BMAC AND PRP ENHANCE MIGRATION OF ENDOGENOUS MSCS

A Thesis

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Master of Science

by

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ABSTRACT

Bone marrow derived mesenchymal stem cells (MSCs) demonstrate promise for musculoskeletal regenerative medicine. An alternate approach to the direct delivery of stem cells is to exploit the concept of *in situ* tissue engineering. Biologics such as bone marrow aspirate concentrate and platelet rich plasma (PRP) enhance recovery from musculoskeletal injuries. These biologics contain growth factors which could act as chemoattractants for stem cells. In this project, we observed the migratory capacity of stem cells in response to biologics using of a microfluidics device. The purpose of this study was to identify the optimal biologic for recruitment of stem cells. Our hypothesis was that PRP would result in the greatest migration of MSC's because of the milieu and concentration of growth factors contained in PRP. We found that all biologics tested resulted in an increase in migration of stem cells compared to the control. This demonstrates that biologics can be used as chemotactic agents to recruit MSCs to a site of injury. This information will reduce the need and therefore the risks and costs associated with direct stem cell delivery.

BIOGRAPHICAL SKETCH

Hannah Holmes graduated from Cornell University College of Agriculture and Life Sciences with a Bachelor's degree in Animal Science in 2011. She began her education at Cornell University, College of Veterinary Medicine in the fall of 2011. In December of 2012, she took a leave of absence from the College of Veterinary Medicine to participate in the NYSTEM training grant and to pursue a Master of Science in Comparative Biological Sciences. After completing the Master of Science she intends to complete her degree in veterinary medicine.

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LIST OF ABBREVIATIONS

ACI – autologous chondrocyte implant

bFGF – bovine fibroblastic growth factor

BMA – bone marrow aspirate

BMAC – bone marrow aspirate concentrate

CXCL12 – C-X-C motif chemokine 12

DMSO – dimethyl sulfoxide

IGF – insulin-like growth factor

FBS – fetal bovine serum

L^{hi}PRP – leukocyte high platelet rich plasma

L^{lo}PRP – leukocyte low platelet rich plasma

MSC – mesenchymal stem cell

NC – neutral control

PBS – phosphate buffered saline

PDGF – platelet derived growth factor

PRP – platelet rich plasma

TGF – transforming growth factor

CHAPTER 1

BACKGROUND

Deficits in Natural Healing

The body's response to injury involves three major steps; inflammation, proliferation and remodeling [44]. After an injury, cellular and molecular signals stimulate the inflammatory response. This results in the recruitment of neutrophils and macrophages. The inflammatory stage of repair is responsible for fighting infection, clearing debris, and release of cytokines that stimulate angiogenesis and recruitment of mesenchymal cells [42]. After the inflammatory stage, proliferation of tissue and remodeling occur resulting in formation of native tissue.

In several tissue types, the body is not able to respond appropriately after an injury. This often results in insufficient rejuvenation of tissue. For example, cartilage does not have its own blood supply and is instead supplied with nutrients by diffusion through synovial fluid [20]. Since cartilage lacks a blood supply, neutrophils and macrophages are not delivered after an injury and inflammation cannot occur. Thus, cartilage has a limited regenerative capacity. Treatments for musculoskeletal injuries should be aimed at supplementing the body's inadequate response to injury, thereby reducing pain, increasing functionality, and minimizing the extent of tissue deterioration.

In situ Tissue Engineering

The concept of *in situ* tissue engineering is to use regenerative therapies to create an environment that exploits the body's natural healing ability. This idea was

popularized by Chang Lee and Jeremy Mao using a rabbit model [24]. In this model, the articular surface of the humeral head was removed and replaced with a control scaffold or one infused with transforming growth factor β3 (TGF-β3). Rabbits treated with a scaffold infused with TGF-β3 regained more weight bearing function and locomotion than controls. The articular surface of TGF-β3 treated rabbits was regenerated by homing of endogenous cells, whereas the controls only had isolated areas of cartilage formation. This study demonstrated the potential of a chemoattractant to promote recruitment of local tissue cells and regional stem cells to the site of injury and promote tissue repair.

This exemplifies the potential of *in situ* tissue engineering for use in musculoskeletal regenerative therapies. Though this model resulted in successful regeneration of the humeral head, use of concentrated TGF- β3 is not a clinical reality. TGF- β3 results in fibrosis, extracellular matrix proliferation and increased chemotaxis of inflammatory leukocytes [13]. The purpose of *in situ* tissue engineering should be to create this same healing environment without subjecting the body to the detrimental effects of cytokines such as concentrated TGF- β3.

Current Therapies in Cartilage Repair

Cartilage injuries have commonly been treated medically using intra-articular drugs. These therapies do not address the causative underlying lesions and do not supplement the natural healing process. Glucocorticoids provide temporary relief for joint pain, but are palliative rather than therapeutic [23]. Hyaluronic acid injections are also used to treat arthritic joints. The goal of this therapy is to restore the viscoelastic properties of the joint to enable normal joint mechanics [23]. Though this

therapy is frequently used, clinical trials indicate that hyaluronic acid therapy has minimal efficacy compared to placebo treatment [31].

Chondral or osteochondral defects do not repair spontaneously or with medical treatment; therefore surgical intervention can be necessary to preserve the joint. The ultimate goal of a surgical procedure, such as microfracture, is to provide the joint with an improved environment for restoration of hyaline cartilage. Microfracture is a procedure that creates a break in the area of cartilage loss through the subchondral bone plate into the bone marrow. Released marrow creates a clot that is enriched in stem cells for cartilage regeneration [27, 38]. Microfracture results in improvement in both pain and function compared to the pre-operative state of the patient [4].

Microfracture tries to emulate *in situ* tissue engineering, but falls short. The clot results in formation of fibrocartilage, which is inferior to native hyaline articular cartilage because it has different biological, structural and mechanical properties [20]. Microfracture causes detrimental changes such as thickening of subchondral bone, and formation of cysts and intra-lesional osteophytes in one third of patients [18]. Additionally, the fibrocartilage does not interdigitate with surrounding tissue, resulting in an area that is weaker and more prone to re-injury [27].

Neither surgical nor non-surgical interventions have been sufficient to restore normal viability to the articular cartilage surface. Both treatment modalities have drawbacks that have yet to be overcome. Ultimately, regenerative therapies should be considered in place of, or to supplement the use of commonly used treatments.

Mesenchymal Stem Cells

Mesenchymal stem cells can be used to enhance musculoskeletal repair after injury. Mesenchymal stem cells (MSCs) proliferate extensively *in vitro* and differentiate into varying tissues of mesenchymal origin, such as chondrocytes, tenocytes, adipocytes, or osteoblasts [7]. Autogenous bone marrow or adipose mesenchymal stem cells are culturally expanded and then reintroduced into the injured patient [15]. This process has realized success both clinically and experimentally.

In one study, seventy-two matched human patients were treated with either autologous chondrocyte implantations (ACI) or bone marrow derived MSCs. Patients treated with MSCs had improved quality of health and sport activity that was comparable with patients treated with ACI [29]. This indicates that MSC treatment is preferential to ACI therapy because it achieves comparable results and does not have potential negative side effects such as cartilage donor site morbidity. Autologous bone marrow MSCs have been culture expanded and applied to cartilage defects of osteoarthritic knees of twelve human patients [43]. Patients who received the cell transplant had improved formation of hyaline cartilage and higher arthroscopy scores than control, untreated patients. Histological observations were made in an *in vivo* study using a rabbit model [45]. Full-thickness cartilage defects of the knee were treated with MSC or autologous chondrocyte implantation. In comparison to defects treated with autologous chondrocyte transplantation, MSC-treated defects showed more complete integration with surrounding cartilage, more normal architecture and presence of a tidemark.

MSCs have also been successfully used to increase healing capacity in tendons. Using horses as a model, culturally expanded mesenchymal stem cells were injected into collagenase-induced tendinopathies. Increased repair and organization of tissue was observed in comparison to scar tissue formation in control limbs [9]. In a case-based twelve month trial, bone marrow mononuclear cells were isolated and administered at the time of surgery in rotator cuff repair of fourteen human patients [17]. All but one of the patients showed increased functional status of the rotator cuff. This study supports the use of MSCs in tendinopathies in humans.

Though the full potential of MSC therapy has yet to be fully understood, some studies indicate that it is not risk-free. MSC derived chondrocytes implanted into muscle express hypertrophy genes that lead to cell death, calcification and vascularization, but articular-derived chondrocytes are stable after implantation into muscle [2]. This indicates that MSC derived chondrocytes are prone to alternations that would not occur with native articular cartilage. Additionally, MSCs can undergo spontaneous differentiation in culture. This is rare and can be controlled *in vitro*, but controlling differentiation *in vivo* might be more difficult [22].

MSC therapy is also time-consuming. It requires extracting bone marrow and then allowing the stem cells to proliferate. This process can take up to six weeks, at which point an acute injury will have had time to deteriorate [15]. One solution to this problem would be the use of allogeneic stem cells. Research has suggested that MSCs are immune-privileged; meaning that they can mediate suppression of T-cell proliferation and can be transferred into mismatched haplotype patients [1]. However, recent reports refute the hypothesis that MSCs are immune-privileged. Autologous

stem cells implanted into infarcted hearts have sustained benefits on heart function, but allogeneic stem cells are unable to preserve heart function long-term [21]. Differentiation of allogeneic cells results in a switch from immune-privileged to immune-reactive, rendering them susceptible to humoral and cell-mediated cytotoxicity.

MSC characteristics also vary based on several factors. The ability of MSCs to differentiate and replicate could be altered depending on where they are isolated from, age of the individual, and growth conditions in tissue culture. Bone marrow derived MSCs from older donors have decreased proliferative capacity and chondrogenic differentiation capacity opposed to MSCs from young donors [32]. In comparison to adipose and skeletal muscle stem cells, bone marrow derived MSCs have superior proliferative capacity, chondrogeneic capacity and osteogenesis [34]. Although synoviocyte derived MSCs have greater proliferative capacity and chondrogenesis than bone marrow derived cells, they are less accessible than bone marrow MSCs. Therefore, bone marrow MSCs are more widely used [22].

An additional confounding factor for MSC therapy is that it lacks FDA approval. To use MSCs regularly as a therapy for musculoskeletal injuries in humans, more time is needed for regulatory approval. Since there are so many variables that can affect stem cells and confound their use, alternate options for treatment of musculoskeletal injuries should be considered.

Biologics

A novel approach to *in situ* tissue engineering is the use of biologics to create a regenerative environment in tissues that lack their own healing capacity. A biologic is

a medicinal agent that is produced by the body rather than artificially synthesized. Biologics contain bioactive growth factors such as TGF-β1 and TGF-β3 and platelet-derived growth factor (PDGF) [13]. As demonstrated by Mao's rabbit model, TGF-β3 is a chemoattractant for cells [24]. Similarly, PDGF acts a chemoattractant for cells of mesenchymal origin [35]**.** Since biologics contain growth factors that can act as chemoattractants, it follows that they could be used to modulate the local tissue environment and promote migration of healthy cells to a site of injury. Platelet rich plasma, bone marrow aspirate and bone marrow aspirate concentrate are biologics that have all been used to this end.

Platelet rich plasma (PRP) is produced through blood aspiration followed by centrifugation. This results in a product that contains all the components of blood, though in different concentrations [6]. According to the Red Cross, PRP must contain at least 200,000 platelets/μl. There are no standards for the other components of PRP which include leukocytes, various proteins and hormones, and erythrocytes. Bone marrow aspirate (BMA) is collected through the use of a bone marrow biopsy needle and no further processing is necessary [36]. Bone marrow aspirate concentrate (BMAC) is prepared through aspiration of bone marrow followed by density gradient centrifugation [14, 38].

Bone Marrow Aspirate

Treatment of meniscal tears with BMA in sheep resulted in improved vascularization and integration of the repair site [11]. In an *in vitro* study using suspensory ligament cells, acellular bone marrow aspirate was subjected to a freeze-

thaw cycle to render the aspirate acellular. This aspirate was more successful than PRP at promoting anabolic responses of the ligament matrix [36].

Bone Marrow Aspirate Concentrate

A prospective study in human patients examined the use of BMAC in a collagen matrix for cartilage defects [16]. The results revealed improved clinical scores and the formation of hyaline-like cartilage. However, this study was performed on 15 people, out of which, only four underwent second-look arthroscopy, and there were no controls. Using an equine model, use of BMAC in conjunction with microfracture resulted in significantly more improvement of full thickness cartilage defects compared with microfracture alone [14].

Platelet Rich Plasma

A controlled study comparing platelet rich plasma to hyaluronic acid injections in patients with chronic knee pain showed that PRP treatment significantly reduced pain in comparison to hyaluronic acid [23]. Closer examination revealed that the difference was only significant in patients younger than 50 that had only moderate degenerative changes. Administration of PRP also resulted in increased improvements in patients with epicondylitis compared with corticosteroid injections [19]. Patients treated with PRP showed continued improvement over a two year period but those treated with corticosteroids returned to baseline. This is in opposition with another study that showed the positive effects of PRP treatment were not long-lived in chronic knee degeneration [12]. PRP use for musculoskeletal injuries has varying degrees of success and longevity. This could ensue from individual differences such as age or severity of disease, though it is also possible that differences in the use of PRP play a

role in the effectiveness of the treatment. The amount of PRP used, whether or not a scaffold was present, if PRP was activated or not and which commercial system was used to make PRP could all play a role in affecting the outcome of studies examining the effects of PRP on tissue regeneration [26].

Though the general protocol for making PRP is consistent, the commercial production systems vary tremendously. Differences include the number of centrifugations, duration of centrifugation and original blood volume. The importance of this is that different systems result in formation of PRPs with different compositions of platelets and leukocytes [26]. More platelets are not necessarily better. The optimal range of platelet concentration for bone regeneration in the rabbit is 3.8*10^5 - 1.8*10^6 platelets/µl [43]. A lower concentration has no effect on regeneration and a higher concentration appears to have an inhibitory effect on regeneration. Commercial PRP systems increase platelet concentrations from 1.5 to 8 times the original concentration in venous blood [26].

The effect of leukocyte concentration on healing is questionable. The presence of neutrophils can be detrimental to tissue, as they release reactive oxygen species during the inflammatory stage of healing [40]. However, the presence of leukocytes during repair helps to remove debris, protect from infection, promote angiogenesis, and activate growth factors [3]. It is unclear if leukocyte high PRP would be beneficial or detrimental to the tissue. In a comparison of leukocyte low and leukocyte high PRP, an increased inflammatory response was seen five days after application in rabbits that received leukocyte high PRP [10]. However, no differences were noted on day fourteen. The lack of standards for use of PRP and lack of

understanding of the healing process makes it difficult to fully assess the effectiveness of this treatment. This highlights the importance of monitoring the composition of PRP to help determine its functionality.

Thesis

In situ tissue engineering strives to create an optimal environment for regeneration of tissues. This concept has been pursued through use of surgical procedures such as microfracture, cell delivery, and delivery of autologous biologic substances. Though stem cell therapy is very promising for *in situ* tissue engineering, it is costly, time-consuming and lacks FDA approval. This makes it necessary to seek other options. Exploiting biologics a source for regenerative medicine is an alternative to direct stem cell delivery.

Biologics contain growth factors that have the ability to modulate the environment and induce migration of cells. While studying the individual effects of growth factors and other biological components such as leukocytes is informative, it does not tell us how the body will respond to the milieu of factors within a biologic. It is still unclear if one of the studied biologics provides more benefit than others.

In the experiments of this thesis, a microfluidics device was used to measure the direct competition of chemoattraction between biologics. The goal for this study was to determine which biologic was capable of causing the greatest migration of stem cells and would therefore be the optimal biologic for use in regenerative medicine. The hypothesis was that all the biologics would attract stem cells but leukocyte low platelet rich plasma (L^{lo}PRP) would be the optimal biologic because of the diminished concentration of inflammatory cytokines compared to the other biologics.

CHAPTER 2

MESENCHYMAL STEM CELL MIGRATION

Methods

All experiments were completed at Cornell University, College of Veterinary Medicine. Animal use was approved by the Institutional Animal Care and Use Committee.

Mesenchymal Stem Cell Isolation

Bone marrow aspirate was collected from eight mature horses aged 2-19 years. Horses were either euthanized or sedated, and the sternum was clipped and scrubbed. Lidocaine anesthesia was administered to horses that were sedated. Bone marrow was drawn using a Jamshidi needle and a syringe containing 10mls of 5000 units/ml acid citrate dextrose. The aspirate was diluted 1:1 with sterile phosphate buffered saline (PBS), and layered on top of Ficoll (GE Healthcare, Piscataway, NJ) in a 50ml conical tube at a ratio of 3:2, respectively. Conical tubes were centrifuged at 400g and 4°C for 20 minutes. The mononuclear cell fraction was retained and rinsed with PBS, and centrifuged again at 500g, 4°C for 5 minutes. This was repeated and the final cell pellet was resuspended in MSC media (Dulbucco's Modified Essential Medium, 10% FBS, penicillin and streptomycin, hepes, L-glutamine, bFGF). Cells were expanded in tissue culture plates at 37°C in a humid, 5% CO_2/air incubator. Cells were washed and medium was changed every third day until they reached 80-90% confluence. Cells were then lifted with Accumax (EMD Millipore Corporation, Billerica, MA) and cryopreserved in complete media with 10% DMSO until needed.

Bone marrow aspirate/ Bone marrow aspirate concentrate (BMAC)

Bone marrow was aspirated as described above. BMA was additionally processed using SmartPReP 2 technology (Harvest Technology Corp, Plymouth, MA) to generate BMAC. Samples were aliquoted into 1ml cryovials and frozen at -80°C. Prior to freezing, complete blood counts for both BMA and BMAC were performed by Cornell University Hospital for Animals.

Platelet rich plasma

Horses were sedated and the jugular vein was clipped and scrubbed. 50mls of blood were drawn using an 18 gauge 1.5 inch needle into a syringe with 10mls of 5000 units/ml acid citrate dextrose. Leukocyte low PRP (L^{lo}PRP) was generated from blood using the Double Syringe Autologous Conditioned Plasma System (Arthrex, Naples, FL). Leukocyte high PRP (L^{hi}PRP) was generated from blood using the GPS III Platelet Separation system (Biomet, Warsaw, IN). Samples were aliquoted into 1ml cryovials and frozen at -80°C. Samples were retained for complete blood counts.

Controls

Human recombinant platelet derived growth factor AB (PDGF-AB) (Life Technologies, Grand Island, NY) was used as the positive control to discriminate between poorly migrating cells and ineffective biologics [35]. PDGF-AB was reconstituted at a concentration of 5000ng/ml according to manufacturer instructions and stored in aliquots at -4°C. MSC media with 10% FBS was used as the neutral control (NC). Negative controls such as serum free media resulted in MSC death during the experiments.

Microfluidics preparation

MSCs were thawed and incubated in medium at 37°C in a humid, 5% CO_2/air incubator the day prior to use. To allow CO_2 equilibration to prevent air bubble formation, the μ-slide chemotaxis device (Ibidi LLC, Verona, WI), 10% FBS media, and 1% FBS media were also incubated a day prior to use.

The following day, MSCs were washed with PBS, lifted using Accumax and centrifuged at 500g for 5 minutes. The cell pellet was resuspended at an average concentration of 166 cells/cm^3 in the 10% FBS media that was equilibrated on the previous day. Caps were placed on ports C, D, E and F (Figure 1) and 6ul of cell suspension was dropped onto port B. Gentle aspiration was applied to port A to pull cells into the observation area. Caps were removed and the device was covered with a lid. The device was placed in a petri dish with a wet paper towel and the dish was wrapped in parafilm and incubated for 2-3 hours to allow the MSCs to adhere. Biologics were thawed and centrifuged at 12,000*g* for 20 minutes to remove cell debris. The biologic supernatant and PDGF were incubated with the device to allow - CO_2 content to equilibrate.

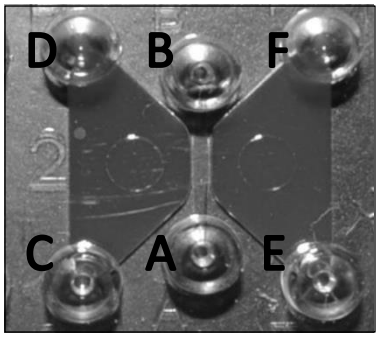


Figure 1: The u-slide chemotaxis device. The area between ports A and B is the observation area where the MSCs are seeded. The areas indicated in dark gray are the reservoir wells where the putative chemoattractants are placed.

After incubation, the reservoir wells of each device were filled with 60µl of 1% FBS media. Caps were placed on ports A, B, E and F and 10ul of PDGF or biologic was placed in port D and 5µl of 1% FBS media was aspirated from port C to draw the biologic into the reservoir well. Caps were then moved to cover ports A, B, C and D and 10% FBS media was placed in port F and 5µl of 1% FBS media was aspirated from port E so that a direct competition of chemoattraction could be measured between a biologic and the NC.

The device was then imaged using live cell imaging on an inverted bright field microscope (Thorlabs, Newton, NJ) with a 4X objective (Olympus America Inc, Center Valley, PA) every five minutes for a period of twenty four hours. Time-lapse images were analyzed by tracking the migratory patterns of individual cells by use of a custom code written in Image J (NIH, Bethesda, MD). The program tracked the location of the computer cursor as it hovered over the cells for the duration of the time-lapse videos. Cell (x, y) coordinates were recorded for every frame. Cells were tracked up to the point of division, death, migration out of the center well, or 24 hours of migration. A custom code written in Matlab (MathWorks Inc, Natick, MA) was used to compile the (x, y) coordinates and determine displacement of each cell from its location at time 0 to the final location at time 24 hours. The program averaged displacement of individual cells towards the biologic or NC to give an overall displacement for each experiment. The number of cells migrating in each direction was also recorded. Cell flux (%migrated * µm/24hrs) was calculated to represent the rate of movement of MSCs within the device.

The percentage of cells migrated was used as a measure of the ability of biologics to act as chemotaxis agents for MSCs. Chemotaxis refers to the ability to cause a directed migration of cells [5]. Cells influenced by a chemotactic factor will move along a chemical gradient instead of randomly (Figure 2). Displacement of MSCs was used as a measure of the ability of a biologic to stimulate chemokinesis. Chemokinesis refers to the ability to cause an increase in rate of migration [5]. Cells influenced by a chemokinetic factor will move further in the same amount of time as cells that were not influenced by the chemokinetic factor, but not in any specific direction.

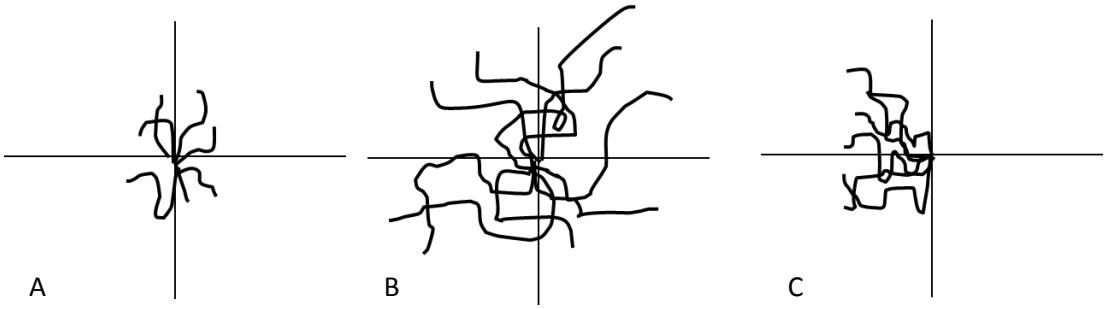


Figure 2: Chemotaxis versus chemokinesis. Black lines indicate cell trajectories. A) Cells are not influenced by any factors, they move randomly. B) Cells are influenced by a chemokinetic factor. They have increased the rate of migration, but do not move in a particular direction. C) Cells are influenced by a chemotactic factor. They have not increased the rate of migration, but directed migration is occurring either up or down a chemical gradient.

Displacement and percentage of cells migrating toward a putative chemoattractant or NC are useful measures since they relate directly to chemokinesis and chemotaxis, respectively. However, these variables fail to give a comprehensive picture of cell migration, therefore cell flux was calculated. Cell flux is a measurement of the rate of flow of the cells. It indicates overall migration of a cell population toward either a putative chemoattractant or a control substance.

Statistics

Outcome variables included the number of cells migrated, cell displacement and cell flux. The number of cells that migrated was normalized by calculating the percentage migrated in order to control for differences in seeding density of the MSCs between experiments. The NCs were compared using a Kruskal-Wallis one-way ANOVA by ranks using JMP Pro 11 (SAS Institute Inc, Cary, NC). Independent variables were NC, PDGF, BMA, BMAC, L^{lo}PRP and L^{hi}PRP. Independent variables were analyzed by using a Kruskal-Wallis one-way ANOVA by ranks. A p-value of < 0.05 was considered significant. Post hoc comparisons between independent variables were made using the Wilcoxon rank-sum test with a downward adjustment in the p-value to compensate for the increased chance of type-I error with multiple comparisons. A p-value of < 0.01 was considered significant.

Results

Visual interpretation of time-lapse videos indicated that cells tended to migrate toward biologics in preference to NC (Figure 3).

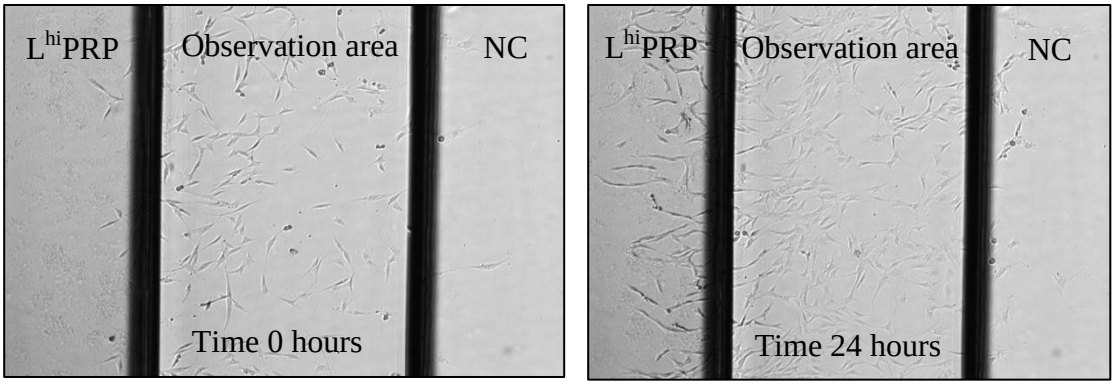


Figure 3: Cell migration images. Image on the left is the μ- slide chemotaxis device immediately after preparation. Image on the left is the μ-slide chemotaxis device after completion of the experiment. The observation area is the area between the thick black lines. The putative chemoattractant is on the left side of the observation area. Putative chemoattractant in this experiment was leukocyte high platelet rich plasma ($L^{hi}PRP$). The NC is on the right side of the observation area.

Every experimental set up on the μ-slide chemotaxis device allowed for direct competition of a putative chemoattractant against the NC. Since each set up contained a NC, at the end of the trial five NC groups existed. NC outcome variables from each experiment were compared (Table 1). NCs were not significantly different from each other, so NC data was averaged for further statistical analyses.

Table 1 – Percentage of cells migrated, displacement and cell flux toward NC. Putative chemoattractant in each experiment is indicated; PDGF – platelet derived growth factor, BMA – bone marrow aspirate, BMAC – bone marrow aspirate concentrate, $L^{lo}PRP$ – leukocyte low platelet rich plasma, $L^{hi}PRP$ – leukocyte high platelet rich plasma. Significance was determined with a Kruskal-Wallis one way ANOVA. A p-value < 0.05 was considered significant.

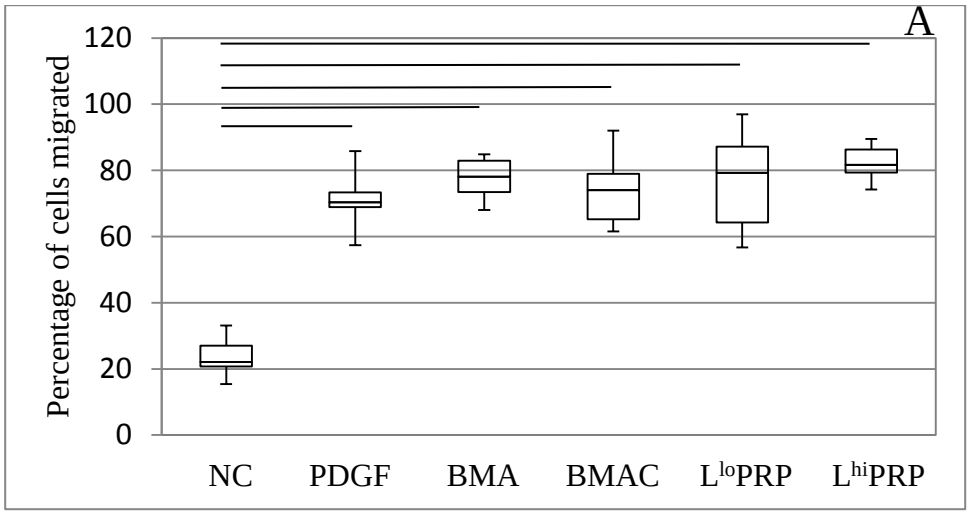
	PDGF	BMA	BMAC	$L^{lo}PRP$	$L^{hi}PRP$	Average	p-value
Percent migrated	28.61	22.63	26.13	23.40	17.84	23.72	0.24
Displacement (μm)	44.83	40.15	31.47	24.59	27.49	33.71	0.15
Cell flux	55.01	36.84	36.99	28.49	21.34	35.73	0.10

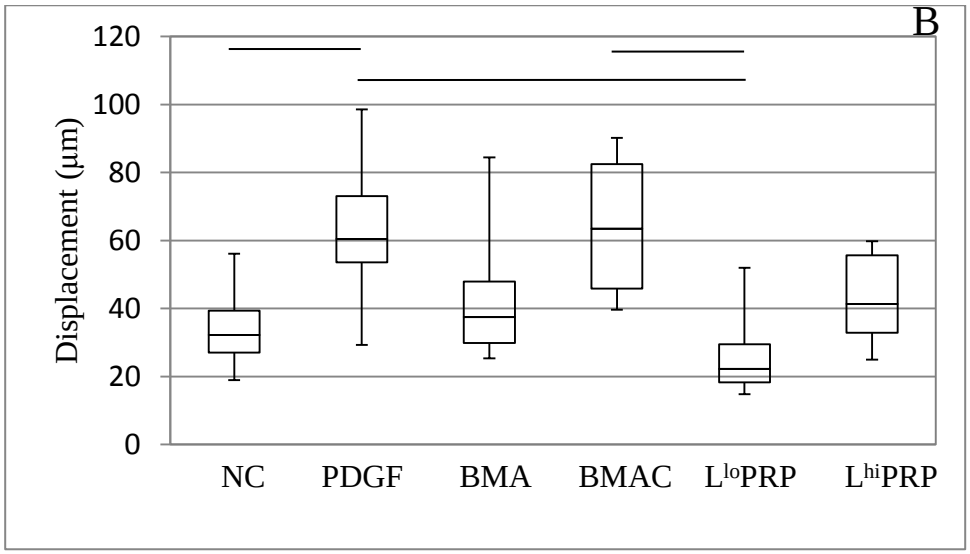
All biologics attracted a greater percentage of cells compared to the NC (Figure 4A; $p < 0.001$). PDGF also attracted a greater percentage of cells compared to the NC ($p < 0.001$). None of the biologics were different from each other. Biologics and PDGF recruited three times more cells compared to the NC group.

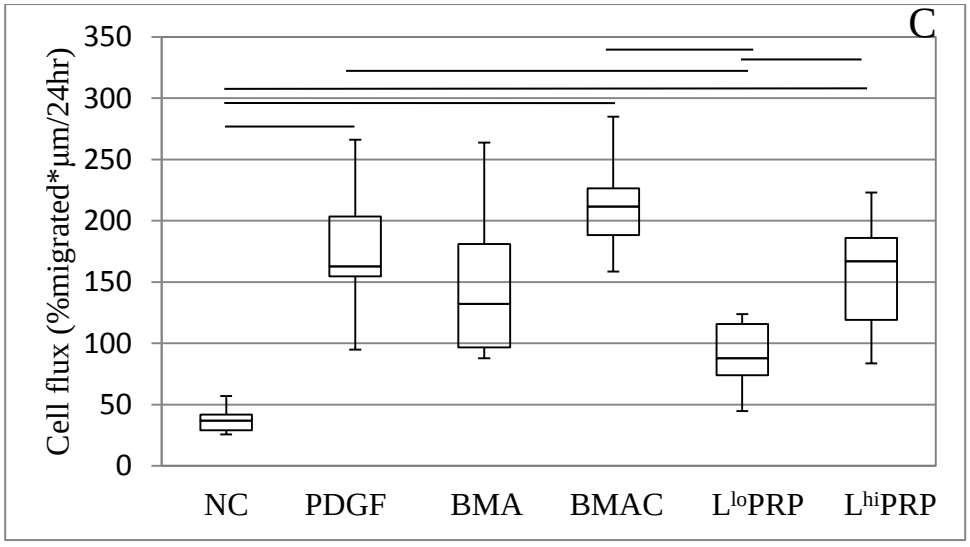
BMAC stimulated significantly more displacement than L^{lo}PRP (Figure 4B, $p=0.005$). Similarly, PDGF stimulated significantly more displacement than L^{lo}PRP, ($p = 0.002$). None of the other biologics were significantly different from each other. BMAC displacement was not significantly different from BMA or L^{hi}PRP. None of the biologics resulted in a significantly different displacement from the NC. Displacement of cells toward PDGF was significantly greater than toward the NC ($p = 0.01$).

Cell flux was greater toward BMAC than all of the other biologics, but only significant with respect to L^{lo}PRP (Figure 4C, $p = 0.002$). Cell flux toward L^{hi}PRP was also significantly greater than toward L^{lo}PRP ($p = 0.01$). L^{hi}PRP resulted in 1.8 times more cell flux compared to L^{lo}PRP. L^{hi}PRP resulted in greater cell flux than the NC ($p = 0.001$). BMAC also resulted in greater cell flux than the NC ($p = 0.0009$). L^{lo}PRP resulted in significantly less cell flux than the NC ($p = 0.01$). Cell flux toward PDGF was significantly greater than the cell flux toward the NC ($p = 0.0009$). PDGF resulted in more cell flux than L^{lo}PRP ($p = 0.002$).

Figure 4: Percentage of cells that migrated (A). Displacement of cells (B). Cell flux (C). Data are represented as median with maximum and minimum values, n=8. NC = neutral control, PDGF = platelet derived growth factor, BMA = bone marrow aspirate, BMAC = bone marrow aspirate concentrate, L^{lo}PRP = leukocyte low platelet rich plasma, L^{hi}PRP = leukocyte high platelet rich plasma. Bars indicate significant differences between groups. Significance was determined by a Kruskal-Wallis followed by Wilcoxon multiple comparison post-hoc test. A p-value < 0.01 was considered significant.


A
Percentage of cells migrated
120
100
80
60
40
20
0
NC
PDGF
BMA
BMAC
LloPRP
LhiPRP


B
Displacement (µm)
120
100
80
60
40
20
0
NC
PDGF
BMA
BMAC
LloPRP
LhiPRP


C
Cell flux (%migrated*µm/24hr)
350
300
250
200
150
100
50
0
NC
PDGF
BMA
BMAC
LloPRP
LhiPRP

CHAPTER 3

DISCUSSION AND CONCLUSION

Discussion

The results from this study did not support the hypothesis that L^{lo}PRP would cause the most migration of mesenchymal stem cells in comparison to other biologics. Each biologic tested contains a unique milieu of growth factors. We proposed that L^{lo}PRP would cause the most migration because of its milieu of bioactive growth factors.

Contrary to our hypothesis, the results showed that BMAC and L^{hi}PRP resulted in more migration than L^{lo}PRP. Though these two biologics were not significantly different from each other, BMAC resulted in a greater overall flux than L^{hi}PRP. From this experiment, it appears that BMAC results in greater chemokinesis, while L^{hi}PRP resulted in slightly more chemotaxis. Since flux incorporates both of these values, there was no significant difference between BMAC and L^{hi}PRP. Though L^{lo}PRP did not result in more cell flux or displacement than other biologics, it has the ability to cause chemotaxis of MSCs, as seen by the percentage of migrated cells.

Both chemotaxis and chemokinesis factors are important in determining the optimal biologic for enhanced stem cell recruitment. The optimal biologic would be able to cause directed migration of cells towards the wound and increase the speed at which cells could reach the wound. Growth factors range in their ability to stimulate chemotaxis and chemokinesis. For instance, C-X-C motif chemokine 12 (CXCL12) can promote chemotaxis but not chemokinesis in human blood cord stem cells [30]. Insulin-like growth factor (IGF) I and II can promote chemotaxis and chemokinesis of

malignant mesothelioma cells [25]. PDGF is a known chemotactic growth factor for cells of mesenchymal origin [35]. Growth factor interactions can also alter the response of a cell. For instance, PDGF results in an increased expression of IGF-I receptors [33].

All biologics used in this study would contain comparable levels of IGF-I, because of the basal level of IGF-I in blood and bone marrow [39]. The differences in concentrations of PDGF and other growth factors in the biologics was likely the cause of the varying responses seen by MSCs exposed to biologics. The percentage of cells migrated toward L^{lo}PRP was just as great as any of the other biologics. However, it induced the least amount of cell displacement. Another way that this can be stated is that the chemotactic ability of L^{lo}PRP was equivalent to the other biologics studied, but it had an inferior ability to cause chemokinesis.

Cell flux is a particularly useful measure of migration because it represents both chemotactic and chemokinetic capability of biologics. Since L^{lo}PRP stimulated the least cell flux of any of the biologics it can be presumed that L^{lo}PRP is not the optimal biologic for *in vivo* use. BMAC and L^{hi}PRP stimulated more cell flux than L^{lo}PRP. Therefore it is suggested to further explore their potential use *in vivo* and to determine if either have greater potential for musculoskeletal regeneration.

Conclusion

One aspect of cell migration that was not measured in this study was the effect of the biologics on cell division. Growth factors not only cause cell movement, but also have mitogenic effects on cells. The mitogenic capability of a biologic could have an effect on cell flux. When a cell divides, it produces two daughter cells. If

both cells migrated toward the chemoattractant, then the percentage of cells migrated would change and cell flux would increase for that direction. Unfortunately, the program that was used to manually track the cells in these studies was limited in this regard. It was not possible to start tracking part-way through the time-lapse video. This would have been necessary if daughter cells were tracked in order to prevent tracking a parent cell twice. An additional confounding factor for tracking daughter cells was the rate of division. In many instances, the daughter cells also divided. One way to overcome this would be to inhibit division of MSCs through irradiation and determine if the flux changes.

In this experiment cells could only be tracked for twenty-four hours. After twenty-four hours, MSCs became too confluent to reliably track individual cell movements. If cells were plated at a lower concentration to allow for longer observation, they did not migrate until they became more confluent. This suggests that cell-cell interactions are necessary to promote migration. This is consistent with studies that showed that cells at lower seeding densities were less capable of differentiation, indicating that cell-cell interactions are important for MSC function [28].

Interesting phenotypes were observed in early experiments that were allowed to run for longer periods of time. Cells exposed to BMA or BMAC tended to roll up into long sheets of cells and then conglomerate into spheroids. Some studies have deliberately induced spheroid formation [8]. This can occur when cells become confluent or are nutrient deprived. Formation of spheroids allows stem cells to maintain viability in serum-free or hypoxic conditions. This is consistent with the

present study, as the cells that reached confluence were imaged for three or more days. Cells exposed to PRP tended to exhibit a streaming behavior that appeared to be similar to behavior shown by fungal hyphae. The reason for this is unknown.

The next step in this experiment should be to determine if the findings of this study are consistent *in vivo.* Specifically, it should be ascertained if either BMAC or L^{hi}PRP is superior *in vivo*. One difficulty in finding this out is that there are minimal standards for processing consistent biologics. This is something that will need to be specified if biologics are used regularly in musculoskeletal injuries. Additionally, a mechanism for tracking endogenous MSC migration *in vivo* has yet to be determined.

Another direction that this project could take would be to study the response of other cell types to the same biologics that the MSCs were exposed to. It is worthwhile to find out if chemotaxis and chemokinesis of cell types that compose musculoskeletal tissues could be promoted by biologics. Results from an initial trial on chondrocytes suggest that BMA is capable of stimulating migration; the other biologics only had a minimal effect on chondrocyte migration.

In conclusion, we found that L^{lo}PRP, L^{hi}PRP, BMA and BMAC all result in chemotaxis and/or chemokinesis of MSCs. Specifically, BMAC and L^{hi}PRP result in significantly more cell migration than L^{lo}PRP. Biologics can be used as chemotactic agents to recruit MSCs to a site of injury. This information has the potential to reduce the need and therefore the risks and costs associated with direct stem cell delivery.

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