Translational Research: The CD34+ Cell Is Crucial for Large-Volume Bone Regeneration from the Milieu of Bone Marrow Progenitor Cells in Craniomandibular Reconstruction

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Purpose: *This study investigated the role of the bone marrow–derived CD34+ cell in a milieu of osteoprogenitor cells, bone marrow plasma cell adhesion molecules, recombinant human bone morphogenetic protein (rhBMP), and a matrix of crushed cancellous allogeneic bone in the clinical regeneration of functionally useful bone in craniomandibular reconstructions. The history and current concepts of bone marrow hematopoietic stem cells and mesenchymal stem cells are reviewed as they relate to bone regeneration in large continuity defects of the mandible.* ***Materials and Methods:*** *Patients with 6- to 8-cm continuity defects of the mandible with retained proximal and distal segments were randomized into two groups. Group A received an in situ tissue-engineered graft containing 54 ± 38 CD34+ cells/mL along with 54 ± 38 CD44+, CD90+, and CD105+ cells/mL together with rhBMP-2 in an absorbable collagen sponge (1 mg/cm of defect) and crushed cancellous allogeneic bone. Group B received the same graft, except the CD34+ cell concentration was 1,012 ± 752 cells/mL. The results were analyzed clinically, radiographic bone density was measured in Hounsfield units (HU), and specimens were analyzed histomorphometrically.* ***Results:*** *Forty patients participated (22 men and 12 women; mean age, 57 years). Eight of 20 group A patients (40%) achieved the primary endpoint of mature bone regeneration, whereas all 20 group B patients (100%) achieved the primary endpoint. CD34+ cell counts above 200/mL were associated with achievement of the primary endpoint. Bone density was lower in group A (424 ± 115 HU) than in group B (731 ± 98 HU). Group A bone showed a mean trabecular bone area of 36% ± 10%, versus 67% ± 13% for group B.* ***Conclusions:*** *The CD34+ cell functions as a central signaling cell to mesenchymal stem cells and osteoprogenitor cells in bone regeneration. The mechanism of bone marrow–supported grafts requires a complete milieu to regenerate large quantities of functionally useful bone. CD34+ cell counts in a concentration of at least 200/mL in composite grafts are directly correlated to clinically successful bone regeneration.* Oral Craniofac Tissue Eng 2012;2:263–271. doi: 10.11607/octe.0059

Key words: *bone regeneration, hematopoietic stem cells, mesenchymal stem cells, recombinant human bone morphogenetic protein*

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The link between cells of hematopoietic lineage and marrow osteoprogenitor cells is not new. As early as 1763, Albrecht von Haller stated that "the origin of bone is the artery carrying blood and in it the mineral content."[1] Additionally, the Conhein hypothesis of 1867 stated that the bloodstream and consequently the bone marrow was the source of cells involved in wound regeneration.[2] These early works established the importance of the vascular system and bone marrow, along with their associated cell

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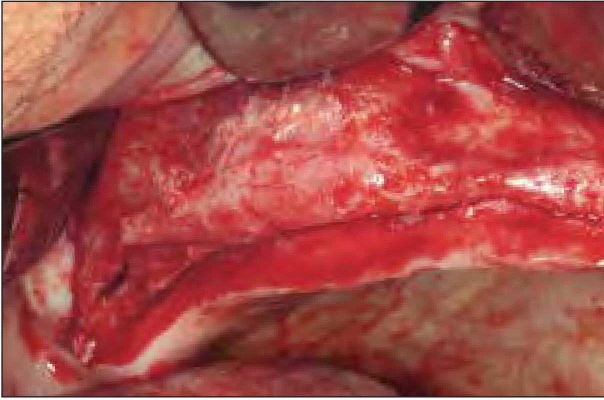

Fig 1 Small-volume alveolar ridge bone regeneration was accomplished with platelet-rich plasma, rhBMP, and cancellous allogeneic bone.

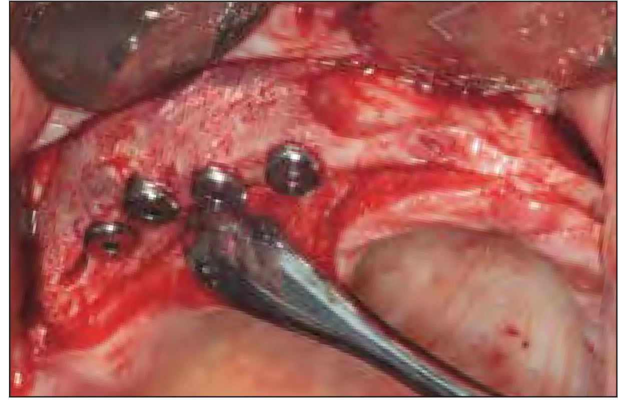

Fig 2 Graft seen in Fig 1; it was capable of receiving dental implants without further augmentation.

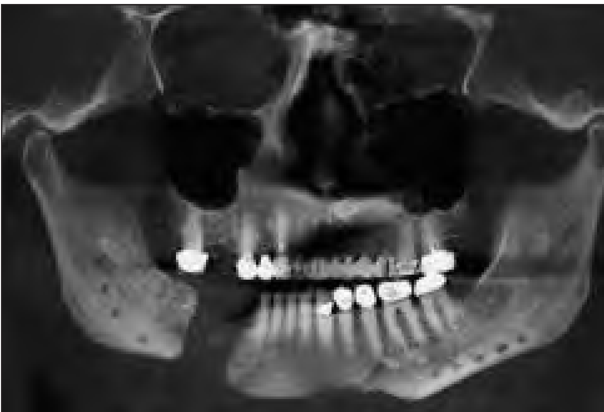

Fig 3 Low numbers of circulating stem cells, even when combined with rhBMP and cancellous allogeneic bone, will not predictably regenerate bone in large mandibular continuity defects.

lines, in the activation of resident cells within bone for the purpose of bone regeneration and resident cells within soft tissue for wound healing.

The current understanding of the cellular composition of bone marrow aspirates (BMA) and bone marrow aspirate concentrates (BMAC) used in today's craniofacial bone regeneration involves both hematopoietic stem cells (HSCs) and mesenchymal (stromal) stem cells (MSCs). However, the term stem cells creates confusion in and of itself, as the term is often used to refer to both true stem cells as well as to progenitor cells.[3,4] This confusion is multiplied by the common belief that an MSC represents a pluripotent stem cell and is all that is required for tissue regeneration,[5] a belief that has not been validated to date.[6,7] However, it has been demonstrated that HSCs are directly involved in both bone regeneration and soft tissue regeneration,[8–10] theoretically supporting the concept of a pluripotent stem cell to regenerate the entire tissue complex. On the other hand, two landmark papers[11,12] (published in 2005 and 2011, respectively) identified the paracrine cellular communications and cell contact between osteoblasts and hematopoietic stem cell/progenitor cells, suggesting that a multicellular mechanism of MSCs in BMA/BMAC is required for bone regeneration.

Marx and Tursun identified four important subsets of stem cells in blood and in BMA (CD34+, CD44+, CD90+, and CD105+) and found by polymerase chain reaction and colony-forming unit (CFU) analysis that their native amounts in the anterior and posterior ilium were equal and were more than twice that of the tibial plateau.[13] This was followed by a randomized clinical study, which demonstrated useful and durable bone regeneration in maxillary alveolar defects of small volume when combined with platelet-rich plasma, which contains only small numbers of these MSCs/progenitor cells, and recombinant human bone morphogenetic protein (rhBMP) (Figs 1 and 2).[14] Although these circulating stem cells/osteoprogenitor cells proved to be adequate in these smaller defects with abundant host MSCs/osteoprogenitor cells, they were found to be incapable of large-volume bone regeneration in continuity defects, where few (if any) host site resident stem cells or osteoprogenitor cells exist (Fig 3). Therefore, it became apparent that large continuity

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defects of the jaws required *(1)* identification of the crucial cells in bone marrow that control bone regeneration and *(2)* a means of increasing their numbers.

Several studies have implied that bone marrow CD34+ cells might be the pivotal cells controlling bone regeneration. The CD34+ cell has been considered an HSC.[9,15–17] However, studies in the early 2000s identified it as a cell capable of plasticity[18]; ie, capable of differentiation between MSC and HSC lineages.[19,20] This concept reinforced the notion that a single cell line can differentiate into the full cellular tissue composite of bone, blood vessels, lymphatic tissue, endosteum, periosteum, and bone marrow once again.[5] Experiments by Matsumoto et al confirmed the multilineage differentiation of circulating CD34+ cells, resulting in endothelial cells for vasculogenesis and osteoblasts for bone regeneration.[8] This finding was confirmed by others,[3] proving that clonal cells of HSC isolated from bone marrow produce CFU-fibroblasts (CFU-F) and fibrocytes that were not of MSC origin but of HSC origin.[21,22]

The issue that remains under debate is whether the bone marrow–derived CD34+ cell controls bone regeneration via its own multilineage differentiation, followed by expansion or by paracrine and autocrine signaling to other bone marrow precursor cells and even by signaling to its parents or progeny to create a multilineage differentiation and expansion to regenerate bone that then undergoes remodeling and renewal.

To explore this issue, the present study used two concentrations of bone marrow–aspirated CD34+ cells in a proven in situ tissue engineering model.[14] This model combines the selected CD34+ concentration and bone marrow osteoprogenitor cells with rhBMP-2/absorbable collagen sponge (rhBMP-2/ACS) and a matrix of crushed cancellous allogeneic bone, thus completing the classic tissue-engineering triangle (Fig 4).

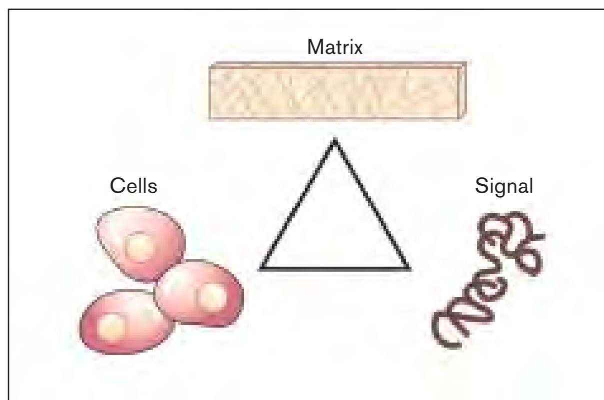

Fig 4 The classic tissue-engineering triangle for predictable tissue regeneration: cells, signal, and matrix.

MATERIALS AND METHODS

With approval from the institutional review board, patients with 6- to 8-cm continuity defects of the mandible were randomized to receive treatment with one of two protocols. Group A patients received *(1)* BMA containing total nucleated cells (TNC) 15.5 ± 10^6/mL and 54 ± 38 cells/mL CD34+ CFU-F; *(2)* rhBMP-2/ACS, 1 mg/cm of defect; *(3)* crushed cancellous allogeneic bone (University of Miami Tissue Bank); and *(4)* BMA containing CD44+, CD90+, and CD105+ osteoprogenitor cells 15.5 × 10^6 TNC and 54 ± 38 cells/mL CFU-F of each. Group B patients received the same materials, except that the CD34+ BMA contained TNC 98 ± 32 × 10^6/mL and 1,012 ± 752 cells/mL CD34+ CFU-F.

Method of Bone Marrow Harvest and Concentration

A total of 60 mL of autologous bone marrow was harvested from each of four puncture sites in the bilateral anterior iliac crest intraoperatively at the point of care using a heparinized trocar to aspirate 15 mL from each site, per the protocol of Marx and Stevens.[23] Each 60-mL marrow harvest was placed into a blood transfer bag into which 4 mL of anticoagulant citrate dextrose-A had been placed. In group A patients, 10 mL of this anticoagulated BMA was used as the CD34+ cellular leg and the CD44+, CD90+, CD105+ cellular legs of the tissue-engineering triangle, with TNC counts and CFU-F cell counts as noted previously. In group B patients, 60 mL of BMA was processed using a Smart Prep2 BMAC system (Harvest), which uses a gradient density centrifugation to provide 10 mL of CD34+ rich BMAC with the TNC and CFU-F cell counts as noted earlier.

Patients

Inclusion criteria for the study were:

1. Age over 18 years
2. Radiographic evidence of healed proximal and distal bone segments in good alignment
3. Sufficient soft tissue to cover a graft without the need for a local or distant flap

Exclusion criteria were as follows:

1. Previous procedure entering bone marrow
2. History or presence of bone marrow pathology
3. Hypersensitivity to heparin, BMP, or anticoagulant citrate dextrose-A
4. Previous radiation to the jaws
5. Metastatic cancer

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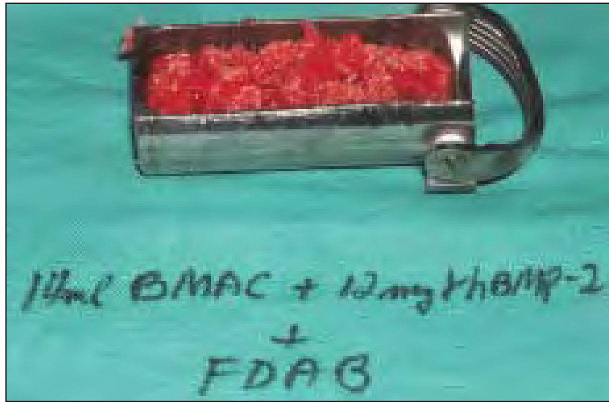

Fig 5 Composite bone graft of BMAC (CD34+ cell–enriched), rhBMP, and cancellous allogeneic bone.

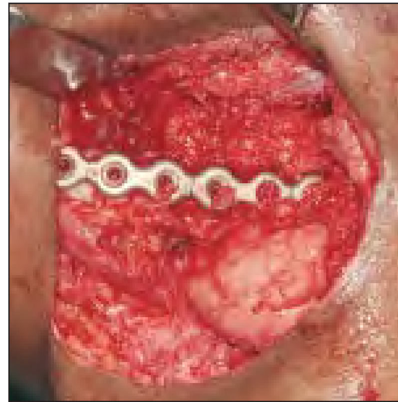

Fig 6 At surgery, the composite graft shows a loose consistency.

6. Exposure to an oral bisphosphonate less than 9 months previous
7. Current regimen or history of receiving an intravenous bisphosphonate, methotrexate, or prednisone

Surgical Procedure

Each patient's continuity defect was exposed through a submandibular incision. The recipient tissue bed was developed by removing scar tissue and reflecting a minimum of 4 cm of periosteum from both the proximal and distal segments and scoring the surface edge of each bone segment to remove the cortex.

The graft was prepared by solubilizing the rhBMP-2 for 5 minutes according to the manufacturer's directions (Infuse Bone Graft, Medtronics) and applying it to the ACS. The rhBMP-2 was allowed a minimum of 15 minutes to bind to the ACS to achieve 95% binding. Cubes of crushed cancellous allogeneic bone (University of Miami Tissue Bank) were passed through a bone mill (Stryker Corp) one time using 10 mL of premilled bone per centimeter of mandibular defect. The respective anticoagulated BMA or bone marrow concentrate was added to the milled crushed cancellous allogeneic bone and mixed thoroughly. Then the saturated rhBMP-2/ACS was cut into 1-cm square pieces and added to the mixture to create a composite graft. Prior to placement, 0.5 mL of a 10% calcium chloride solution containing 5,000 IU of bovine thrombin was added to the composite graft to reverse the anticoagulant, release the growth factors inherent in the platelet fraction, and activate the cell adhesion molecules in the plasma fraction.

This composite graft (Fig 5) was placed and condensed into the prepared continuity defect using Penfield bone packers (Fig 6). The graft was contained with either a titanium mesh or an allogeneic bone strut, as appropriate to the morphology of the defect. Four weeks of maxillomandibular fixation followed for each patient, along with 1 week of postoperative antibiotics and appropriate analgesics.

Assessments and Follow-up

All patients underwent examination and release of maxillomandibular fixation and arch bar removal under local anesthesia at 4 weeks. A baseline cone beam computed tomographic (CT) scan was taken at that time. Examinations and cone beam CT scans were also performed at 3 and 6 months. At 6 months, the principal investigator determined whether there was sufficient bone and sufficient mineral density to accommodate dental implants (primary endpoint). Those patients in whom the clinical and radiographic assessments indicated implant placement received implants (Biomet 3i), and a core or open bone biopsy specimen was harvested. All implants were allowed 6 months for osseointegration before functional loading.

Study Endpoints

The primary endpoint was sufficient bone to place dental implants without the need for additional grafting. Patients who regenerated sufficiently dense bone capable of implant primary stability were considered responders. One secondary endpoint was the radiographic density of the bone in Hounsfield units (HU) taken from the 6-month cone beam CT scan. Another secondary endpoint was the analysis of trabecular bone density obtained from histomorphometric analysis of the bone cores or biopsy specimens taken at implant placement using the Image-Pro Plus 5.0 computer-based analyzer.

Statistical Analysis

Data evaluation was accomplished using the statistical software package SPSS 13.0 (SPSS Inc). Discrete variables were presented as cell counts and percentages. A paired *t* test was used to analyze the

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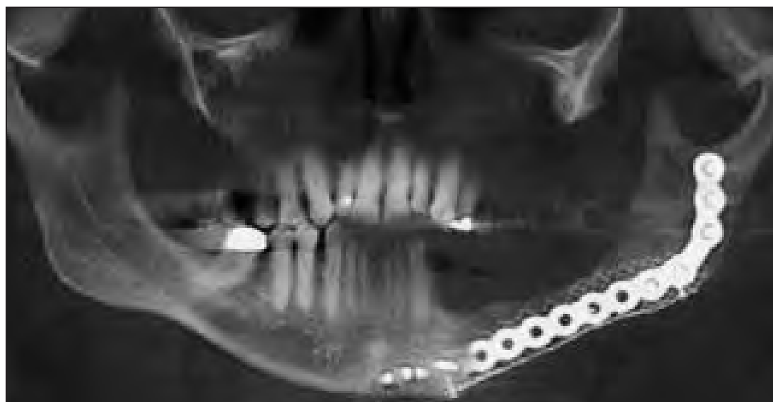

Fig 7 Group A graft that did not achieve the primary endpoint. Bone height, quantity, and maturity were inadequate at 6 months.

Fig 8 Group A graft cone beam CT slice indicating bone regeneration with voids and the absence of a cortical rim.

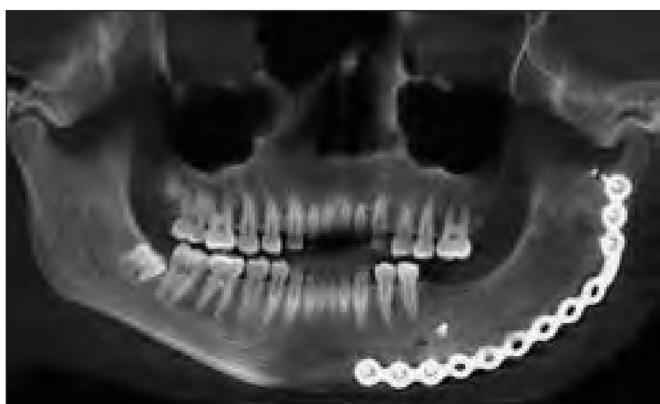

Fig 9 Group B graft that met the primary endpoint. Bone height, quantity, and maturity are present at 6 months.

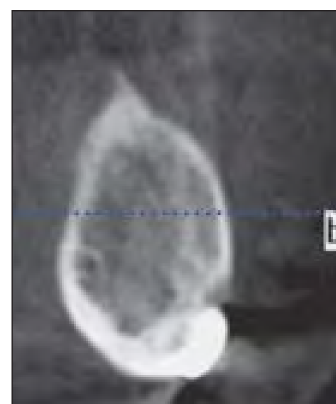

Fig 10 Group B graft cone beam CT slice indicating complete bone regeneration with a cortical rim and trabecular bone without voids.

BMA and BMAC cell counts. A multivariate logistic regression analysis was used to study predictors of clinical benefit after BMA and BMAC graft applications. For all analyses, $P < .05$ was considered significant.

RESULTS

Forty patients (mean age, 57 years; range, 19 to 78 years; 22 men, 12 women) participated in the study. All patients proceeded through the postoperative course without significant complications and showed evidence of new bone regeneration by 6 months.

Table 1 identifies achievement of the primary endpoint in each group. Eight of 20 group A patients (40%) achieved the primary endpoint (Figs 7 and 8), whereas all 20 group B patients (100%) achieved the primary endpoint (Figs 9 and 10); this difference was statistically significant ($P = .006$). This correlates to a CD34+ cell count of 54 ± 38 cells/mL in group A versus a CD34+, cell count of 1,012 ± 752 cells/mL in group B, while the concentrations of progenitor cells of CD44+, CD90+ and CD 105+ were nearly equal between the two groups.

According to univariate analysis, a CD34+ cell count of 200/mL CFU-F was associated with a clinical outcome of sufficient bone regeneration to accommodate

Table 1 Results of Treatment

Endpoint	Group A (n = 20)	Group B (n = 20)	*P*
Regeneration of implantable bone	8/20 (40%)	20/20 (100%)	.006
Mean radiographic density	424 ± 115 HU	731 ± 98 HU	.01
Mean trabecular bone area	36% ± 10%	67% ± 13%	.01

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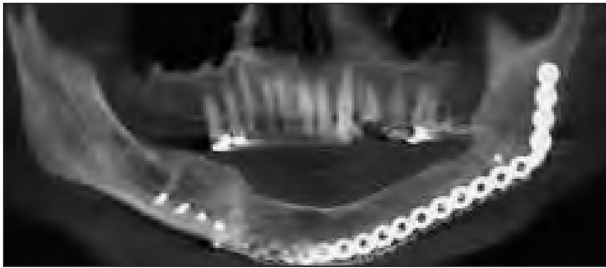

Fig 11 Complete bone regeneration occurs if the CD34+ cell count exceeds 200/mL. Here, a CD34+ cell count of 292/mL provided the required crucial cell count in the tissue-engineering triangle.

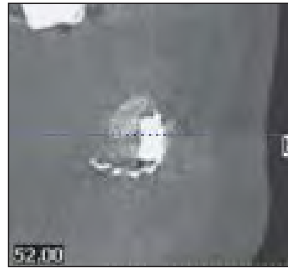

Fig 12 Radiographic bone density of a group A graft (512 HU).

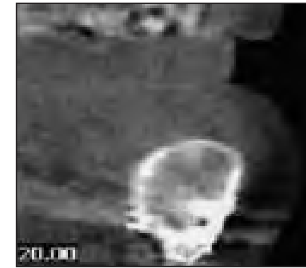

Fig 13 Radiographic bone density of a group B graft (794 HU).

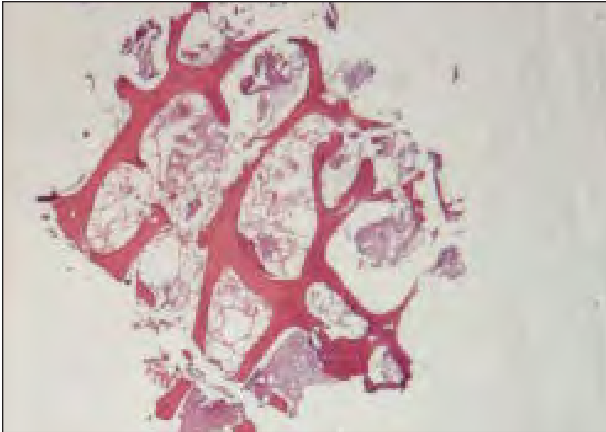

Fig 14 Group A graft with trabecular bone area of 28%. Note the thin trabecular bone struts and the fibrous-fatty marrow spaces that occupy much of the volume instead of bone (hematoxylin-eosin; original magnification ×1.6).

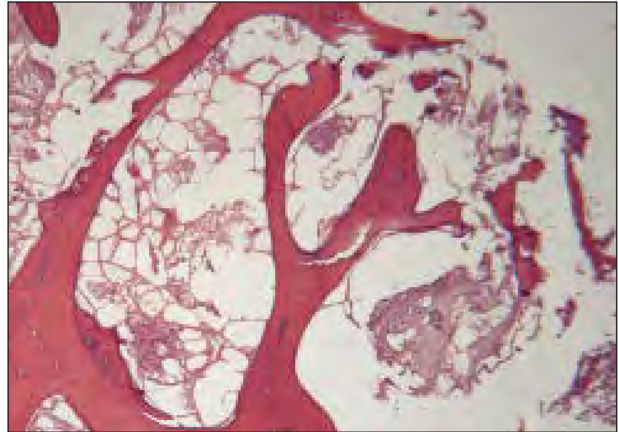

Fig 15 Group A graft at higher power. Mature bone with lamellar architecture but insufficient quantity and thin trabecular struts is present; haversian canals are absent (hematoxylin-eosin; original magnification ×10).

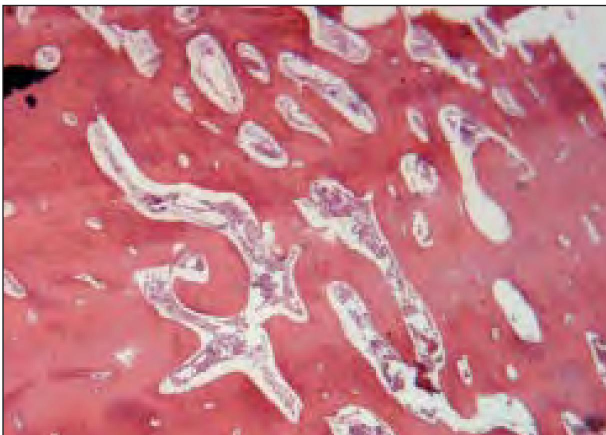

Fig 16 Group B graft with trabecular bone area of 74%. Note the thick bone trabecular struts and regeneration of marrow cells rather than fibrous-fatty marrow (hematoxylin-eosin; original magnification ×1.6).

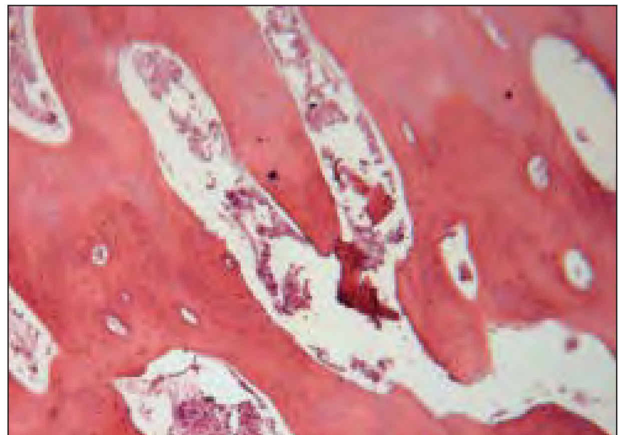

Fig 17 Group B graft at higher power. Mature bone shows lamellar architecture, small cellular marrow spaces, and haversian canals (hematoxylin-eosin; original magnification ×10).

dental implant placement and gain primary stability without the need to augment the regenerated bone (P = .011, odds ratio 5.1, 95% confidence interval 1.25 to 20.52) (Fig 11).

Table 1 also illustrates the mean radiographic density of the regenerated bone in both groups. Group A sites showed only 58% of the density of group B sites (424 ± 115 HU and 731 ± 98 HU, respectively) (Figs 12 and 13) (P = .01). The trabecular bone area of group A (36% ± 10%) was only 54% of the group B trabecular bone area (67% ± 13%) (Figs 14 to 17) (P = .01).

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DISCUSSION

This study investigated the role of the bone marrow–derived CD34+ cell in a milieu of osteoprogenitor cells, bone marrow plasma cell adhesion molecules, rhBMP, and a matrix of crushed cancellous allogeneic bone in the clinical regeneration of functionally useful bone in craniomandibular reconstructions. The main findings can be summarized as follows:

1. The CD34+ cell as an HSC is the crucial cell among the other stem cells/osteoprogenitor cells in regenerating bone in an in situ human tissue-engineering model.
2. A CD34+ cell count of 200/mL CFU-F or greater is directly correlated to a successful clinical outcome.
3. The correlation of extensive bone regeneration with higher counts of CD34+ cells as compared to lower counts of CD34+ cells together with equal counts of other MSC/osteoprogenitor cells and cell adhesion molecules in the milieu strongly supports the mechanism of action proposed by others; that is, a master signaling cell using paracrine and autocrine cellular cross talk to up-regulate other bone marrow stem cells/progenitor cells of either HSC or MSC origin, resulting in significant bone regeneration within the environment of the graft.[12,24–26]

CLINICAL IMPLICATIONS

This study demonstrates that BMA by itself is insufficient to predictably regenerate clinically useful bone in large bony continuity defects of the mandible. Concentrations of bone marrow to achieve a CD34+ cell count of at least 200 CFU-F/mL are necessary to provide predictable bone regeneration in the context of the composite graft system used in this study. Therefore, devices must be capable of concentrating anticoagulated bone marrow to five to eight times baseline levels and to particularly focus on the CD34+ cell population while still retaining baseline levels of CD44+, CD90+, and CD105+ cells.[27]

Improved Understanding of Adult Bone Marrow Stem Cells

Beyond using the results of this stem cell study to improve clinical outcomes, this study also sheds some light on the understanding of cell-to-cell, cell-to-matrix, and cell-to-microenvironment interactions within human adult bone marrow that may result in further improvements in clinical outcomes.

Although hematopoeisis was once thought to be the sole function of bone marrow,[28] work by Friedenstein et al in 1966 identified bone marrow MSCs other than HSCs, thereby defining two distinct classes of multipotent human bone marrow stem cells: HSCs and MSCs.[29] They isolated CFU-F multipotent cells from marrow plastic adherent cells and found that they could differentiate into osteoblasts, chondrocytes, adipocytes, and supporting stroma for hematopoietic cells. Hence, they have often been referred to as mesenchymal stromal cells as well as mesenchymal stem cells.

HSCs were originally thought to solely produce the lineage of red and white blood cells and thrombocytes/platelets and reconstitute the bone marrow stem cell population. However, more recent studies have identified that HSCs are significantly plastic, producing and up-regulating MSCs and their progeny, and are not limited to blood cell production.[20,30,31] Researchers now realize that these two classes of stem cells are not independent of each other but are actually very interdependent.[31,32] Furthermore, each is influenced by its own anatomical niche,[10,11,33] various growth factors,[25,34] and by the local microenvironment into which it may be placed.[35]

In the context of this study, the HSCs were represented by the CD34+ cell and the MSCs by the CD44+, CD90+, and CD105+ cells. Each cell responded to the peripheral signals in the microenvironment, represented by the rhBMP-2 as well as the inherent hypoxia in the wound, the growth factors from the platelets and macrophages, and the cell adhesion molecules present throughout the graft. The subsequent cellular proliferations were directed by the CD34+ cell, and the osteoblast differentiation and osteoid synthesis were directed, up-regulated, and stimulated by all of the cellular components, growth factors, and matrix proteins in the graft. These cell-to-cell, cell-to-matrix, and growth-factor-to-cell interactions resulted in the useful bone regeneration observed and emphasize the importance of cellular heterogeneity in graft systems.

Although significant research efforts have been made to identify one specific cell type or one specific growth factor that would significantly increase tissue regeneration, this study and others demonstrate that a heterogenous population of HSCs and MSCs, delivered with numerous but specific growth factors in a conducive microenvironment, provides more predictable bone regeneration. The importance and supportive actions of the various cellular components in human bone marrow are outlined in Table 2, and the supportive actions of growth factors and the microenvironment are outlined in Table 3. Although the CD34+ cell is a crucial and perhaps outcome-determining cell, it does not act alone and cannot regenerate bone without a diversity of cells in the milieu and the various signals from growth factors and the microenvironment.

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Table 2 Activity of Various Cellular Components of Human Whole Bone Marrow

Component	Role
MSC	Differentiate into osteoblasts; bone formation; respond to HSCs and growth factors
HSC	Drive regeneration via angiogenesis/vasculogenesis; orchestrate bone formation; convert to MSC "plasticity"
Endothelial progenitor cells	Stimulate angiogenesis; release BMP-2 and BMP-6; up-regulate BMP-2 production
Platelets	Mediate cell-to-cell adhesion and cell-to-matrix adhesion via cell adhesion molecules; release growth factors
Lymphocytes	Support migration and proliferation of endothelial progenitor cells
Granulocytes	Release vascular endothelial growth factor to support angiogenesis and vasculogenesis

Table 3 Activities and Roles of the Growth Factors and the Microenvironment in a Graft

Factor	Action
Hypoxia	Driving force for wound healing via macrophage chemotaxis
Fibronectin and fibrin (from plasma)	Cell adhesion molecules; matrix for osteosynthesis
Vitronectin (from platelets)	Cell adhesion molecule; matrix for osteosynthesis
Stromal-derived activating factor 1-alpha (from platelets)	Homing signal for MSCs and HSCs
Platelet-derived growth factors aa, ab, bb (from platelets)	Protein isomers that are mitogenic and angiogenic
Transforming growth factor beta-1 and beta-2 (from platelets)	Protein isomers that are mitogenic, angiogenic, and support MSC differentiation toward bone and cartilage
Vascular endothelial growth factor (from platelets and macrophages)	Angiogenesis
BMP	Attraction for MSCs and HSCs; proliferation and osteoblast differentiation; stimulation of osteoid synthesis
Macrophages	Secretion of growth factors for completion of wound healing

Therefore, concentrating the cellular components of whole bone marrow, rather than isolating and expanding selected MSCs and/or HSCs, optimizes and promotes both osteogenesis and angiogenesis. The authors suggest that the use of the full diversity of human nucleated bone marrow cells and increasing their numbers translates into enhanced bone regeneration that is superior to outcomes obtained by single cell expansions or single growth factor applications.

The long-awaited goal of rebuilding lost skeletal parts of significant size without the morbidity of open bone harvesting (eg, a long hospital stay, a long convalescent period, scarring, disability, and higher costs) has now become a reality. Although researchers have just begun to understand the complexities of human bone marrow cell functions and their interactions, the value of rhBMPs, and the matrices upon which bone can regenerate, it is known that composite grafts of an appropriate cellular milieu, a signal, and a suitable matrix can regenerate up to 8 cm of missing bone. The challenge now is to expand the knowledge and understanding of adult bone marrow cells, the signal of rhBMP, and the best matrix for bone growth to apply these results to larger skeletal defects and those associated with a hostile tissue environment (eg, radiated tissue, scar tissue, bisphosphonate-compromised bone).

ACKNOWLEDGMENTS

Dr Marx is a paid consultant for Harvest Technologies and Medtronic, Inc. Dr Harrell is the chief research scientist at Harvest Technologies.

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