

MARROW CELLS

Quarterbacks of Bone Formation

2016

TREATING COMPOSITIONS SOURCED FROM MARROW ASPIRATE—A CELL DRIVEN THERAPY

Through cytokine release and cell-to-cell contact, bone marrow stem cells are the quarterbacks of the injury site

Paracrine signaling to create synergistic interactions between cells in wound healing requires a coordinated interplay among cells, growth factors, and extracellular matrix proteins. (9,10,13,14,15,16,21,31) Central to the coordinated interplay among cells, and the extracellular matrix is the MSC (mesenchymal stem cell), which coordinates the repair response by producing growth factors that orchestrate the healing cascade by recruiting cells, making resident cells more plastic and changing the growth factor production of resident cells. (31,54) The standard measurement for MSC's from marrow is a cfu-f test. (1) MSC's have substantial involvement in the initial stage of bone healing by controlling the fate of inflammation. (25,26) By responding to changes in their environment, and using complex growth factor mediated signaling circuitry, MSC organize site-specific regenerative responses. (9,10,13,14,15,16,21,31) Recent studies have reported the ability of MSCs to modulate the phenotype of macrophages by inducing a shift from inflammatory M1 macrophages to anti-inflammatory M2 macrophages, thereby transitioning the wound healing cascade from inflammatory to proliferation and remodeling. Thus, the growth factor profile of cells from marrow is different than that produced by blood cells. (54) Mature resident cells, under the influence of migrating stem cells, demonstrate a plasticity that allows them to make a significant contribution to the healing cascade. (31,54)

Dose Response of Marrow Cells

Critical to successful healing are adequate numbers of immature stem cells and complimentary cells that have migratory capability and whose growth factor profile can influence migrating and resident cells to move into a tissue proliferation and regeneration profile. (19,20,35,52,53) The growth factor profile of a biologic that has a greater proportion of cells from marrow is different from PRP that is made entirely from peripheral blood cells and platelets. (18) Hernigou et al in non-union and osteonecrosis demonstrated that clinical results were linked to the stem cell content of the graft as measured by CFU-f. (43,52,53) This correlation between the CFU-f content of the biologic and outcomes has been repeated by other groups. (5) Interestingly, in the Hernigou work, CFU-f was the only variable that rose to statistical significance; not total nucleated cells or platelets. (43,53) This is consistent with bone marrow rescue therapy in oncology where the stem cell content of the graft, not the number of nucleated cells, is the driver of clinical success.

RELATIONSHIP BETWEEN CFU-F, ENDOTHELIAL PROGENITOR CELLS (EPC'S) AND CD 34+ CELLS

Stem Cell Marker CD34+ and CFU-f Test

CD34+ cells from marrow aspirate are a heterogeneous population of cells that are found in both marrow and blood and include mature endothelial cells, certain monocytes and macrophages, hematopoietic stem cells and endothelial progenitor cells. (22) Cells that mark for CD34+ typically account for 1-2% of nucleated cells from a marrow aspirate. Various medications, such as statins, can influence the number and types of these cells

found in marrow and blood. (38) A majority of these cells are committed blood lineage cells. (39,60) Trauma causes a small subset of CD34+ cells, called endothelial progenitor cells (EPC's), to be mobilized from marrow and home to the site of injury. (32) However, no definitive marker for EPC's exist. (32) Combination markers that include CD34+, as well as CD133+, CD 184+, ckit, VEGF-2 denote a smaller population of cells within the overall population of CD34+ cells that have a greater proportion of endothelial progenitor cells. (24,34) However, markers used in flow cytometry that are based on CD34+ with a parsing based on various combination markers, do not identify and discern exclusively EPC's. (32)

Consequently, CD34 is considered a first pass surface antigen suitable for capture of a large population of heterogeneous cells, that will include a smaller subpopulation of stem and progenitor cells, including mesenchymal cells; CD34+ is not associated only with hematopoietic cells. (50) Certain sub-populations of CD34+ cells reside in marrow and not blood. (33) Early stage, rare CD34+ cells, cannot be counted using flow cytometry, but are capable of forming a CFU-f. (33) Lin et al demonstrated that CD34 is not a negative marker of MSC (mesenchymal stem cell) and that freshly isolated CD34+ / BM MSC form greater proportions of CFU-f colonies than their CD34-counterparts. (33) Therefore the CFU-f test is the appropriate analysis to determine how many cells from the heterogeneous population of CD34+ cells from the aspirate are early stage stem cells to include mesenchymal stem cells (50) Counting cells that reside only in marrow and not blood is a key measure to determine the quality of the biologic. Given the limitations of flow cytometry and the fact that CFU-f reside in marrow and not blood, having a high CFU-f count will correlate with other rare marrow and accessory cells; the full complement of these marrow cells is what drives the transition from inflammation to proliferation and remodeling. (37)

Aspiration Technique and Implications of Centrifuging Marrow

It is well known that the highest quality bone marrow aspirations (greatest quantity of stem/progenitor cells) require aspirating small volumes of bone marrow (1-2 mL) from different locations. (1,2,3,4) It is also known that peripheral blood infiltrates bone marrow

aspirates when greater than 1-2 mL is drawn from any single location. (1,2,3,4) Stem and progenitor cells are enriched in the spongy marrow that is located within the pockets created by the honeycomb of trabecular bone within the medullary space. (1,2,3,4) Only a finite number of stem cells reside within any given pocket of spongy marrow. (1) Volume over 1 mL retrieved from a single site introduces significant peripheral blood into the aspiration. (1,2,3,4) This peripheral blood dilutes further aspiration volume and significantly reduces the stem/progenitor cell quantity of the aspiration per mL. (1,2,3,4) Performing multiple punctures in a clinical setting is often not practical.

To overcome the limitations of lower-quality (reduced cellularity) high volume marrow aspirations from traditional needles, clinicians attempt to enhance the marrow biologic by using a centrifuge-based system. (42) Centrifuge systems discard 85% of the aspirate by removing lower density plasma and higher density cells composed primarily of red cells while retaining 15% of the starting volume that contains a majority of the platelets, lymphocytes, monocytes, granulocytes and young red cells from both the marrow and the infiltrated peripheral blood components of the aspiration. (42) These systems do not distinguish between nucleated cells from the peripheral blood component of the aspirate compared to the marrow component of the aspirate (both sets of cells have the same density). (42) In the case of older patients, such systems increase inflammatory peripheral blood macrophages, neutrophils, and related cells within the treating biologic. In addition, within the discarded higher density red cells are a great number of very potent, cycling, high-density, proliferating anti-inflammatory progenitor cells. (6,7,8,42) These cells increase in density as they build up nucleic mass prior to cell division and are always found in the red cell component after centrifugation and consequently, are discarded by all centrifuge protocols. (6,7,8,42) In the case of a poor aspirate comprised primarily of peripheral blood, the only difference between the biologic that a PRP kit produces compared to what a bone marrow concentrate (BMC) kit produces is that the BMC kit has a higher red cell content and more macrophages and granulocytes. Centrifugation protocols **1.** require larger aspiration volumes that are associated with excess peripheral blood and related inflammatory macrophages, **2.** have

inherent inefficiencies that leaves significant numbers (approximately 40%) of stem cells behind in the discarded red cell portion of the processed marrow, 3. require at least 10% dilution by volume for the addition of anti-coagulant to allow the sample to separate, and 4. and require another 10% dilution in the form of a neutralizing agent such as thrombin and calcium chloride in order for the marrow to clot in the graft. (26,31,42,55,57,58) Finally, centrifugation protocols require the marrow to be filtered prior to centrifugation.

The cell viability of un-manipulated aspirate after 24 hours is typically between 99% and 100% compared to centrifuged marrow that is typically 93% to 95%. This raises a concern that the stress from the manipulation due to filtering and centrifugation that led to increased cell apoptosis in the filtered and centrifuged biologic, has potentially damaged the remaining living cells; making them less productive post transplant. Because marrow based therapies are driven by the stem cell content of the biologic, the sentiment against manipulation, including centrifugation, is best summarized by Muschler et al who concluded “A larger-volume of aspirate (more than 2 mL) from a given site is contraindicated with the additional volume contributing little to the overall number of bone-marrow cells and results principally in unnecessary blood loss” (p 1707). (1)

Clinical Implications

In older or healing impaired patients a chronic condition results when the cytokine profile from naturally aggregating platelets and white blood cells that home into the clot is not sufficient to stimulate the marrow to cause an adequate vasculogenic response to complete the tissue regeneration process. (9,12) PRP is often used as an adjunctive therapy for the addition of platelets, white cells, and resulting growth factors beyond what would naturally aggregate at the newly injured site. (51) The scientific basis for the intervention is that the enhanced chemotaxic profile from the PRP will create an adequate vasculogenic response to move the healing cascade beyond the inflammatory phase. (51) PRP is therefore a growth factor driven mechanism. When a PRP enhanced therapy is not sufficient, adding additional blood cells and platelets in an attempt to start a new healing cascade is not as reliable as mechanically

aspirating and transplanting marrow cells in sufficient quantities to move the cascade beyond the inflammatory phase. (25,38) Moving from the initial inflammatory phase into the proliferation and remodeling phase requires stem cells and complimentary cells to create an anti-inflammatory immune cascade to alter the cell type and growth factor profile in a site-specific manner. (9,10,11,13,14,16,17,21,27,31,56) Therefore marrow-based strategies are dependent on transplanting adequate numbers of stem cells and complimentary cells from marrow at the site. (5,43,48,52,53) For example, in a tibia non-union setting, the only variable that rose to significance was the number of stem cells in the graft, as measured by cfu-f, not platelets or white blood cells. (63)

MARROW CELLUTION

Marrow Cellution is a novel bone marrow access and retrieval device, co-developed by Endocellutions Corp (475 School Street, suite 12, Marshfield MA) and Ranfac Corp, (30 Doherty Dr. Avon MA) that incorporates features designed to minimize the limitations of traditional needles. Flow into the aspiration system is collected mainly laterally because the tip of the aspiration cannula is closed. (49) This design allows for collection of marrow perpendicular to and around the channel created by the tip of the device; traditional needles, even ones with side ports, aspirate primarily through an open-ended cannula which leads to excess peripheral blood in the aspirate. (49) Additionally, Marrow Cellution incorporates technology to precisely reposition the retrieval system to a new location in the marrow after each 1 mL of aspiration. (49) The effects of these two features are that multiple small volumes of high quality bone marrow aspiration are collected from a number of distributed sites within the marrow geography while also retaining clinicians’ desire for a single entry point. (49) The design minimizes peripheral blood infiltration and enables a total volume of approximately 10 mL to be collected per puncture. In effect, a single puncture with Marrow Cellution appears to be functionally equivalent to repeated small aspirations (1 mL) from a number of puncture sites using traditional needles, but with substantial savings of time, effort, and reduced patient trauma and risk of infection. (49)

CONCLUSION

Vasculogenesis is a key driver of tissue regeneration

PRP is a growth factor dependent strategy based on the additional growth factors from the platelets and white cells, beyond what would naturally aggregate at the wound site. (23,36,51) These additional growth factors from the PRP causes greater stem cell migration with a resulting enhancement of the proliferation and remodeling phase of the healing cascade. (23,36,51) The heightened inflammatory profile caused by aging on **1.** the micro-environment of the wound bed and **2.** peripheral blood macrophages, combined with **3.** the age dependent diminished vasculogenic capability of marrow, suggests that PRP may be a strategy better suited for younger patients. (41,44,46,47,48)

Marrow-based interventions are a cell dose driven strategy. (45,52,53) Marrow-based treating compositions take advantage of marrow stem cells and marrow complimentary cells to alter the type and function of local cells to create an anti-inflammatory immune driven cascade to transition and amplify the cellular inventory needed to complete the remodeling phase of the healing cascade. (35,45,52,53,54,56) Consistent with oncology models of marrow stem cell transplantation, the only variable that rose to significance in an orthopedic clinical setting using marrow as the biologic, was the number of stem cells in the graft, as measured by cfu-f, not platelets or white blood cells. (5,43,52,53)

A poor marrow aspirate will be comprised of predominately peripheral blood. (1,2,3,4) Nucleated marrow cells and blood cells have the same density. Concentrating the cells from a poor aspirate by density centrifugation results in a high proportion of peripheral blood cells in the biologic.

All cells found at the site of surgical trauma can play a beneficial role in the tissue regeneration process (23,40) The number of platelets and white blood cells in peripheral blood and their ability to home to sites of tissue damage and form a platelet fibrin clot is an efficient process in a majority of patients and does not diminish with age. Using PRP to further amplify the stem cell homing signals of SDF-1a, ATP, and VEGF provided from naturally aggregating platelets and white cells can have a clinical benefit. (23,36,51)

The ability of ones body to mobilize marrow cells to the site of trauma diminishes greatly over time. (28,30) In older patients or healing impaired patients, the vasculogenic signals from PRP is often not sufficient to complete the healing cascade. (29,38) In such cases, marrow rich in CFU-f has been shown to have clinical success. (45,52,53) Central to the coordinated interplay among cells, and the extracellular matrix is the mesenchymal stem cell (MSC), which coordinates the repair response. (19,20,35,52,53,54) CD34 is not a negative marker of MSC (mesenchymal stem cell) and that freshly isolated CD34+ / BM MSC form greater proportions of CFU-f colonies than their CD34– counterparts. (33) The CFU-f test is the appropriate analysis to determine how many cells from the heterogeneous population of cells to include CD34+ cells, are early stage stem cells, to include mesenchymal stem cells. (43,48,52,53) In a clinical setting, CFU-f is the only variable that rose to statistical significance. (43,52,53)

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