomography, and mechanical testing were used to define outcome. The union score for the enriched bone matrix plus bone marrow clot composite was superior to the enriched bone matrix alone and the bone matrix plus bone marrow clot. The enriched bone matrix plus bone marrow clot composite also was superior to the enriched bone matrix alone in fusion volume and in fusion area. These data confirm that the addition of a bone marrow clot to an enriched cell-matrix composite graft results in significant improvement in graft performance. Enriched composite grafts prepared using this strategy provide a rapid, simple, safe, and inexpensive method for intraoperative concentration and delivery of bone marrow-derived cells and connective tissue progenitors that may improve the outcome of bone grafting.

Autogenous cancellous bone is currently the most biologically effective graft material. However, harvest of autogenous bone is associated with significant morbidity: surgical scars, blood loss, pain, prolonged surgical time and rehabilitation, increased exposure to blood products, and infection risk.[39] Effective alternatives to autograft could significantly improve the outcome of these procedures and reduce the cost.

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Contemporary dogma suggests that the effectiveness of any successful bone graft material generally can be attributed to one or more of three core properties: osteoconduction, osteoinduction, and osteogenic cells.[3,11,18,27,28] Autogenous cancellous bone, the current gold standard, delivers all three to a grafted site. In addition to these biologic properties of the graft, optimal graft site incorporation generally requires that the surgeon provide a viable tissue surface from which revascularization of the graft site can take place and a mechanical environment in the graft site that promotes or allows successful bone formation.

Osteoconduction can be defined as the properties of a scaffold that facilitate the attachment and migration of cells transplanted in the graft or cells arising from neighboring bone or soft tissues, so that the bone healing response is distributed throughout the grafted volume.[27,28] Osteoinduction broadly refers to biologic stimuli from diffusible peptide growth factors and cytokines which promote osteoblastic progenitors to proliferate and differentiate to express a bone phenotype.[27,28] The prototypical stimuli are the family of BMPs, but many other factors also contribute, such as TGF-βs, IGFs, FGFs, PDGFs, and EGFs.[9,27,28] Osteoblastic cells are critical in this process, but other cells, such as vascular endothelial cells, inflammatory cells, and others also play important roles. Osteogenic and nonosteogenic cells, including endothelial cells,[38] may elaborate inductive factors that contribute to osteoinduction in the graft site.[5,11,18,20,28]

Although osteoconduction and osteoinduction must be present in any successful graft site, no osteoconductive material or osteoinductive stimulus will be effective in the absence of osteogenic cells. Osteogenic cells may be transplanted into the graft in an auto-genous graft of bone or bone marrow.[11,28] They also may migrate into the graft site from surrounding tissues, or be delivered to the graft site through the vascular system during and after revascularization. Regardless of the pathway, all successful graft procedures require some minimal number of osteogenic progenitors within the graft site.

Fortunately, osteogenic cells are present in most, if not all, graft sites. They are found in viable local bone and periosteum, and may arise from vascular pericytes[6,10,33,34] in adjacent soft tissues, muscle,[4] or fat.[14,15,40] The presence of osteogenic progenitors in systemic circulation is speculative, but the capacity of osteoblastic progenitors to survive in systemic circulation and be delivered to sites of bone formation is suggested by evidence that bone marrow transplantation has a beneficial and systemic skeletal effect in children with osteogenesis imperfecta.[17]

Despite the fact that osteoblastic progenitors are present in or can be recruited into virtually any graft site, one can predict that many clinical graft sites in many disorders are deficient or suboptimal for the number of osteoblastic progenitors. These include atrophic nonunited fractures; large or segmental bone defects; regions of scarring after infection, trauma, or prior surgery; regions of prior radiation therapy; regions of osteonecrosis; and sites in patients compromised by systemic illness or chemotherapy. The concept that the number of osteoblastic progenitor cells in many graft sites is suboptimal is supported by the large volume of data from animal models indicating that the addition of bone marrow-derived cells to almost any osteoconductive or osteoinductive material results in a significant improvement in outcome.[11,19,20,28,35]

Recognizing the likely biologic value and low risk, some surgeons have used bone marrow aspirated from the iliac crest as an adjuvant to their bone grafting procedures.[8,12,13,16,32,36,37] Surgeons have been guided in this effort by reports showing that osteoblastic progenitors can be harvested by aspiration in essentially every patient[21,24] as a mixture of bone marrow-derived cells diluted in blood and that the concentration of bone

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marrow-derived cells in a bone marrow aspirate can be enhanced by limiting the volume of aspiration from a given needle site to 2-cc or less.[24]

Additional advance in the use of bone marrow-derived cells may be achieved in the operating room by rapidly concentrating osteoblastic progenitors to form a cellular composite graft enriched in bone marrow-derived cells and osteoblastic progenitors.[23] However, optimal use of such an enriched composite graft requires delivery of cells in an optimized environment. One readily available means of changing the local environment in a graft site is to control whether the graft site includes the physiologic environment created by formation of a blood clot. The current study was designed to test the hypothesis that the efficacy of a cellular composite graft that is enriched in bone marrow-derived cells will be improved significantly by including clot formation in the milieu of the graft site.

MATERIALS AND METHODS

Materials

Canine allograft cancellous bone matrix was prepared as 3 × 3 × 3 mm cubes from Beagle femurs and humeri (Osteotech, Inc, Eatontown, NJ) using cleaning and processing techniques similar to those used for human bone in clinical use and in accordance with the 1998 standards of the American Association of Tissue Banks.[1] Mineralized and demineralized cubes were prepared. Graft matrices used in each dog were derived from one donor.

Graft Composites

The following cellular composite grafts were evaluated: (1) cancellous bone matrix enriched with bone marrow cells (2 cc); (2) cancellous bone matrix (2 cc) plus aspirated bone marrow clot (2 cc); and (3) enriched cancellous bone matrix (2 cc) plus aspirated bone marrow clot (2 cc).

Posterior Segmental Canine Spinal Fusion Model

Composites of allograft bone matrix and bone marrow cells were evaluated in an established canine posterior segmental spinal fusion model.[25,26,29] Twenty-four Beagle dogs were used (Marshall Farms, North Rose, NY) (age, 9–17 months; weight, 9.4–12.8 kg). In 12 dogs, the grafts were prepared using mineralized cancellous matrix. In another 12 dogs, the grafts were prepared using demineralized cancellous matrix.

Detailed methods for the surgical procedure, animal care, and specimen harvest have been published.[26] Briefly, localized fusions are done at three separate spinal fusion sites in each animal (L1–L2, L3–L4, and L5–L6). The fusion site includes the facet joints at each level and the space between the lamina at each site. Each animal is grafted with all of the three materials being evaluated using a randomization scheme that limits the potential for surgical bias and ensures equal distribution of materials at each of the three graft sites. Internal fixation is applied to each segment using stainless steel plates (0.125 × 0.4 × 1.4-inch) placed on either side of the spinous processes. These plates are fixed using threaded bolts (size 2; thread 56/inch; 0.5 inches long) passing through each spinous process with locking nuts. No external immobilization is used. Study animals are cared for in accordance with the Guide for the Care and Use of Animals.[31]

All animals were euthanized at 12 weeks. The lumbar spine was harvested intact. Plate fixation was removed. Quantitative assessment of the bone formation in each fusion segment was done using helical xray computed tomography (CT) and three-dimensional 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-

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sectional area of the fusion mass bridged with firm mineralized bone tissue was assessed as a union score for each site.

Bone Marrow Aspiration and Composite Preparation

After induction of general anesthesia, a 3-mm stab incision was made using a Number 11 blade over the anterior aspect of the proximal humerus. The authors' previous experience has shown that the canine proximal humerus 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 canine iliac crest.

A Lee-Lok bone marrow aspiration needle (Lee Medical, Ltd, Minneapolis, MN) was inserted through one penetration in the cortex. Twelve 2-cc aspirates were collected from bone sites separated by approximately 1 cm by changing the direction and depth of the needle placement, six from the left and six from the right humerus. Samples were aspirated into 10-mL syringes. The first aspirate of each side was harvested without heparin to provide a 2-cc clot of bone marrow-derived cells mixed with blood. The following five aspirates of each side were harvested into syringes containing 1-cc heparin solution (1000 units/mL) to provide a single cell suspension of bone marrow-derived cells mixed with blood. The 10 heparinized aspirates were pooled (approximately 30-cc total volume). A 1-cc aliquot of the pooled sample was used to assay the number of nucleated cells and connective tissue progenitors.

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A 2-cc volume of mineralized or demineralized cancellous chips were loaded into a 20-cc syringe (approximately 27 chips) and wetted with saline. The remaining marrow suspension (29-cc) was divided into equal parts and passed through the matrix samples using a flow rate of 0.5 mL/minute to prepare two cellular composite grafts enriched in bone marrow-derived cells and osteoblastic progenitors. This process exposed the surface of the 2-cc volume of cancellous matrix to the cells and osteoblastic progenitors present in a 10-cc volume of fresh marrow aspirate volume, a fivefold excess of marrow to matrix by volume. The number of cells and osteoblastic progenitors present in the effluent solution was assayed to allow determination of the number of cells and osteoblastic progenitors retained in the matrix.

The clotted samples of bone marrow-derived cells mixed with blood then were mixed physically with the cancellous bone matrix or the enriched bone matrix to form the final bone matrix plus bone marrow clot and the enriched bone matrix plus aspirated bone marrow clot composites, respectively. This preparation took approximately 20 minutes.

Assay of Osteoblastic Progenitors using a Colony Forming Unit Assay for Osteoblastic Progenitors

The number of osteoblastic progenitors in each sample was determined using an established colony forming assay.[21,24,30] Colonies expressing alkaline phosphatase were counted as positive, approximately 90% of all colonies. Colonies assayed in this way include cells which are capable of differentiation into phenotypes other than bone (cartilage, fat, muscle, fibrous tissue). This population of cells has been defined broadly as connective tissue progenitors.[11,28]

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Heparinized marrow suspensions were centrifuged at 400 × g for 10 minutes. The buffy coat was pipetted and resuspended in 5-mL alpha minimal essential medium (Gibco BRL, Grand Island, NY). Nucleated cells were counted using a hemocytometer. Cells are plated at a density of 125,000 cells/cm^2 on 4-cm^2 Lab-tek chamber slides (Fisher Scientific, Pittsburgh, PA). The culture medium consisted of 90% alpha minimum essential medium, 10% fetal bovine serum (Lot Number 6MO109, BioWhittaker, Walkersville, MD), dexamethasone (10^{-8} mol/L), and ascorbic acid (50 μg/mL). Slides were incubated at 37°C in 5% CO_2. Medium was changed

once at Day 7. On Day 9, the slides were stained in situ for determination of alkaline phosphatase activity. Cell clusters containing eight or more cells also expressing alkaline phosphatase were counted as connective tissue progenitors.

Calculation of the Number of Cells and Connective Tissue Progenitors Delivered in the Composite Graft

The matrix loading process consists of four steps: (1) prepare a heparinized suspension of bone marrow-derived cells in blood; (2) pass the heparinized suspension through the bone matrix allowing interaction between the suspended cells and the bone matrix surface; (3) collect the effluent containing cells that do not adhere to the bone matrix; and (4) assay the initial sample and the effluent sample for nucleated cells and connective tissue progenitors.

Knowing the number of nucleated cells in the original sample (N_O) and load effluent (N_{LE}) and the number of connective tissue progenitors in the original sample (CTP_O) and load effluent (CTP_{LE}), several calculations can be done. The number of cells retained in the bone matrix (N_R) and the number of connective tissue progenitors retained in the bone matrix (CTP_R) can be determined using the equations: $N_R = N_O - N_{LE}$ and $CTP_R = CTP_O - CTP_{LE}$. The binding efficiency for connective tissue progenitors (CTP_{BE}) or nucleated cells (N_{BE}) can be calculated as $N_{BE} = N_R/N_O$ and $CTP_{BE} = CFU_R/CTP_O$. The selection ratio (SR) for connective tissue progenitors versus nucleated cells is calculated as a ratio of binding efficiency, $SR = CTP_{BE} / N_{BE}$, where a selection ratio greater than 1 implies positive selection or enrichment of connective tissue progenitors over other marrow cells. The fold increase in concentration (ΔC) for cells and connective tissue progenitors in the matrix compared with the starting concentration in the bone marrow aspirate is calculated as $N_{\Delta}C_{=}$ (N_R / graft volume) ÷ (N_O / aspirate volume) and $CTP_{\Delta}C_{=}$ (CTP_R / graft volume) ÷ (CTP_O / aspirate volume).

Quantitative Computed Tomographic Image Analysis of Fusion Mass

Quantitative assessment of the fusion mass was done using helical xray CT 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 (area at center slice), and the mean bone mineral density of the fusion mass (bone density).

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

Original software (D – image Dog 2 for Unix system), developed in the Department of Biomedical Engineering at the authors' institution was used. The inferior boundary of the fusion mass was a plane defined by the dorsalmost points of the left and right neural foramen one segment above and below the fusion site. The center slice of the fusion mass was defined in the transaxial plane as the level of the intervertebral disc space. The area of the fusion mass at the center slice (center slice area) was calculated by summing the number of segmented pixels in the transaxial plane at the middle section with a value over 1400 (366 Hounsfield units) and then multiplying this sum by the pixel area (1-mm^2). The volume of the fusion mass (fusion volume) was calculated by summing the segmented voxels with a value over 1400 units within the specified region of interest in 11 contiguous slices (the center slice plus five slices above and five slices below the middle disc cross section) and multiplying this sum by the voxel volume (1-mm^3). The mean mineralization density (bone density) was calculated for the entire fusion mass and referenced to density phantom.

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Mechanical Testing

As previously described,[26] before testing, each specimen was thawed overnight at room temperature. Testing was done on an MTS Bionix 858 Materials Testing System (MTS, Minneapolis, MN) using a custom 4-point bending device. After three sinusoidal conditioning cycles, load displacement data were collected nondestructively in the planes of flexion, extension, and lateral 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 found to be most closely correlated with union status in prior studies.[25,26,29] Load displacement curves were used to derive 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.

Union Score

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 is scored from 0 to 4 based on a regional grid system described in previous publications.[25,26,29] A score of 4 is defined as 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 concerning the material grafted at each side.

Statistical Analysis

An ordered logistic regression using the proportional odds model[22] that adjusted for the correlation of observations within the same animal and potential differences between graft sites was used to compare union scores for three cell-matrix composites. Pair-wise 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 Institute, Cary, NC), which provided estimates of the means for each cell-matrix composite. The reported probability values were two-sided.

RESULTS

Complications

A deep wound infection developed in the region of plate fixation in one animal grafted with mineralized cancellous allograft chips and one animal grafted with demineralized allograft chips. These animals were euthanized 4 weeks after surgery and excluded from analysis. This left data from 22 animals and 66 fusion sites for analysis.

Union Score

Tables 1 and 2 summarize the union score for each composite in each animal and graft site for mineralized cancellous bone matrix and demineralized cancellous bone matrix, respectively.

These data support the hypothesis that the addition of a marrow clot to the bone graft environment significantly enhances the efficacy of the composite graft. When mineralized cancellous bone matrix was used, the union score was greatest for the Enriched Bone Matrix Plus Bone Marrow Clot Group (mean, 1.9). Furthermore, the union scores for the Enriched Bone Matrix Plus Bone Marrow Clot Group were significantly greater than those for the Enriched Bone Matrix Group without the marrow clot (mean, 0.6) ($p = 0.008$). The Enriched Bone Matrix Plus Bone Marrow Clot Group was also statistically superior to the Bone Marrow Clot Group (mean, 1.2) ($p = 0.04$). However, the difference between the Bone Matrix Plus

Bone Marrow Clot Group and the Enriched Bone Matrix Group was not significant (p = 0.09). The overall union rates (union score > 0) for the three composites, enriched bone matrix plus bone marrow clot, bone matrix plus bone marrow clot, and enriched bone matrix alone were nine of 11 (81%), seven of 11 (63%), and four of 11 (36%), respectively. Fusion sites with higher fusion scores were clustered in the Enriched Bone Matrix Plus Bone Marrow Clot Group. In the Enriched Bone Matrix Plus Bone Marrow Clot Group, four of 11 fusion sites (36%) were graded 3.0 or higher. However, only one of 11 (8.3%) sites in the Enriched Bone Matrix Group, and two of 11 sites (18%) in the Bone Matrix Plus Bone Marrow Clot Group were graded at this level.

When demineralized cancellous bone matrix was examined alone (Table 2), the union score also was greatest for the Enriched Bone Matrix Plus Bone Marrow Clot Group. However, union scores in the Enriched Bone Matrix Plus Bone Marrow Clot Group (mean = 1.9) were not statistically greater than those in the Enriched Bone Matrix Group (mean = 0.8) (p = 0.09), nor were they statistically superior to the Bone Matrix Plus Bone Marrow Clot Group (mean, 1.5) (p = 0.18). Similarly, the union score for the Bone Matrix Plus Bone Marrow Clot Group was not statistically superior to the Enriched Bone Matrix Group (p = 0.59). The overall union rates for the three materials, enriched bone matrix plus bone marrow clot, bone matrix plus bone marrow clot, and enriched bone matrix alone were seven of 11 (63%), six of 11 (54%), and five of 11 (45%), respectively. Again, fusion sites with higher fusion scores were clustered in the Enriched Bone Matrix Plus Bone Marrow Clot Group. In the Enriched Bone Matrix Plus Bone Marrow Clot Group, five of 11 fusion sites (45%) were graded 3 or higher. However, only two of 11 (18%) fusion sites in the Enriched Bone Matrix Group and three of 11 fusion sites (27%) in the Bone Matrix Plus Bone Marrow Clot Group were graded at this level.

When pooled data from mineralized and demineralized matrices were assessed, enhancing the power of these comparisons, union scores in the Enriched Bone Matrix Plus Bone Marrow Clot Group were statistically greater than those in the Enriched Bone Matrix Group (p = 0.002). The Enriched Bone Matrix Plus Bone Marrow Clot Group was also statistically superior to the Bone Matrix Plus Bone Marrow Clot Group (p = 0.04). However, there was no statistically significant difference between the union scores in the Bone Matrix Plus Bone Marrow Clot Group and those in the Enriched Bone Matrix Group (p = 0.11). The overall pooled fusion rate for the Enriched Bone Matrix Plus Bone Marrow Clot Group was 16 of 22 (73%), compared with 13 of 22 (59%) for the Bone Matrix Plus Bone Marrow Clot Group and nine of 22 (41%) for the Enriched Bone Matrix Group.

Mechanical Testing

Stiffness data were available for all specimens. Data for maximum load, deformation to failure, and energy to failure could be calculated only for specimens in which a bony union provided a defined yield point. The data presented in Tables 3 and 4 show a trend for greater stiffness in the Enriched Bone Matrix Plus Marrow Clot Group. The stiffness in the Enriched Bone Matrix Plus Bone Marrow Clot Group was significantly greater than that in the Enriched Bone Matrix Group (p = 0.05). However, the differences between materials did not approach statistical significance for any other mechanical parameter.

Computerized Tomographic Image Analysis

Tables 5 and 6 show data obtained using CT image analysis for each composite graft for fusion volume, fusion area, and mean bone density within the fusion mass. Fusion volume and fusion area were greatest for the Enriched Bone Matrix Plus Bone Marrow Clot Group.

For the mineralized matrix, data from the Enriched Bone Matrix Plus Bone Marrow Clot Group were superior in fusion volume and fusion area to the Enriched Bone Matrix Group (p = 0.004

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and p = 0.02, respectively). For the demineralized matrix, data from the Enriched Bone Matrix Plus Bone Marrow Clot Group were also superior in fusion volume and fusion area to the Enriched Bone Matrix Group (p = 0.03 and p = 0.02, respectively).

Pooling data from bone matrices enhanced the power of the comparison between the Bone Matrix Plus Bone Marrow Clot Group and the other two groups. This analysis showed that fusion volume and fusion area were greater in the Bone Matrix Plus Bone Marrow Clot Group than the Enriched Bone Matrix Group (p = 0.03 and p = 0.008, respectively). However, the difference in fusion volume and fusion area between the Enriched Bone Matrix Plus Bone Marrow Clot Group and the Bone Matrix Plus Bone Marrow Clot Group did not rise to the level of statistical significance (p = 0.16 and p = 0.28, respectively).

Concentration of Cells and Connective Tissue Progenitors in Mineralized and Demineralized Cancellous Bone Matrix

Tables 7 and 8 summarize data related to marrow cell loading and delivery using cancellous bone matrix. In each table, the concentration of cells in the initial pooled aspirate (column 2) is determined experimentally. Because it is not possible to directly measure the number of cells and connective tissue progenitors in the individual clotted marrow samples that were used in preparing each graft, it was necessary to estimate the concentration of cells in the bone marrow clot used during graft preparation. Because the number of cells and progenitors harvested by aspiration of a 2-cc marrow sample into an empty syringe should not be systematically different than the number of cells and progenitors in a marrow sample of equal volume that is aspirated into a heparin containing solution, the number of cells and progenitors in the clotted marrow samples in each subject was estimated based on the concentration of cells in the pooled heparinized sample, correcting for the 3:2 dilution caused by the 1-cc heparinized saline included in each aspiration syringe.

In the case of mineralized bone matrix (Table 7), these data show that mean concentration (total number) of marrow-derived cells implanted in the Enriched Bone Matrix Plus Bone Marrow Clot Group was increased by a factor of 2.7 ± 0.3. The total number of cells implanted with the graft was 489 ± 148 million cells, in contrast to an estimated 184 ± 54 million cells in the Bone Matrix Plus Bone Marrow Clot Group. The enrichment process alone resulted in delivery of 305 ± 100 million nucleated cells in the Enriched Bone Matrix Group.

In the case of the demineralized bone matrix, the mean concentration of marrow-derived cells implanted in the Enriched Bone Matrix Plus Bone Marrow Clot Group was increased by a factor of 2.5 ± 0.7. The number of cells implanted with the graft was 448 ± 177 million cells, in contrast to an estimated 178 ± 46 million cells in the Bone Matrix Plus Bone Marrow Clot Group. The enrichment process resulted in delivery of 270 ± 148 nucleated cells.

Tables 9 and 10 show data related to loading and delivery of connective tissue progenitors using cancellous bone matrix.

In the case of mineralized cancellous bone matrix, the mean number and concentration of connective tissue progenitors implanted in the Enriched Bone Matrix Plus Bone Marrow Clot Group was increased by a factor of 3.1 ± 0.7 by the loading process. The mean number of connective tissue progenitors implanted with the graft was 20,000 ± 12,600, in contrast to an estimated 6000 ± 3000 connective tissue progenitors in the Bone Matrix Plus Bone Marrow Clot Group.

Connective tissue progenitors tended to be retained in mineralized cancellous bone with slightly greater frequency than other bone marrow-derived cells. Overall, 57% (± 18%) of connective tissue progenitors that were exposed to the matrix were retained. However, only

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49% (± 8%) of the other nucleated cells in bone marrow were retained. This represents a selection of connective tissue progenitors over other marrow-derived cells of only 1.2 ± 0.3.

In the case of demineralized cancellous bone matrix, the mean number and concentration of connective tissue progenitors implanted in the Enriched Bone Matrix Plus Bone Marrow Clot Group was increased by a factor of 3.4 ± 0.5. The mean number of connective tissue progenitors implanted with the graft was 27,000 ± 14,900, in contrast to an estimated 8260 ± 4040 connective tissue progenitors in the Bone Matrix Plus Bone Marrow Clot Group.

In contrast to mineralized cancellous bone, connective tissue progenitors were retained in demineralized cancellous bone with significantly greater frequency than other bone marrow-derived cells. Overall, 70% (± 13%) of connective tissue progenitors that were exposed to the matrix were retained. However, only 44% (± 19%) of the other nucleated cells in bone marrow were retained. This allows calculation of a selection ratio of 1.9 ± 1.2.

DISCUSSION

Aspiration of bone marrow is a simple, safe, and effective means of harvesting connective tissue progenitors in significant numbers.[21,24,30] Bone marrow-derived cells also provide a rich growth factor environment that may enhance the bone healing response.[2] Methods for harvesting bone marrow by aspiration have been defined and can be taught and learned.[24] If done properly, bone marrow aspiration presents little risk of injury or morbidity at the site of aspiration. As evidence of this, the senior author (GFM) has done more than 600 bone marrow aspiration procedures in the past 10 years. No significant complications have been reported. Only two patients have described a bruise at the aspiration site, which resolved before their first followup. No patient has reported clinically significant pain, swelling, scarring, infection, or hematoma at the aspiration site.

Connolly et al[7] described methods that might be used intraoperatively to concentrate cells from bone marrow. They showed that concentration of low density cells obtained from rabbit bone marrow using density gradient separation resulted in increased bone formation in diffusion chambers and proposed that intraoperative processing of bone marrow might be used to improve the efficacy of bone marrow grafts.

The methods described here using a porous implantable matrix, such as allograft bone, as a means of cell selection and concentration have significant advantages over density separation as a means of concentration. These methods can be done rapidly, with a minimum of specialized or expensive equipment. The technique does not require a trained technician and does not expose cells to other agents, such as Percoll or Ficol.

However, the data presented here show that simple concentration of cells from bone marrow and delivery of an increased number of cells in a graft site, are not enough. These data confirm the hypothesis that the biologic environment provided to the graft site by addition of a clot of bone marrow-derived cells and blood significantly improved the biologic result. A cellular composite graft that contained 50% to 70% more nucleated cells and more than twice the number of osteogenic cells (the Enriched Bone Matrix Group) was inferior to a graft containing fewer cells and progenitors but included the clot environment in the graft site (the Bone Matrix Plus Bone Marrow Clot Group). Only when the enriched cell population was combined with a bone marrow clot to deliver 2½ times the number of marrow cells and more than three times the number of osteogenic cells (the Enriched Bone Matrix Plus Bone Marrow Clot Group) were the results significantly better than either technique alone.

There are several reasons why inclusion of a bone marrow clot might have an important effect on the efficacy of a composite. The fibrin clot that is formed within the graft site may provide

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important mechanical stability to the graft site. It also may serve as a scaffold in which transplanted osteogenic cells and other cells within the site can attach and migrate. Formation of the marrow clot also will include degranulation of platelets. This will deliver many osteotropic cytokines and growth factors into the graft site, which might be limited in a site containing only the enriched matrix. Important bioactive factors released in this process include PDGF, EGF, FGFs, and TGF-beta. Fibrinolytic activity that occurs during the first several days within a clot may provide an additional source for angiogenic factors (fibrin split products) during early stages of graft incorporation. One additional possibility in this study is that the process of concentrating cells by selective attachment results in loss or exclusion of some cells that are important to the process of successful bone healing. Adding a sample of unselected marrow might restore this deficiency.

The optimal number of cells or progenitors required in a graft site is not known. The current study only evaluated an enrichment of slightly more than threefold for osteogenic connective tissue progenitors and only a 2.5-to 2.7-fold for other nucleated cells. This enriched composite achieved a mean union score of 1.9, which is close to the range for union scores that were experienced using autogenous cancellous bone in this model (2.0–3.0).[26,29] One might speculate that delivery of a larger number of cells or connective tissue progenitors might result in greater improvements in efficacy. However, this is only speculation. Furthermore, there is reason to think that there may be an upper limit of cell density that is desirable in a wound site. This theoretical limit is imposed by the limited capacity for oxygen, carbon dioxide, and other nutrients to diffuse within a graft composite having dimensions greater than a few millimeters across. As the number of cells delivered to a graft site is increased, the metabolic demand on the site also will rise. When this metabolic demand exceeds the capacity of diffusion to support an environment in which cells can survive, additional increases in the number of cells in a graft site may reduce rather than improve the efficacy of the graft.[11,28] This theoretical limit can be calculated based on empiric assumptions. The number of cells delivered in an autogenous cancellous bone graft may exceed this limit in many settings. This potential limitation on desired concentration of cells in a graft site provides some additional rationale for the concept of bone graft expanders which may distribute autogenous cells, lowering their density throughout a graft site. However, determination of the optimal number and concentration of cells and connective tissue progenitors needed in a graft site can be defined only experimentally, and will vary depending on the composition of the transplanted cells, the architectural and surface properties of the matrix in which they are transplanted, and the dimensions of and physiologic environment within the graft site. The relationship between graft performance and the concentration of transplanted cells and connective tissue progenitors and additional refinement of optimal parameters for the design of matrices used for transplantation are being investigated.

The combination of methods presented for preparation of composite grafts containing an enriched population of bone marrow-derived cells in an implantable matrix represent a new strategy for optimizing the performance of bone grafting materials. The key elements of this method are rapid concentration and selection of a population of bone marrow-derived cells that includes cells which are capable of osteoblastic differentiation using the surface of an appropriate porous implantable matrix as the initial means of cell selection; the delivery of these cells at a concentration that is sufficient to repopulate the graft site with bone-forming cells; delivery of cells in an environment in which their survival will not be precluded by the metabolic environment present in the graft site after implantation; choice of a matrix with three-dimensional and surface properties that facilitate the 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 the proliferation and differentiation of transplanted cells.

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Similar methods might be applied to various substrate materials and other tissue engineering applications using marrow- or blood-derived cells.

List of Abbreviations

BMP, bone morphogenetic protein; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TGF-beta, transforming growth factor-beta; IGF, insulinlike growth factor.

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TABLE 1

Union Score for Each Composite by Animal and Graft Site for Mineralized Cancellous Bone Matrix Cubes

Animal	Enriched Bone Matrix			Bone Matrix + Bone Marrow Clot			Enriched Bone Matrix + Bone Marrow Clot		
	L1–L2	*L3–L4*	*L5–L6*	*L1–L2*	*L3–L4*	*L5–L6*	*L1–L2*	*L3–L4*	*L5–L6*
1			0		1.5		2.0		
2	0				0				0.5
3			4.0	4.0				4.0	
4		1.0				3.5	2.0		
5		0		1.0					4.0
6	0					0.5		3.0	
7		0				0	0		
8			1.0		2.5		1.5		
9			0	0				0	
10	0					0.5		1.0	
11		1.0		0					3.0
Subtotal	0	2.0	5.0	5.0	4.0	4.5	5.5	8.0	7.5
Total		7.0			13.5			21.0**	
Mean		0.6			1.2			1.9	
Median		0			0.5			2.0	

** Significantly greater than union scores for bone matrix + Bone Marrow Clot Group ($p = 0.04$) and Enriched Bone Matrix Group ($p = 0.008$)

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TABLE 2

Union Score for Each Composite by Animal and Graft Site for Demineralized Cancellous Bone Matrix Cubes

	Enriched Bone Matrix			**Bone Matrix + Bone Marrow Clot**			**Enriched Bone Matrix + Bone Marrow Clot**		
Animal	***L1–L2***	***L3–L4***	***L5–L6***	***L1–L2***	***L3–L4***	***L5–L6***	***L1–L2***	***L3–L4***	***L5–L6***
1		3.0				4.0	4.0		
2			0		4.0		0.5		
3	0					1.5		4.0	
4	0				3.0				1.0
5		3.0		1.0					4.0
6			1.5	2.5				4.0	
7			1.0	0				0	
8	0				0				0
9		0				0	0		
10			0		0		0		
11	0.5				0				3.0
Subtotal	0.5	6.0	2.5	3.5	7.0	5.5	4.5	8.0	8.0
Total		9.0			16.0			20.5	
Mean		0.8			1.5			1.9	
Median		0			1.0			1.0	

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TABLE 3

Mechanical Stiffness for Each Cell-Matrix Composite Using Mineralized Cancellous Bone Matrix

Composite	Stiffness (N/mm)	
	Mean ± Standard Deviation	**Median**
Enriched bone matrix (n = 11)	6.9 ± 2.4	6.4
Bone matrix + bone marrow clot (n = 11)	7.9 ± 2.3	7.1
Enriched bone matrix + bone marrow clot (n = 11)	8.2 ± 4.2*	7.5

*Significantly greater than the stiffness in the Enriched Bone Matrix Group ($p = 0.05$)

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TABLE 4

Mechanical Stiffness for Each Cell-Matrix Composite Using Demineralized Cancellous Bone Matrix

	Stiffness (N/mm)	
Composite	***Mean ± Standard Deviation***	**Median**
Enriched bone matrix (n = 11)	9.1 ± 6.0	7.0
Bone matrix + bone marrow clot (n = 11)	9.4 ± 6.2	6.9
Enriched bone matrix + bone marrow clot (n = 11)	9.6 ± 4.8	9.5

*No significant differences between groups

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TABLE 5

Computed Tomographic Data for Each Cell-Matrix Composite Using Mineralized Cancellous Matrix

Outcome Parameter	Enriched Bone Matrix (n = 11)	Bone Matrix + Bone Marrow Clot (n = 11)	Enriched Bone Matrix + Bone Marrow Clot (n = 11)
Fusion volume (mm^3)	869 ± 196*	961 ± 115	1006 ± 185
Fusion area (mm^2)	81 ± 17**	91 ± 14	95 ± 27
Bone density	1848 ± 62	1840 ± 43	1824 ± 66

*Significantly less than Bone Matrix + Bone Marrow Clot Group (p = 0.03) and the Enriched Bone Matrix + Bone Marrow Clot Group (p = 0.004)

**Significantly less than Enriched Bone Matrix + Bone Marrow Clot Group (p = 0.02)

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TABLE 6

Computed Tomographic Data for Each Cell-Matrix Composite Using Demineralized Cancellous Matrix

Outcome Parameter	Enriched Bone Matrix (n = 11)	Bone Matrix + Bone Marrow Clot (n = 11)	Enriched Bone Matrix + Bone Marrow Clot (n = 11)
Fusion volume (mm^3)	872 ± 288*	1010 ± 268	1115 ± 406
Fusion area (mm^2)	85 ± 31**	95 ± 27	98 ± 31
Bone density	1901 ± 35	1858 ± 29	1854 ± 77

*Significantly less than the Enriched Bone Matrix + Bone Marrow Clot Group (p = 0.03)

**Significantly less than Enriched Bone Matrix + Bone Marrow Clot Group (p = 0.02)

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TABLE 7

Retention and Delivery of Bone Marrow Cells in Mineralized Cancellous Bone Chips

Dog Number	[Cells] in Pooled Heparinized Sample ($\times 10^6$/cc)	[Cells] Nonheparinized ABM Sample ($+10^6$/cc)	Cell Number Loaded in Enriched Matrix ($\times 10^6$)	Cell Number Retained in Enriched Matrix ($\times 10^6$)	Efficiency of Cell Attachment (%)	[Cells] in Enriched Matrix ($\times 10^6$/cc)	Total Cell Number Implanted Enriched Group ($\times 10^6$)	Total Cell Number Implanted Enriched + ABM Group ($\times 10^6$)	Fold Increase in [Cell] Enriched Group	Fold Increase in [Cell] Enriched + ABM Group
1	32	48	319	177	55	89	177	273	1.8	2.8
2	57	85	566	282	50	141	282	452	1.7	2.7
3	66	99	662	329	50	165	329	528	1.7	2.7
4	83	125	826	468	57	234	468	717	1.9	2.9
5	57	86	570	200	35	100	200	371	1.2	2.2
6	93	140	934	421	45	211	421	701	1.5	2.5
7	40	60	402	149	37	75	149	270	1.2	2.2
8	67	101	670	323	48	162	323	524	1.6	2.6
9	61	92	614	367	60	184	367	550	2.0	3.0
10	62	93	742	359	48	180	359	545	1.9	2.9
11	56	84	558	280	50	140	280	448	1.7	2.7
Mean	61	92	624	305	49	153	305	489	1.7	2.7
SD	18	26	175	100	8	50	100	148	0.3	0.3

ABM, aspirated bone marrow clot; [Cells], cell concentration; SD, standard deviation

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TABLE 8

Retention and Delivery of Bone Marrow Cells in Demineralized Cancellous Bone Chips

Dog Number	[Cells] in Pooled Heparinized Sample ($\times 10^6$/cc)	[Cells] Nonheparinized ABM Sample ($+10^6$/cc)	Cell Number Loaded in Enriched Matrix ($\times 10^6$)	Cell Number Retained in Enriched Matrix ($\times 10^6$)	Efficiency of Cell Attachment (%)	[Cells] in Enriched Matrix ($\times 10^6$/cc)	Total Cell Number Implanted Enriched Group ($\times 10^6$)	Total Cell Number Implanted Enriched + ABM Group ($\times 10^6$)	Fold Increase in [Cell] Enriched Group	Fold Increase in [Cell] Enriched + ABM Group
1	86	130	860	630	73	315	630	889	2.4	3.4
2	46	70	464	312	67	156	312	451	2.2	3.2
3	44	66	441	142	32	71	142	274	1.1	2.1
4	50	75	500	327	65	163	327	477	2.2	3.2
5	58	87	575	200	35	100	200	374	1.1	2.1
6	87	131	872	284	33	142	284	546	1.1	2.1
7	59	88	765	259	34	129	259	435	1.5	2.5
8	62	94	749	333	44	166	333	520	1.8	2.8
9	47	71	466	269	58	135	269	410	1.9	2.9
10	50	75	498	177	36	89	177	327	1.2	2.2
11	60	90	481	44	9	22	44	224	0.2	1.2
Mean	59	89	606	270	44	135	270	448	1.5	2.5
SD	16	23	170	148	19	74	148	177	0.7	0.7

ABM, aspirated bone marrow clot; [Cells], cell concentration; SD, standard deviation

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TABLE 9

Concentration and Delivery of Connective Tissue Progenitors in Mineralized Cancellous Chips

Dog Number	[CTP] in Pooled Heparinized Sample CTPs/cc	[CTP] in Nonheparinized ABM Sample CTPs/cc	CTPs Loaded in Enriched Matrix	CTPs Retained in Enriched Matrix	Efficiency of CTP Attachment (%)	[CTP] in Enriched Matrix CTPs/cc	Total CTP Number Implanted Enriched Group	Total CTP Number Implanted Enriched + ABM Group	Fold Increase in [CTP] Enriched Group	Fold Increase in [CTP] Enriched + ABM Group	Selection Ratio CTPs Versus Cells
1	637	956	6372	3523	55	1762	3523	5434	1.8	2.8	1.0
2	632	948	6314	2196	35	1098	2196	4092	1.2	2.2	0.7
3	2052	3078	20516	8851	43	4426	8851	15007	1.4	2.4	0.9
4	2645	3968	26440	15009	57	7505	15009	22944	1.9	2.9	1.0
5	1368	2052	13680	6656	49	3328	6656	10760	1.6	2.6	1.4
6	2746	4119	48558	25721	53	12861	25721	33959	3.1	4.1	1.2
7	724.5	1087	7245	2131	29	1066	2131	4305	1.0	2.0	0.8
8	3615	5423	36154	28347	78	14174	28347	39192	2.6	3.6	1.6
9	2333	3500	23332	20856	89	10428	20856	27855	3.0	4.0	1.5
10	2537	3806	30422	20352	67	10176	20352	27963	23.7	3.7	1.4
11	2788	4182	27880	19594	70	9797	19594	27958	2.3	3.3	1.4
Mean	2000	3000	22400	13900	57	6970	13900	20000	2.1	3.1	1.2
SD	1000	1500	13500	9660	18	4830	9660	12600	0.7	0.7	0.3

ABM, aspirated bone marrow clot; CTP, connective tissue progenitors; [CTP], CTP concentration; SD, standard deviation

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TABLE 10

Concentration and Delivery of Connective Tissue Progenitors in Demineralized Cancellous Chips

Dog Number	[CTP] in Pooled Heparinized Sample CTPs/cc	[CTP] in Nonheparinized ABM Sample CTPs/cc	CTPs Loaded in Enriched Matrix	CTPs Retained in Enriched Matrix	Efficiency of CTP Attachment (%)	[CTP] in Enriched Matrix CTPs/cc	Total CTP Implanted Enriched Group	Total CTP # Implanted Enriched + ABM Group	Fold Increase in [CTP] Enriched Group	Fold Increase in [CTP] Enriched + ABM Group	Selection Ratio CTPs Versus Cells
1	5585	8378	55559	46627	84	23314	46627	63382	2.8	3.8	1.1
2	3332	4998	33312	26501	80	13251	26501	36497	2.7	3.7	1.2
3	1380	2070	13804	9657	70	4829	9657	13797	2.3	3.3	2.2
4	3414	5121	34000	28544	84	14272	28544	38786	2.8	3.8	1.3
5	3248	4872	32200	23067	72	11534	23067	32811	2.4	3.4	2.1
6	4812	6417	42753	18145	42	9073	18145	30979	1.4	2.4	1.3
7	1529	2294	19881	12833	65	6417	12833	17420	2.8	3.8	1.9
8	1374	2061	16485	11898	72	5949	11898	16020	2.9	3.9	1.6
9	2276	3414	22755	17628	77	8814	17628	24456	2.6	3.6	1.3
10	1948	2922	19482	13858	71	6929	13858	19702	2.4	3.4	2.0
11	1923	2885	15387	7415	48	3708	7415	13184	1.3	2.3	5.3
Mean	2750	4130	27800	19700	70	9800	19700	27900	2.4	3.4	1.9
SD	1350	2020	13100	11200	13	5610	11200	14900	0.5	0.5	1.2

ABM, aspirated bone marrow clot; CTP, connective tissue progenitors; [CTP], CTP concentration; SD, standard deviation