Long-term bone marrow culture and its clinical potential in chronic wound healing

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ABSTRACT

Bone marrow-derived cells have long been regarded to play a crucial role in the homeostasis of skin. We have previously described the clinical benefit of directly applying autologous bone marrow aspirate and cultured bone marrow cells to recalcitrant chronic skin wounds. The initial response to treatment appears to be vascular in nature with the formation of new blood vessels. The difficulty in consistently growing adequate numbers of cells for delivery to patients was, however, a limiting factor. Here, in a subsequent protocol, we describe an improved bone marrow culture system yielding a reliable growth of bone marrow cells and leading to a greater clinical response. Cells expressing markers of endothelial progenitors including CD133, CD146, and particularly CD14 are enhanced in these cultures. CD14-isolated cells produced colonies in endothelial cell assays and sprouting in matrigel assays. Angiogenic cytokines, including angiogenin, epithelial neutrophil-activating protein-78, growth-regulated oncogene, growth-regulated oncogene-α, Interleukin-8, CXCL16, and monocyte chemoattractant protein-1, were found to be elevated in these cultures. Administration of improved culture cells to patients with chronic wounds present for > 1 year lead to an enhanced clinical response.

The contribution of bone marrow cells to cutaneous wound healing has long been acknowledged. Inflammatory cells help to orchestrate hemostasis and initiate healing through the release of cytokines. Scavenger cells such as macrophages remove cellular debris and damaged tissue. Several recent findings have, however, raised questions as to the role of other bone marrow-derived cells in providing the cellular substrate for tissue reconstruction. In a prior report,1 we described healing in chronic wounds with apparent tissue rebuilding following the administration of autologous fresh bone marrow and cultured bone marrow cells. Several histologic and clinical changes were noted including improvements in the wound bed and new vascular growth. Stem and/or progenitor cells present in the bone marrow have been reported to give rise to several structures and related tissues found in the skin. It thus seems plausible that the bone marrow may contribute cells that give rise to tissues present in the skin such as vasculature and perhaps stromal cells. There are multiple reports supporting the notion that the bone marrow contains endothelial cell progenitors and/or cells that stimulate vessel growth.2,3 Among these cells of interest are those expressing cluster domain (CD)133, CD146, and CD14. A reliable method for growing these types of endothelial progenitors and/or cells that stimulate vascular growth could be of considerable clinical value.

Our initial report illustrated the beneficial effect of autologous bone marrow in stimulating recalcitrant wounds to heal.1,4 Several subsequent studies have supported this
effect of the bone marrow to heal complex wounds.\(^7\)\(^{-11}\) In our studies, healing was achieved after application of whole fresh autologous bone marrow aspirate and cultured derived cells from the aspirate. We have, however, observed difficulties in consistently growing adequate amounts of autologous bone marrow cells for delivery to patients. Unlike bone marrow cultures derived from healthy donors, cultures from chronic wound patients grow slower and in several cases fail to reach maximal growth. This inability to grow some patients’ cells also makes characterization of delivered bone marrow cells difficult due to the limited number of cells available for treatment and analysis. By modifying our culture system, we are able to considerably improve the growth of bone marrow cells from chronic wound patients. Utilizing these modifications has the additional benefit of decreased weekly manipulations of the cultures that will limit the potential for contamination of the cultures. A greater clinical response was noted following administration of cells grown with these modifications.

In this report, we also describe the initial events that occur following the application of autologous cultured bone marrow cells to chronic wounds. There appears to be a vascular response with new blood vessel growth noted clinically and histologically. Analysis of the cells applied to wounds revealed that they express markers for endothelial progenitor cells and are considerably enriched in CD14\(^+\) cells. In addition, cytokine analysis of culture supernatants revealed a profile consistent with stimulatory components of angiogenesis as well as maintenance of endothelial cell progenitor cells.

**METHODS**

**Clinical protocol**

The patients described in this report participated in a newly designed randomized study for chronic wound patients. All patients had wounds present for more than 3 years and had failed numerous standards of care and advanced wound treatments including autologous and/or bioengineered skin grafting. The patients then served as their own control for failure to respond to standard therapy. The patients described here were randomized to treatments where their wounds received autologous cultured bone marrow cells alone or autologous bone marrow aspirate, followed by autologous cultured bone marrow cells. Two patients received cultured bone marrow cells only. Two patients received fresh bone marrow aspirate, followed by cultured bone marrow cells. In this report, four chronic wounds were treated in four individuals. The ulcer types included one due to scleroderma, an ischemic/postsurgical wound of more than 7 years duration, a pressure ulcer in a paraplegic patient, and a diabetic foot ulcer. The protocol was approved by the Roger Williams Medical Center Institutional Review Board (RWMC IRB). A separate donor-based protocol, also approved by the RWMC IRB, was used to obtain bone marrow aspirates from healthy individuals.

**Culture method**

Total bone marrow (including red blood cells) from iliac crest aspirates were placed in modified Dexter cultures as described previously.\(^1\) In order to achieve growth surpassing the original culture method described,\(^3\) several culture parameters were altered. Modifications included the hypotonic lysis of red blood cells in the fresh bone marrow aspirate with 0.8% ammonium chloride solution containing 0.1 mM EDTA (Stem Cell Technologies, Vancouver, BC) before placing cells in culture. Cultures were incubated at 33°C and 5% CO\(_2\) in 175 or 225 cm\(^2\) Corning flasks. In previously described cultures,\(^1\) half of the media was removed and supplemented with fresh media each week. Here, cells were fed with new media without removing any media during the first 8–9 weeks of culture.

**Administration of bone marrow cells to wounds**

Fresh bone marrow aspirate was collected in heparinized syringes. Cultured cells were prepared and washed as described previously.\(^1\) Bone marrow aspirate and cultured cells to be administered to the wound were placed in a syringe fitted with a 19-gauge needle. These bone marrow cells were then injected into and applied directly over the wounds. The material was held in place with an occlusive dressing (Opsite or Tegaderm). Fresh aspirate or cultured cells administered to wounds were left in place under dressings for 24–48 hours. Wound closure was monitored by direct measurement, digital photography, and tracings using the Visitrak wound measurement system (Smith and Nephew, Largo, FL).

**Functional assays**

Depletion of hydrocortisone in cultures leads to the development of adipose cells. This was reversible on the re-introduction of hydrocortisone to cultures. Endothelial cell colony-forming assays were performed under standard methods using Endocult Media (Stemcell Technologies, Vancouver, BC, Canada) and fibronectin-coated plates (BD Scientific, Franklin Lakes, NJ).\(^12\) Fibroblast colony-forming units were shown by placing cells in Mesencult media (Stemcell Technologies) with 10% fetal calf serum (Cambrex, Manassas, VA). Matrigel assays were performed by placing 1 x 10\(^5\) human umbilical vein endothelial cells (HUVEC, ATCC, Rockville, MD) suspended in endothelial basal media (EBM, Cambrex) or bone marrow cells were placed on matrigel-coated plates and incubated at 37°C as described previously.\(^13\)

**RESULTS**

**Comparison of growth in cultures derived from normal donors versus chronic wound patients**

Initial experiments were directed at defining the in vitro growth characteristics of normal marrow in contrast to marrow derived from our chronic wound patients. Fresh bone marrow aspirates were obtained from normal donors and the wound patients enrolled in our study. Bone marrow derived from healthy donors was obtained under a separate protocol. Bone marrow aspirates were placed in culture and evaluated by their ability to form an adherent “feeder” layer. The time to reach confluence, of this adherent layer, was the chosen measure of culture.
maturation. As methods were optimized, bone marrow from healthy donors consistently reached confluence in 2–4 weeks of culture while cells derived from chronic wound patients, however, showed marked variability in their growth potential in culture. The fastest growing bone marrow cultures from chronic wound patients reached confluence in 5–6 weeks. While not all cultures from every aspirate reached confluence, it is important to note that all aspirates derived from two particular study patients failed to reach confluence when placed in culture. Cultures derived from these two patients never exceeded a maximum of 60% confluence even after optimization of the culture methods (as described below). These two patients differed clinically from others in the protocol, in that their wounds were associated with extensive scarring and fibrosis. This might suggest a systemic process affecting the bone marrow in chronic wound patients with extensive fibrosis.

Improved growth following ammonium chloride lysis and sequential feeding of cells

In performing our preliminary experiments, as described previously, consistent growth of bone marrow cells in culture was elusive. These cultures characteristically establish an adherent layer with loosely attached overlying cells. Growth proceeds as an organotypic culture and cannot be disrupted for sampling without risk of destroying the culture microenvironment. A main objective of the clinical protocol was the administration of as many cultured cells as possible back to the patients. Toward this goal, a non-invasive method for analysis of assessing culture maturity was developed. The confluence of the adherent layer, over time, was chosen as the parameter to determine the maturity of the cultures.

Utilizing our original culture methods, bone marrow cells derived from both chronic wound patients and normal healthy donors were unable to reach confluence, even after 12 weeks in culture. When red cells, susceptible to hypotonic lysis, were removed from fresh bone marrow cultures with ammonium chloride and subsequently placed in culture, there was a significant increase in growth (Figure 1).

The weekly nutrient supplementation protocol was also varied to minimize the potential for contamination. This is an important point as cultured cells are ultimately given back to patients. Originally, half of the supernatant from cultures was removed and the cells contained in the removed media were isolated by low-speed centrifugation. The supernatant removed was discarded and the pelleted cells were resuspended in fresh media and placed back in culture flasks to restore the original volume in each flask. This degree of manipulation is a potential pitfall that could lead to contamination. We examined a method of sequential feeding of cultures without removal of media.

By removing red cells from the starting material (by hypotonic lysis with ammonium chloride as described above), we were able to begin with smaller volumes in larger flasks. In our original culture method, 1 × 10^6 cells/mL were placed in T-75 flasks, which included red blood cells. This corresponded to ~1 × 10^9 nucleated cells/mL. Using ammonium chloride lysis, 1 × 10^6 nucleated cells/mL were plated, thus enabling a reduction in the starting volume by 40%. Given this volume reduction, we were able to sequentially feed cultures without removal of media for several weeks. Weekly, 58% of the original volume of fresh media could be added to the flasks for feeding without concern for excessive volume of culture media in the flask for up to 10 weeks. If media needed to be removed after 10 weeks due to volume limitations, the media could then be discarded as it contained few suspended cells at that point. This, however, was not a concern in our clinical protocol as no patients received cells after 10 weeks in culture.

Initially, all cells placed in culture are in suspension. After 2–3 weeks, cells begin to attach. Once an adherent layer is established, most cells not firmly attached to the plastic are more loosely attached to the adherent layer. Few cells are present in suspension after 6 weeks.

There was a minor growth advantage seen during the first 2–3 weeks in (ammonium chloride treated) bone marrow cultures derived from normal donors when comparing sequentially fed cultures with those fed by our previous method of media removal. Using our previously described culture methods, we found that some wound patients’ bone marrow cultures grew very poorly. When these poorly growing aspirates were placed in the modified cultures (ammonium chloride lysis plus sequential feeding), there was a significant growth advantage (Figure 2). Because switching to these methods, bone marrow cultures from chronic wound patients prepared in this manner reliably produced a greater number of cells for delivery to patients.

The heterogeneity of cells grown in culture was then evaluated. The multipotent nature of cultured bone marrow cultures prepared as above was shown by the ability to grow several cell types including adipocytes,
endothelial cells, and fibroblasts in functional assays using appropriate media (Figure 3).
Comparison of fluorescent-activated cell sorting analysis of fresh bone marrow aspirate and cultured cells was performed. Several markers of endothelial cell progenitors were noted to increase in cultured cells as compared with the initial bone marrow aspirate (Figure 4). Bone marrow aspirate contained an average of 2.0% CD146 cells. After culture for 5–7 weeks, the number of CD146 cells increased to an average of 15.0% (7.5× increase). On average, CD133 cells increased in culture (over bone marrow) by a factor of 2.0. A substantial increase was seen in CD14 cells that showed an average increase in culture by a factor of 11.5.

CD14 cells were next isolated from 3-week bone marrow cultures derived from a normal healthy donor by cell sorting. CD14+ and CD14− cells derived from 3-week-old cultures from normal donors were placed in colony forming unit-endothelial cell (CFU-EC) assays. Distinct colonies were noted in CD14+ CFU-EC assays in 2–5 days (Figure 5) but not in the CD14− CFU-EC assays. CD14+ and CD14− cells were also placed in matrigel assays. In 2–5 days, CD14+ cells showed sprouting whereas CD14− cells showed no growth (Figure 6).

Clinical response

Patients receiving bone marrow aspirate and/or cultured bone marrow cells exhibited a “vascular” response.

Figure 2. Growth in cultures before and after modifications of lysis and sequential feeding. Patients in A and B both exhibited poor growth in their cultures. (A) Comparison of culture growth at week 5 for Patient 1. Pre- and postmodification cultures were derived from the same bone marrow aspirate. (B) Comparison of culture growth at week 7. Pre- and postmodification cultures were derived from the same bone marrow aspirate.

Figure 3. Functional analysis of cultured bone marrow cells. (A) Adipocytes in culture after depletion of hydrocortisone from cultures. (B) Colony forming unit (CFU)-endothelial cell on a fibronectin-coated plate. (C) CFU-F were readily established from the bone marrow in Mesencult media.

Figure 4. Bone marrow aspirates and cultures derived from those aspirates were analyzed by fluorescent-activated cell sorting. Increases in CD14, CD133, and CD146 cells were noted following 5–7 weeks in culture.
marrow cells exhibited brisk bleeding to an extent that was not evident before treatment. Histologically, wounds had areas of increased blood vessel growth (Figure 7). This occurred in patients receiving bone marrow aspirate and cultured cells or cultured cells alone.

The clinical response to administration of cultured cells appeared to be more dramatic when improved growth cultures (modified as above) were used. In two patients receiving cultured cells only, one (diabetic foot ulcer) was only given cells from ammonium chloride sequentially fed cultures and the other (ulcer due to scleroderma) received most of their treatments (five of seven total) with poorly growing cultures (without ammonium chloride lysis and sequential feeding). The patient with a diabetic foot ulcer receiving ammonium chloride sequentially fed cultures had a greater than 50% closure following only two administrations of cultured cells. Unfortunately, this patient subsequently underwent a surgical procedure on the affected foot that met exclusion criteria for our protocol and they had to be discontinued from treatment. The scleroderma patient’s wound did not respond to therapy with failure to close of the ulcer. There was, however, a 10% closure within 4 weeks after receiving ammonium chloride lysed sequentially fed cultures, which was this patient’s greatest response to a particular treatment. Biopsy of the scleroderma ulcer during treatment showed focal increased blood vessel growth.

In patients receiving bone marrow aspirate and cultured cells, there was closure in an ischemic surgical wound after 1 year. This patient had received cultured cells grown by both the previously described method and improved culture method. Using the previously described methods, their cells were among the poorest with respect to growth. The patient received four bone marrow aspirations in total. Cultures were attempted from each aspirate. The first two aspirates were prepared for culture using our original culture methods. The first aspirate sample produced

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**Figure 5.** Day 5 colony forming unit-endothelial cell assays. Cells used to prepare these assays were derived from a 3-week bone marrow culture (normal donor). (A) CD14+ cells. (B) CD14− cells.

clinically represented by increased erythema with telangiectasia at the wound edge. Debridements following treatment with bone marrow aspirate or cultured bone

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**Figure 6.** Matrigel assays. Cells used to prepare these assays were derived from a 3-week bone marrow culture (normal donor). (A) Day 2 human umbilical vein endothelial cells. (B) and (C) Day 2 CD14+ cells. (D) CD14− cells did not show evidence of growth even after 14 days in Matrigel.
cultures with < 1% growth and were determined to be inadequate for administration to the patient. The second aspirate sample grew to an average of 7% confluence and was administered to the patient. The total number of cells delivered from these cultures was $1.9 \times 10^6$. Administration of these poorly growing cells resulted in only a 21% closure as determined by wound surface area before the administration of cultured cells. The last two aspirates were prepared by ammonium chloride lysis and sequential feeding. These cultures grew to an average confluence of 27.5% (with a range of 20–50%). The total number of cells delivered by these cultures was $1.1 \times 10^7$. There was an 81% wound closure following administration of the improved culture cells as determined by comparison with wound surface area before administration of these cultured cells. With administration of improved cultured cells, this patient had a particularly dramatic vascular response with the development of an angioma during treatment (Figure 8). The angioma spontaneously resolved and epithelialized during treatment. Biopsy of the treated ulcer also showed areas of new vessel growth. The other patient receiving bone marrow aspirate and cultured cells had a pressure-related sacral cavity wound. This patient’s cells were processed by our modified culture method. Overall, this patient showed a dramatic decrease in wound size (81% closure) with dermal rebuilding and new tissue growth easily seen at the wound edges (Figure 9).

**Cytokine analysis**

Bone marrow cells delivered to wounds might exert their effect by delivering cytokines to the wound. We therefore hypothesized that the growth of the cultures as well as aspects of the observed clinical response may correspond to an underlying cytokine growth factor expression profile. To characterize our bone marrow cultures derived from chronic wound patients, we submitted supernatants from cultures that exhibited fairly robust growth and cultures that did not grow well. Their data were examined to find a preliminary broad-spectrum profile that could possibly correlate with the growth characteristics of the cultures and the clinical responses. Samples were analyzed by an

![Figure 7. (A) and (B) Pre-treatment. (C), (D), and (E) posttreatment.](image-url)
immunoblot assay (Raybiotech Inc., Norcross, GA) for 174 known human cytokines. Cytokines with angiogenic effects including angiogenin,14 epithelial neutrophil-activating protein (ENA)-78,15,16 growth-regulated oncogene (GRO),17 GRO-α,17 IL-8,18–20 CXC16, monocyte chemotactic protein (MCP)-1 (CCL2)22–25 were elevated in patient cultures. Levels of angiogenin, GRO, and ENA-78, were, however, higher in better growing cultures than poorly growing cultures. Supernatants from growing cultures also had significantly increased levels of cytokines produced by bone marrow stromal cells, which are important for maintenance of hematopoietic and mesenchymal stem cells including IL-7,26 IL-6,27,28 hepatocyte growth factor (HGF),29,30 and NAP-2 (CXC7).31 IL-7 and NAP-2 (CXC7) were lower in poor-growing cultures indicating potential deficiency in their stromal cells, which might account for their observed poor growth.

Soluble CD14 receptor was elevated in all cultures, likely reflective of an increasing population of CD14+ cells. Cytokines involved in macrophage growth and development including PARC (CCL18),32,33 macrophage inflammatory protein (MIP)-1β (CCL4),34 LIGHT,35 and MCP-122–25,36,37 were also increased.

**DISCUSSION**

We have previously reported on the potential of bone marrow cells to support healing in recalcitrant chronic wounds.1,6 This report and others7–11 continue to confirm the benefit of using bone marrow cells to heal difficult wounds. In our original observations and herein, we have observed an initial and enduring vascular response during the treatment of the wounds. In this study, we report an improved culture system that appears to enhance cells enriched for endothelial progenitors and/or cells that may stimulate endothelial cell growth. In these improved cultures, there is an overall increase in cells expressing markers for endothelial cell progenitors including CD14, CD133, and CD146. The most dramatic increase was noted in the increased number of CD14+ cells. Administration of these cells to wounds produced a clinical “vascular” response as evidenced by telangiectasia and erythema at the wound edge and in the wound bed. Angioma formation as well as rapid and more brisk bleeding on subsequent debridements was also noted. This response could be noted within 3 weeks following the administration of bone marrow aspirate and cultured cells. Histologically, new blood vessel formation was documented. We are continuing to treat chronic wound patients with these cells and are monitoring their progress.

Elevated angiogenic cytokines such as vascular endothelial growth factor (VEGF)38,39 have been reported in chronic wounds. These findings suggest that chronic wounds are able to express angiogenic cytokines; however, due to proteolysis39 and/or the inability of circulating angiogenic cells to reach critical areas, this increased expression is ineffective. Fibrin cuffs, while characteristically having been described in venous ulcerations, have also been reported in other chronic ulcerations.40 These fibrin
cuffs likely interfere with the ability of cytokines to reach their target cells and/or for the target cells to reach the wound bed. By delivering appropriate cells directly to the chronic wound, one may bypass this obstacle. In our approach, we may be delivering the missing angiogenic stimulatory and substrate cells that cannot reach the wound by normal migratory mechanisms. Our improved culture system should expand these effects.

The CD14+ cells in our improved culture system may be adept at carrying out this vascular change. The VEGF receptor kinase insert domain receptor (KDR) is present on endothelial progenitors12,41,42 and multipotent stem cells.43-45 A significant number of circulating KDR+ leukocytes are CD14+.12 These CD14+ cells have been identified to be endothelial progenitors and have been shown to express several early stem cell markers.2,12,46 Injection of a subpopulation of circulating human CD14+ cells was shown to improve healing and stimulate angiogenesis in ischemic limbs of diabetic mice.47 Many of the cells in our cultures were CD14+. When cultured CD14+ cells were placed in CFU-EC assays containing high levels of VEGF, they produced distinct colonies. As these CD14+ cells appear to be VEGF responsive, they may then be quick to act when delivered to a VEGF-rich environment such as a chronic wound.

In utilizing cultured cells, good growth in culture correlated with an improved clinical outcome. Increased growth in cultures also meant delivery of a greater number of cells to the patient. These findings support the idea that cells, and not soluble factors in the bone marrow aspirate, are responsible for the healing effect. Improvements in our culture methods will likely lead to a greater clinical effect in patients to be treated. No toxicity or significant adverse effects were noted with the delivery of autologous bone marrow or cultured cells at doses up to 9 x 10⁷ cells per treatment. Differences in culture growth were noted between patients and in comparing chronic wound patients with normal donors. The two patients with poor growth in culture had prominent fibrotic wounds. This may reflect a systemic effect in chronic patients, particularly those with fibrotic wounds. Cytokine variation in poorly growing patients, particularly IL-7 and NAP-2 (CXCL7), may have accounted for this growth differential. We are continuing to gather data on bone marrow cultures in order to better elucidate these differences.

The angiogenic cytokines elevated in patients’ cultures including angiogenin, ENA-78, GRO, GRO-α, IL-8, CXCX16, and MCP-1 (CCL2) are important in angiogenesis and have been shown to be expressed in angiogenic assays.25,48 While these are cytokines produced by bone marrow cells in our cultures, it is not yet known how the expression patterns of cells once delivered to a wound will change. Nevertheless, we have shown that these cells are capable of producing these cytokines and help characterize the delivered cells as having angiogenic potential.

Cultured bone marrow cells are, however, a heterogeneous population. Cytokines important in macrophage development (PARC/CCL18, MIP-1β, LIGHT, and MCP-1) and in the maintenance of stromal and hematopoietic cells (IL-6, IL-7, HGF, and NAP-2/CXCL7) were also noted in the cytokine analysis of culture supernatants. Many of the cytokines we detected as elevated have been described as being important in normal wound healing.49 Administrations of bone marrow cells to chronic wounds might then replace missing or depleted cytokines and restore a more normal wound environment, allowing for healing to occur. In our protocol, we administered only cultured cells and not the cytokines present in the culture supernatant. It is possible that administering some of the cytokines produced by these cultures with bone marrow cells might yield a greater response.

We have developed a method where bone marrow cells capable of stimulating wound healing can be grown in an improved long-term culture system. Autologous cells may then be grown for later administration to patients. This approach may also have additional benefits for the treatment of other disorders where a vascular response would be beneficial.

ACKNOWLEDGMENTS

The authors have no financial relationship or conflict of interests related to this work. The authors would like to thank Drs. Larry Lum, Peter Quesenberry, and Gerald Colvin for their technical assistance in harvesting and growing bone marrow cells. This work was supported by grants from the National Center for Research Resources (P20RR018757) and the National Institute on Aging (R01AG027874). The National Center for Research Resources (NCRR) and the National Institute on Aging (NIA) are components of the National Institutes of Health (NIH).

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