Hypothesis: Transplanted osteoprogenitor cells derived from cultured bone marrow stromal cells (BMSCs) can be used to fabricate pedicled bone flaps.

Design: Prospective, randomized experimental trials.

Setting: Basic science research laboratory.

Materials: Immunodeficient female NIH-Bg-Nu-Xid mice, aged 3 months.

Intervention: The BMSCs were harvested from the long bones of C57Bl/6 transgenic mice carrying the type Iα(1) collagen–chloramphenicol acetyl transferase reporter gene construct; their numbers were expanded in tissue culture. Treated mice received BMSC transplantations around the common carotid artery and internal jugular vein, the aorta and its venae comitantes, or the saphenous artery and vein; control mice received a sham transplant in comparable recipient sites.

Main Outcome Measures: Mice underwent harvesting from 4 weeks to 2 years after transplantation. Transplants were evaluated via histological, immunohistochemical, and angiographic analyses.

Results: Compared with the controls, which formed no bone, 32 of 37 BMSC-containing transplants formed a vascularized bone island that was perfused specifically and solely by its common carotid artery vascular source. Mature transplants consisted of well-developed lamellar, corticocancellous bone whose osteocytes were derived from the grafted BMSCs; hematopoietic tissue derived from the recipient mouse. Transplants formed as early as 4 weeks and remained stable in size as late as 108 weeks.

Conclusions: Bone marrow stromal cells can be used to create vascularized bone flaps in mice; these bone constructs are vascularized by their pedicle and therefore can potentially be transferred to a recipient site using microsurgical techniques. These findings provide proof of principle of an additional clinical application of BMSC transplantation techniques.

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FRIEDENSTEIN and Owen 1,2 demonstrated the presence of a population of bone marrow stromal cells (BMSCs) with a pluripotent capability. These cells could be distinguished from most hematopoietic elements in the marrow by their high adherence to the substrate plastic in tissue culture flasks and by a number of morphologic, histochemical, and biochemical characteristics. Phenotypically, BMSCs have been found to be capable of differentiating into bone, cartilage, muscle, and adipose and neural tissue elements. 1-6 Populations of BMSCs that include osteoprogenitor cells have been expanded in tissue culture and transplanted into recipient animals. Such heterotopic transplants, whose donor BMSCs have been isolated from species ranging from rodents to humans, form bone in mouse recipients. 1,7,13 Bone marrow stromal cells also have been shown to repair induced bone defects in various animal models. 14,15

Successful repair of bone defects, whether arising from trauma, tumor resection, or congenital disorders, continues to be a major concern to reconstructive surgeons. 16 Free flaps are often the only viable option when the recipient site is scarred by infection or radiation injury; however, their use is associated with greater risk to and discomfort for the patient. 16-19 In an effort to avoid the problems associated with standard bone-flap harvests, investigators have sought to fabricate flaps. Pre-fabricated flaps, whether used to reconstruct bone or soft tissue, can be created in an anatomic location that minimizes donor site morbidity. They can be designed to fit a particular recipient site, since their size and shape is not limited to those found naturally. Techniques for flap fabrication that are already in clinical
MATERIALS AND METHODS

PREPARATION OF MOUSE BMSCs

Bone marrow cells were harvested from the long bones of 8-week-old C57Bl/6 transgenic mice carrying the type Iα(1) collagen–CAT construct using a technique described elsewhere. All studies were completed in accordance with an approved small animal protocol (National Institutes of Health protocol 97-024). Briefly, mice were killed humanely with inhaled carbon dioxide; the femurs, tibias, and humeri were removed and cleaned of all adjacent soft tissue. The epiphyses were removed, and the bone marrow from each medullary cavity was flushed with α-minimal essential media (αMEM; Gibco, Grand Island, NY). Bone marrow cells from 6 long bones (6 × 10^7 to 8 × 10^7 nucleated cells) were plated to a single culture flask (T-75; Becton Dickinson; Lincoln Park, NJ) in complete medium consisting of αMEM, 2-mmol/L L-glutamine, 100-µg/mL penicillin, 100-µg/mL streptomycin sulfate (Biofluids, Rockville, Md), 10−5-mol/L dexamethasone (Sigma-Aldrich Corp, St Louis, Mo), 10−4-mol/L L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, Osaka, Japan), and preselected lots of 20% fetal bovine serum (Atlanta Biologicals, Atlanta, Ga). The cells were incubated at 37°C in 5% carbon dioxide. The medium was changed completely on day 2. When the adherent cell layer had reached confluence, typically at day 10 to day 12, the cells were rinsed with Hanks balanced salt solution (Gibco), detached with 2 portions of 1× trypsin-EDTA (Gibco), and again plated at 1:3 dilution in new flasks. Subsequent passages were performed in a similar manner.

TRANSPLANT PREPARATION AND ANIMAL OPERATIONS

After 2 to 6 passages, cells were released from tissue culture flasks by treatment with trypsin, and they were prepared for transplantation by pelleting at 1000 rpm and resuspending in a small volume of complete medium. From 2 × 10^5 to 3 × 10^6 BMSCs were absorbed into sponges of cross-linked porcine collagen type I (Gelfoam; Upjohn, Kalamazoo, Mich) measuring 125 mm^3. The sponges were incubated at 37°C for 60 minutes. A control group of sponges was prepared under similar conditions; these were moistened with full medium but received no cells. Three-month-old immunodeficient NIH-Bg-Nu-Xid female mice (Harlan-Sprague Dawley, Indianapolis, Ind) served as transplant recipients. Mice were anesthetized using a combination of intraperitoneal ketamine hydrochloride (Fort Dodge Animal Health; Fort Dodge, Iowa) at 140 mg/kg body weight and intraperitoneal xylazine hydrochloride (Butler, Columbus, Ohio) at 7 mg/kg body weight. The bilateral common carotid arteries, saphenous vessels, or abdominal aorta were exposed. The arteries and their adjacent veins were isolated and encircled with the graft. The grafts were bilayered constructs. The inner layer, closest to the vessels, consisted of a sponge that did or did not include cells. The sponge was encircled by a strip of polytetrafluoroethylene (PTFE) (Gore-Tex; WL Gore & Associates; Flagstaff, Ariz) vascular graft material, constituting the graft’s outer layer (Figure 1). The PTFE served to prevent vascularization of the transplant by the surrounding soft tissue. The incisions were closed in layers.

In an effort to confirm that the pedicle is the source of any vascularization of the transplants, some BMSC transplants (a small subset) were wrapped around pedicles that had first been encircled by an impermeable plastic membrane. This membrane served to prevent all vascularization to demonstrate the feasibility of transplanting BMSCs to mice with intact immune systems.

RESULTS

A total of 48 transplants were delivered to 28 recipient mice. Five of the mice received BALB/c isografts, whereas the remainder were immunodeficient mice receiving transplants of BMSCs derived from C57Bl/6 transgenic mice harboring the type Iα(1) collagen–CAT 3.6 construct. Transplants were harvested from 4 to 108 weeks postoperatively. Thirty-two of 37 transplants with BMSCs formed bone, while all 11 cell-free transplants formed only fibrous connective tissue (Figure 2). Bone was formed as early as 4 weeks after transplantation and persisted, with no signs of degeneration, senescence, or sarcomatous transformation in transplants harvested at 108 weeks (Figure 3). Transplants free of BMSCs were harvested at 4, 8, 92, and 100 weeks; bone formation was equally absent at all 4 time points.

TRANSPLANT MORPHOLOGIC FEATURES

Transplants that had received cells from C57Bl/6 transgenic mice had characteristic morphologic features,
whether harvested early or late after transplantation (Figure 4A). The vascular pedicle lay at the heart of the transplant. It was encircled by 2 concentric layers of lamellar cortical bone; the first of these sat immediately adjacent to the pedicle, whereas the second lay further out against the inner wall of the PTFE sheath. Each layer of cortical bone was nearly intact, except for small and sporadic fenestrae. The space between the 2 layers of bone demineralized in Bouin fluid (Sigma-Aldrich Corp), embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

To confirm that osteoblasts and osteocytes within the transplants were of donor origin, immunohistochemical analysis for CAT was performed on the transplants. The transplants were fixed overnight at 4°C in 4% formalin in phosphate-buffered saline solution freshly prepared from paraformaldehyde; they were then decalcified with 10% EDTA (pH, 8.0) at 4°C for 2 days. The transplants were embedded (Tissue-Tek O.C.T. 4583 Compound; Sakura Finetecchnical Co Ltd, Tokyo, Japan), frozen rapidly in ethanol–dry ice, and sectioned with a frozen microtome (Jung Frigocut; Leica Instruments GmbH, Wetzlar, Germany). Frozen sections of 7 to 10 µm in thickness were immersed in phosphate-buffered saline solution for 10 minutes. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide for 30 minutes. An avidin–biotinylated peroxidase complex was completed using rabbit anti–CAT antibody at a 1:500 dilution (5Prime-3Prime Inc, Boulder, Colo) as the primary antibody, and biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, Calif) as the second antibody. The immunolocalized antigen was visualized by means of diaminobenzidine (Histomark; Kirkegaard and Perry Laboratories, Gaithersburg, Md). Normal rabbit serum (1:500) and normal rabbit IgG (17 µg/mL) served as negative controls.

To confirm that osteoblasts and osteocytes within the transplants were of donor origin rather than originating from skeletal elements of the new bone, confirming that the osteogenic cells were of donor origin rather than originating from the local microenvironment (Figure 4F). Immunoreactivity of CAT was restricted to the new bone and was not present in the peritransplant tissues, suggesting that the BMSCs did not migrate outside the confines of the transplant. No signs of inflammation or dysplasia were detected in the peritransplant region of any of the implants analyzed.

**EVALUATION OF BMSC TRANSPLANT RECIPIENTS**

The mice and their transplants were evaluated using several techniques. To confirm that bone had formed in the transplants, recipient mice underwent radiography (Faxitron MX-20 Specimen Radiography System; Faxitron X-ray Corporation, Wheeling, Ill) at an energy of 30 kV for an exposure duration of 90 seconds (Kodak X-OMAT TL film; Eastman Kodak Company, Rochester, NY). In an effort to confirm pedicule patency and its contribution to transplant vascularization, mice underwent angiography. The mice were injected systemically via the left heart with sodium nitroprusside (Nipride; Gensia Laboratories, Inc, Irvine, Calif) and ethiodized oil (Ethiodol; Savage Laboratories, Melville, NY), then underwent radiography. To further confirm that the transplants were perfused by the vascular pedicle, the transplants were dissected free of surrounding soft tissues, remaining attached to the mice solely via the vascular pedicle. The mice were systemically injected with methylene blue via the left cardiac ventricle. The transplants, which were noted to immediately turn blue, were harvested, subjected to radiography, and processed for histological analysis.

**TRANSPLANT ANALYSIS**

Transplant recipients were killed humanely, and transplants were harvested from 4 to 108 weeks after transplantation. Following angiography, transplants were fixed and demineralized in Bouin fluid (Sigma-Aldrich Corp), embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

To confirm that osteoblasts and osteocytes within the transplants were of donor origin, immunohistochemical analysis for CAT was performed on the transplants. The transplants were fixed overnight at 4°C in 4% formalin in phosphate-buffered saline solution freshly prepared from paraformaldehyde; they were then decalcified with 10% EDTA (pH, 8.0) at 4°C for 2 days. The transplants were embedded (Tissue-Tek O.C.T. 4583 Compound; Sakura Finetechncal Co Ltd, Tokyo, Japan), frozen rapidly in ethanol–dry ice, and sectioned with a frozen microtome (Jung Frigocut; Leica Instruments GmbH, Wetzlar, Germany). Frozen sections of 7 to 10 µm in thickness were immersed in phosphate-buffered saline solution for 10 minutes. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide for 30 minutes. An avidin–biotinylated peroxidase complex was completed using rabbit anti–CAT antibody at a 1:500 dilution (5Prime-3Prime Inc, Boulder, Colo) as the primary antibody, and biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, Calif) as the second antibody. The immunolocalized antigen was visualized by means of diaminobenzidine (Histomark; Kirkegaard and Perry Laboratories, Gaithersburg, Md). Normal rabbit serum (1:500) and normal rabbit IgG (17 µg/mL) served as negative controls.
CONFIRMATION OF TRANSPLANT PERFUSION

Mice that had undergone systemic injection with ethiodized oil before radiography demonstrated the presence of radiopaque dye within the pedicle on coronal and axial views (Figure 5D-E). Transplants from mice that had received a systemic arterial injection of methylene blue before humane killing were examined histologically. These implants demonstrated a blue coloration arising from the arterially delivered dye. Similarly, gross visual examination of the in situ transplants confirmed the absence of any other vascular connections to the peri-transplant tissues.

Transplants containing an impermeable plastic membrane immediately encircling the vascular pedicle were evaluated at 59 (1 transplant) and 70 (2 transplants) weeks. These transplants were prevented from establishing vascular connections to the pedicle or to surrounding soft tissues because of the plastic membrane and the PTFE, respectively. All 3 failed to form bone. Only minimal soft tissue remained of the original transplant, without any evidence of a mineralized structure.

BONE FORMATION IN ISOGENIC TRANSPLANTS

Isogenic transplants were harvested at intervals of 4 (1 transplant), 46 (3 transplants), 56 (1 transplant), and 70 (3 transplants) weeks. Seven of the 8 transplants formed bone; only the 4-week-old transplant failed. The transplant shape, size, and bone morphologic features were comparable to transplants derived from C57BL/6 transgenic mouse cells that had been placed in immunocompromised recipients.

BONE FORMATION AT OTHER VASCULAR SITES

Bone formed among 7 of the 9 cell-containing transplants at the saphenous artery and 2 of the 3 cell-containing aortic transplants (Figure 6). Bone in these sites was comparable in shape, size, and morphologic features to that found in neck-based transplants.

COMMENT

Transplanted BMSCs have been shown to repair calvarial defects in mice and femoral defects in rats and dogs.14,15,27 Such transplants have depended on a hospitable recipient bed, undamaged by scarring, radiation, or infection. Transplantation of BMSCs into an inhospitable location may require the transfer of a vascularized construct. In this study, we have developed a new model combining the bone-forming ability of culture-expanded BMSCs with prefabrication of a vascularized bone flap. We transplanted CAT-expressing cultured mouse BMSCs into immunosuppressed recipient mice around a vascular pedicle. Pedicles included the paired common carotid artery and internal jugular vein, the saphenous artery and vein, and the aorta and its venae comitantes. From periods of 4 to 108 weeks after transplantation, mice underwent histological, plain radiographic, and angiographic evaluation. Our data demonstrate that BMSCs can form bone that is perfused by a specific vascular pedicle. Perfusion was confirmed using arteriography and tracer dye injection. Interposition of an impermeable membrane between the cells and the pedicle at the time of transplantation resulted in no bone formation, further establishing the importance of the pedicle to vascularization and bone formation. Mature transplants were characterized by a double layer of cortical
bone, the outer layer forming the outer layer of the transplant, the inner layer surrounding the vascular pedicle, and the intervening space filled with hematopoietic tissue. The donor origin of the cells forming new bone was established using immunohistochemistry, with an antibody to CAT; in contrast, hematopoietic cells in such transplants are of recipient origin. The persistence of bone in the latest time point (108 weeks) confirmed the stability of these transplants over time; in fact, the transplants maintained their shape and size despite the absence of mechanical stimuli of remodeling. We also demonstrated the feasibility of forming bone in mice with intact immune systems by using syngeneic transplants.

Of special interest is the difference in morphologic features between these pedicle-associated constructs and that of subcutaneous mouse BMSC transplants that are not associated with discrete vessels. The subcutaneous transplants consist of a shell of cortical bone that surrounds a space filled with hematopoietic elements and trabecular bone. Such transplants do not have the inner layer of cortical bone that typifies the pedicled constructs. This inner cortical shell, and the outer as well,
may arise in those portions of the transplant in greatest proximity to regions of higher oxygen tension or nutrient load. Alternatively, cortical bone may develop in those portions of the transplant that are immediately adjacent to host tissue structures; these structures may offer directionality to the osteogenic cells. Developing a greater
understanding of this behavior may aid in developing bone constructs with a higher density of cortical elements and improved biomechanical characteristics.

Prefabrication of vascularized bone constructs is a steadily expanding field of investigation, although most studies involve preparation of the new bone using corticocancellous grafts, bone morphogenetic protein–2, or biocompatible ceramics. Also, the transplantation of BMSCs and ceramic blocks into mouse latissimus dorsi muscle has been described. The muscle, which provided a vascular-rich environment for bone formation, could be detached and transferred to another anatomic site, carrying the new bone construct along with it. In contrast, our model does not rely on muscle, but instead depends on a paired, expendable artery and vein. Optimum vessels, such as the superficial epigastric, would be superficial and easily dissected with minimal scarring of the patient; they could be moved without the patient losing strength or mobility. As well, our use of hydroxyapatite/tricalcium phosphate particles is an advantage over the use of the blocks; in conjunction with the patient losing strength or mobility. As well, our use of hydroxyapatite/tricalcium phosphate particles is an advantage over the use of the blocks; in conjunction with patient losing strength or mobility. As well, our use of hydroxyapatite/tricalcium phosphate particles is an advantage over the use of the blocks; in conjunction with

The system presented herein is clinically beneficial because it may obviate the need to harvest the vascularized fibula or iliac crest in select patients undergoing a bone reconstruction. A patient undergoing a staged reconstruction of the mandible in an irradiated site, for instance, could undergo a bone marrow harvest, ex vivo BMSC expansion, and establishment of an engineered bone flap in the groin. These relatively quick procedures would expose the patient to minimal morbidity. The flap could then be transferred from groin to face in the same way that a standard bone flap is handled. The remaining challenge, that of creating large-volume BMSCs transplants, is being addressed steadily. Cylindrical BMSC transplants 20 mm in length and 13 mm in diameter have formed bone in dogs, relying solely on peripherally based vascularization. If readily vascularized is the limiting factor in transplant size, the use of exogenously administered angiogenic factors may allow for larger transplants.

This system could theoretically be used as an in situ biogenerator. Autogenous, culture-expanded BMSCs that have been engineered to express a specific product could be transplanted around a pedicle. The factors they express could directly enter the systemic circulation. In addition, the transplant could be removed if the factor was no longer required by the patient. Efforts are already under way to transfect human BMSCs to express factor IX, although to date none of these efforts have resulted in long-term expression.

In summary, we have created a new model that combines the bone-forming ability of culture-expanded BMSCs with surgical prefabrication techniques. The successful formation of such a vascularized bone flap offers new clinical and research opportunities. Relying on an expendable vascular pedicle, such transplants in patients could offer a method for obtaining vascularized autogenous bone without sacrificing the traditional donor sites. Alternatively, this technique may increase the feasibility of using transplanted BMSCs as autogenous biogenerators.

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**REFERENCES**


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An Overview of the 4 Randomized Trials of Aspirin Therapy in the Primary Prevention of Vascular Disease

Patricia R. Hebert, PhD; Charles H. Hennekens, MD

Background: In the primary prevention of cardiovascular disease, in contrast to the recommendations of the American College of Chest Physicians and the American Heart Association, the US Food and Drug Administration recently stated that there was insufficient evidence to judge whether aspirin therapy decreases the risk of a first myocardial infarction.

Objective: To perform an overview of the 4 primary prevention trials of aspirin therapy to obtain the most reliable estimates of the effects of aspirin therapy on various vascular disease end points.

Methods and Results: These 4 trials included more than 51,000 subjects and 2,284 important vascular events. Those assigned to aspirin therapy experienced significant reductions of 32% (95% confidence interval [CI], 21%-41%) for nonfatal myocardial infarction and 13% (95% CI, 5%-19%) for any important vascular event. There were possible small but nonsignificant increases in risks of vascular disease–related death (1%; 95% CI, −12% to 16%) and nonfatal stroke (8%; 95% CI, −12% to 33%). When strokes were subdivided by type, there was no significant effect of aspirin therapy on the risk of ischemic stroke, but, while based on small numbers, there was a 1.7-fold apparent increase (95% CI, 6%-269%) in the risk of hemorrhagic stroke, which did achieve statistical significance.

Conclusions: For the primary prevention of vascular disease, aspirin therapy confers significant beneficial effects on first myocardial infarction and, as a result, on any important vascular event; these effects are clinically important. Whether there is any reduction in vascular disease–related death or stroke associated with treatment remains unclear because of inadequate numbers of events in the primary prevention trials completed to date. More data on hemorrhagic stroke are also needed. In addition, randomized trial data, especially in women but also in men, are needed to help to formulate a rational public health policy for individuals at usual risk. Meanwhile, these data provide evidence for a significant benefit of aspirin therapy in the primary prevention of myocardial infarction.

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