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Chapter 18

Platelet Lysate to Promote Angiogenic Cell Therapies

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Additional information is available at the end of the chapter

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Abstract

Cellular therapies for patients with ischemic muscle have been limited by poor cell retention and survivability. Platelets are a robust source of growth factors and structural proteins, and extracts from this peripheral blood component may be manipulated to improve both cell retention and survivability in percutaneous delivery methods. Human platelet lysate is generated from pooled human platelets and contains a growth factor milieu that promotes robust human mesenchymal stem cell (MSC) proliferation without risk of xenogenic contamination. As such, platelet lysate is a practical alternative to animal serum for MSC culture and, with minor adjustments to the production process, can also be used as a scaffold for cell delivery. Human platelet lysate is a promising substrate that can provide nutritive delivery both in vitro and during cell implantation, potentially improving retention and survivability of MSCs that may improve angiogenic function for cell therapy in *treatment* of ischemic tissues.

Keywords: critical limb ischemia, mesenchymal stromal cells, platelet lysate, angiogenic cell therapy

1. Introduction

The legs are a site of ischemic muscle that are particularly attractive to application of angiogenic therapies. Decreased perfusion of the legs is known as peripheral arterial disease (PAD), and PAD is pandemic with extreme costs to society. In its most severe form, it is called critical limb ischemia (CLI). CLI patients do not have adequate perfusion to their resting nutritional needs resulting in rest pain or even tissue loss. While only 1–2% of PAD patients will develop CLI, CLI affects 1 million individuals annually [1]. Surgical revascularization of CLI patients can prevent major amputation. Current treatment for limb salvage includes endovascular therapy (angioplasty or stent placement) and surgical bypass. However, despite improvements in medical and surgical therapies, successful management of CLI is difficult with ~0.5

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of patients with CLI dying or undergoing major amputation within 1 year [2]. Since many patients with CLI either fail revascularization therapy or are not candidates for these procedures, amputation is commonly performed. In addition to the impaired quality of life that results from lower limb amputation, historically mortality rates for this procedure have been reported to approach 40% [3]. New and innovative therapies are imperative to improve mortality and quality of life in patients with CLI and provide an alternative to limb amputation.

Cell therapy is a promising new treatment strategy for patient with CLI. The concept of cell therapy for treatment of critical limb ischemia coincided with the discovery of a circulating endothelial progenitor cell (EPC) in 1997, which described a population of circulating peripheral blood mononuclear cells that presumably arise from the bone marrow yet is also capable of displaying characteristics of a mature endothelium [4]. This discovery challenged the prevailing paradigm of postnatal neovascularization [5] in which blood vessel growth was the result of angiogenesis (the formation of new blood vessels from the existing endothelium through sprouting or intussusception) and arteriogenesis (the expansion of preexisting collateral vessels due to an increase in blood flow in response to changes in shear stress [6]). The work by Asahara et al. was significant for two reasons. First, it introduced the concept of postnatal vasculogenesis, suggesting that bone marrow-derived cells could contribute to the formation of new blood vessels in the adult similar to the process of vasculogenesis seen in embryogenesis, where primitive hemangioblasts give rise to de novo blood vessels in the developing fetus. Second, it pioneered the idea of cell therapy, through experiments in which the ischemic limbs of nude mice were injected with a population of human peripheral blood mononuclear cells enriched for CD34+ and Flk-1 (VEGFR-2), and found that the transplanted cells incorporated into the blood vessel endothelium at sites of neovascularization.

The notion that bone marrow-derived cells incorporate directly into native endothelium is controversial. Despite several initial reports suggesting differentiation of bone marrow cells into endothelial cells [7–9], several subsequent studies demonstrated that endogenous and transplanted bone marrow cells did not directly incorporate into the endothelium but instead support neovascularization through a paracrine mechanism [10–13]. Numerous stem and progenitor cell populations derived from the bone marrow, adipose tissue, and embryonic stem cells and induced pluripotent stem cells have been shown to enhance blood vessel growth in animal models of hind limb ischemia, suggesting a role for cell therapy in therapeutic neovascularization [4, 14–16]. Mesenchymal stromal cells (MSCs) are a potential cell source that can be easily isolated, rapidly expanded ex vivo, and have potent proangiogenic qualities [17] mediated through paracrine stimulation of endogenous tissues [18]. The use of MSCs for cell therapy is advantageous over other cell types because they can be derived from an autologous source, thereby avoiding the immunogenicity and loss of tolerance observed when allogeneic MSCs are exposed to an inflammatory environment [19, 20].

Despite numerous animal studies and small clinical pilot trials demonstrating the ability of MSCs to promote angiogenesis [17, 21, 22], the biology and clinical benefit of this approach has not yet been demonstrated in patients with CLI. Two major limitations have prevented the translation of MSC therapy from the laboratory to the clinical arena. First, the use of an autologous cell source in this demographic is complicated by the fact that progenitor cell populations

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are depleted or functionally impaired in patients with coronary artery disease [23], stroke [24], and diabetes mellitus [25–27] and who are smokers [28]; all risk factors or comorbidities are highly prevalent in patients with CLI. A second major limitation of stem cell therapy thus far has been maintaining a clinically significant cell number in target tissues, as direct intramuscular injection or intra-arterial infusion alone typically does not enable adequate cell delivery [29–31]. In order to fully realize the potential of cell therapy, these limitations must be addressed before successful clinical deployment of MSC therapy can be achieved.

As cell therapy is translated from preclinical animal studies to human clinical trials, strict cell culture techniques must be employed to ensure human safety. The vast majority of clinical trials to date have utilized fetal bovine serum (FBS) for ex vivo expansion and growth of MSCs. However, FBS has considerable xenogenic potential and transplanted autologous MSCs can be rapidly rejected after culture in FBS [32]. Therefore, new human-derived alternatives have been evaluated as possible cell culture supplements to ensure that growth and expansion of MSCs are complain with current good manufacturing processes (GMPs).

Platelets are small enucleated cell fragments derived from megakaryocytes in the bone marrow and play a critical role in initiating hemostasis by binding and adhering to extracellular matrix components after endothelial injury, which in turns leads to platelet activation. Activated platelets then subsequently aggregate and become crosslinked with fibrin through activation of the coagulation cascade, thereby generating a platelet plug capable of blocking the flow of the blood. Platelets normally represent 0.1–0.25% of the blood and typically circulate for 5–9 days. Platelets are also an abundant source of growth factors, accounting for the majority of growth factors found in serum [33, 34]. As a result, various platelet extracts have been used for regenerative medicine applications. Platelet lysate (PL) is one such supplement that has been utilized as a supplement for culture of MSCs and has been shown to be superior to FBS [35, 36]. We and several collaborators have performed extensive characterization of platelet lysate as a cell culture supplement for expansion of human MSCs suitable for cell therapy trials. More recently, we have modified the PL production process such that PL may be used to form a 3D scaffold for MSC growth and invasion. In this chapter, we elaborate on our experience with PL as it pertains to culture of MSCs as well as describe a novel scaffold for cell delivery derived from PL extract.

2. Platelet lysate

2.1. Soluble platelet lysate for expansion of human MSCs

2.1.1. Generation of fibrinogen-depleted platelet lysate

The platelet lysate supplement for cell culture used by our group and our collaborators is manufactured through the Emory Personal Immunotherapy Center (EPIC). The specific production strategy was initially optimized and described by Copland et al. The current protocol employed by EPIC emphasizes good manufacturing process (GMP) technique and results in a fibrinogen-depleted form of platelet lysate (dPL) that is a soluble media supplement for ex vivo expansion of human MSCs. EPIC has provided numerous research groups with dPL and has

received FDA approved for use of dPL for human cell therapy trials using MSCs for treatment of Crohn's disease (NCT01659762) and graft vs. host disease following allogenic bone marrow transplantation (NCT02359929). The production process utilizes outdated plateletpheresis products obtained from the Emory University Hospital blood bank. For each lot of dPL, five plateletpheresis products are exposed to sequential freeze-thaw cycles to ensure adequate membrane fracturing. The platelet products are first stored at -20°C, then thawed at 4°C, and aliquoted into smaller volumes of 20–25 mL. The aliquots are then refrozen at -80°C and filtered through a 40 μm filter. The filtered PL is then centrifuged at 4000×g for 20 min at room temperature and refiltered in 40 μm filters. The PL is then mixed with $CaCl_2$ and heparin solution and stored at 4°C overnight to allow formation of a fibrin clot. Following this, the dPL samples are centrifuged again at 4000×g at room temperature, filtered at 0.2 μm, and then stored at -80°C [37]. These final aliquots are then thawed and immediately ready for use in cell culture.

The standard plateletpheresis process employed by the American Red Cross involves the addition of 10% v/v acid citrate dextrose (ACD) to platelet products, which serves as a calcium chelator to disrupt the coagulation cascade and prevent clot formation. In order to generate fibrin clot for depletion of the fibrinogen from the platelet lysate supplement, 20 mM $CaCl_2$ is added to the platelet lysate, which enables the protease-driven conversion of fibrinogen to fibrin, thus leading to spontaneous clot formation. The addition of heparin at a concentration of 2 U/mL also stabilizes clot formation and increases yield of the soluble fraction of the platelet lysate. Under these conditions, over 85% of the platelet lysate is recovered with a final fibrinogen concentration of less than 5 μg/mL (compared to a fibrinogen concentration of ~120 μg/mL in the unfractionated platelet lysate) [37].

The growth factor content of dPL has been well characterized and contains numerous abundant mitogens for cell culture. Specifically, dPL contains ample amounts of PDG-BB, TFG-β1, VEGF, EGF, and BDNF. These growth factors exist in levels significantly higher than standard serum and are preserved in both unfractionated and fibrinogen-depleted PL preparations. Furthermore, dPL is remarkably stable, with room temperature preparations of PL capable of maintaining consistent levels of PDGF-BB and EGF for up to 3 weeks [37]. The stability of dPL and low interlot variability are extremely desirable qualities in a cell culture supplement and therefore are well suited for ex vivo growth and expansion of human therapeutic cell lines.

2.1.2. Immunomodulatory effect of PL fibrinogen depletion on MSCs

The use of dPL as a cell culture supplement is advantageous because it not only decreases the risk of xenogenic contamination but dPL also induces a robust proliferative response in human MSCs. When compared to cells grown in FBS, low passage MSCs cultured in equivalent concentrations of dPL had significantly decreased doubling times and decreased cell volumes. Despite the increase in proliferation, cells grown in dPL maintained expression of typical MSC markers including CD44, CD90, CD73, HLA-I, and CD105 and lacked expression of CD45 and CD34 [37].

In addition to impacting proliferative capacity of MSCs, platelet lysate composition may also affect the immunomodulatory properties of MSCs. MSCs can have a profound immunosuppressive effect on various inflammatory processes. Exposure of MSCs to fibrinogen

upregulates integrin and non-integrin fibrinogen-binding complexes. Furthermore, the analysis of the secretome of MSCs exposed to fibrinogen in vitro demonstrated a significant increase in pro-inflammatory cytokines IL-8, MCP-1, and IL-6 [37]. The impact of fibrinogen content in various PL formulations on T-cell activation and proliferation has also been examined. Recently, upregulation of indoleamine 2,3-dioxygenase (IDO) in MSCs has been shown to correlate with suppression of T-cell proliferation [38]. MSCs cultured in unfractionated PL had decreased IDO expression compared to cells cultured in FBS; however, the IDO response was restored when dPL was used as a culture supplement. This functional effect of MSCs on T-cell proliferation was also examined through a coculture assay where MSCs were cocultured with PBMCs exposed to CD3/CD8. T-cell proliferation was noted to be higher in the unfractionated PL compared to dPL and standard FBS. These data suggest that an increased fibrinogen content promotes a pro-inflammatory phenotype of MSCs in vitro, and conversely fibrinogen depletion of PL preserves the immunosuppressive properties of MSCs.

2.1.3. Ex vivo enhancement of MSCs with dPL

The ex vivo expansion for MSCs is essential for cell therapy, as a large number of cells are needed to achieve a desired therapeutic effect. Bone marrow aspirates and peripheral blood cultures provide a relatively low yield of MSCs, and this necessitates prolonged in vitro expansion in order to obtain an adequate number of cells for autologous cell therapy. An unfortunate consequence of this process is that prolonged culture leads to MSC senescence, loss of plasticity, and loss of self-renewal capacity. Typically, human MSCs grown in media supplemented with FBS begin to show signs of senescence after 10 passages, so either increasing proliferation at early passages or delaying the effect of senescence would improve the yield of MSCs for treatment of CLI.

The enriched milieu of growth factors contained within dPL can potentially overcome the senescence associated with long-term MSC culture. Griffith et al. demonstrated that proliferation in senescent MSCs (passage 13 or greater) could be reversed with culture in dPL. MSCs cultured in 5% dPL showed reduction in cell size and a decrease in doubling time while maintaining MSC markers and reducing β-gal production compared to control MSCs cultured in FBS. These effects were short-lived, however, and by passage 16, MSCs failed to proliferate adequately regardless of culture conditions. The transient increase in proliferative response was also evident in *late* passage MSCs (passages 10–11). Culturing in dPL led to a marked decrease in doubling time, but by passage 16, MSCs showed severe signs of senescence in both dPL and FBS treatment groups. Consistent with these results, no changes in telomerase activity were noted between groups [39]. This study clearly established that poor-quality MSCs could be rescued and restored to a robust proliferative state using dPL, even if the effect was only temporary.

2.1.4. Expansion of MSCs from patients with CLI

Expanding on the research of our collaborators, we have worked to exploit the benefits of dPL for cell therapy in patients with CLI. By generating patient-specific MSC cell lines

from individuals with CLI, we were able to test the feasibility of expanding MSCs with dPL for future cell therapy trials. In our initial series, we isolated MSCs from bone marrow of amputated limbs of four patients with critical limb ischemia and four patients with critical limb ischemia and concomitant diabetes mellitus. This cohort of patients had no further surgical or endovascular revascularization options and therefore is representative of the population of patients who would benefit from new cell therapy strategies. Additionally, we obtained MSCs from four healthy donors through EPIC. All MSCs were initially plated in media containing FBS; however, at passage 4 they were transitioned to either media containing FBS or dPL. As demonstrated in previous studies, MSCs grown in dPL maintained a similar phenotype based on cell surface markers compared to cells grown in FBS (**Figure 1**), with no notable differences detected across all patient groups. Additionally, differentiation capacity was preserved in MSCs from all patient groups and culture conditions, with all cells differentiating toward osteogenic or adipogenic lineage under appropriate assay conditions [40].

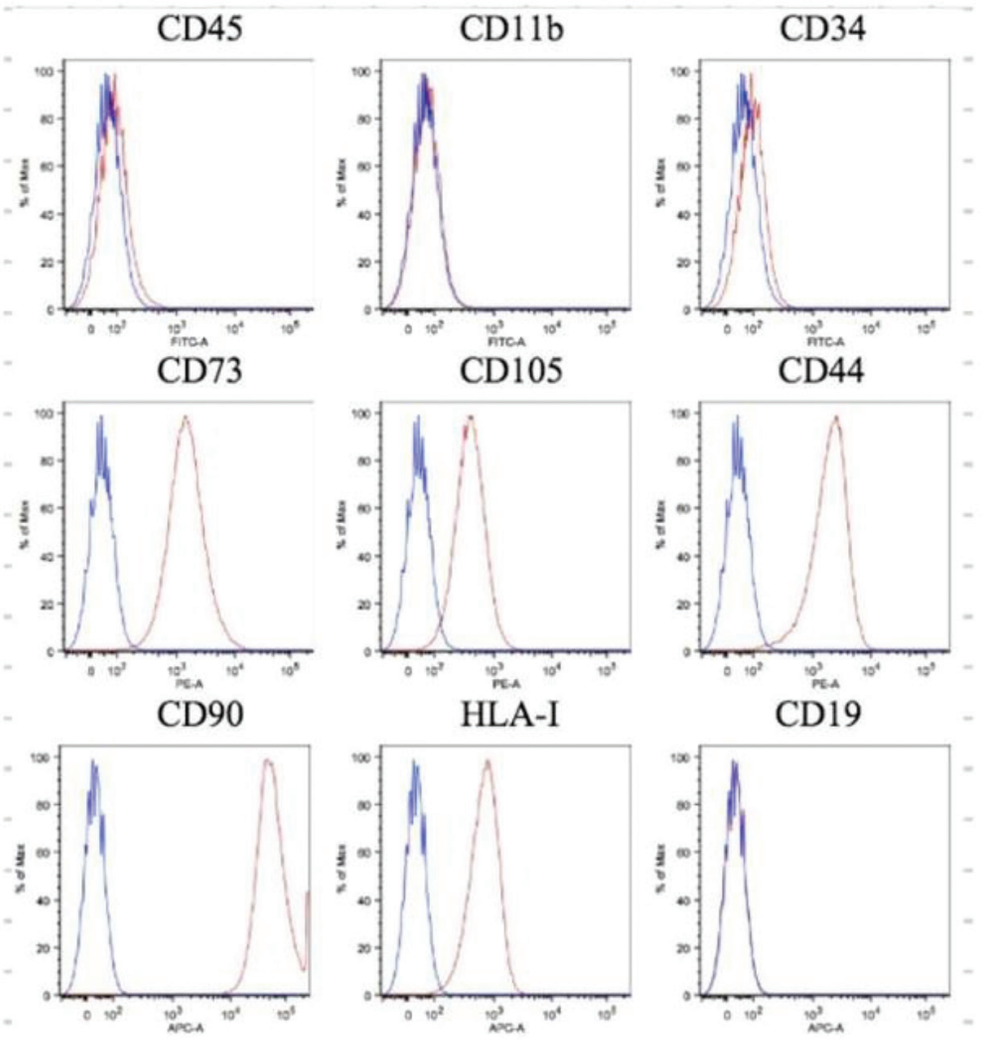


Figure 1. Representative flow cytometry of human MSCs cultured in dPL expresses typical MSC markers. Reprinted from Brewster et al. [40] with permission from Elsevier.

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The proliferative response of MSCs from all patient groups and in culture conditions containing both FBS and dPL was examined by quantifying population doublings over multiple passages. For these experiments, a direct comparison of FBS and PL was performed, such that cells were cultured in either 5% FBS or 5% dPL. Cell counts were recorded at 7-day intervals, at which point MSCs were replated up to passage 11. We found that when all patient groups were analyzed together, the number of mean population doublings was increased in the dPL group at earlier passages, but by mid to late passage, the benefit of culturing cells in dPL had disappeared (**Figure 2**). When stratified according to disease state, cells cultured in dPL were non-inferior to cells cultured in FBS, and at early passage, cells cultured in dPL had increased number of population doublings across all groups. At early passage, there was also no difference when disease states were compared, suggesting that proliferative capacity of MSCs was preserved regardless of patient characteristics. From a practical standpoint, it should be noted that MSCs grown in dPL grew so rapidly at early passages that they quickly became confluent prior to subculturing, and thus proliferation was likely impaired by contact inhibition, so our experimental design may not have captured the full proliferative effect of dPL [40].

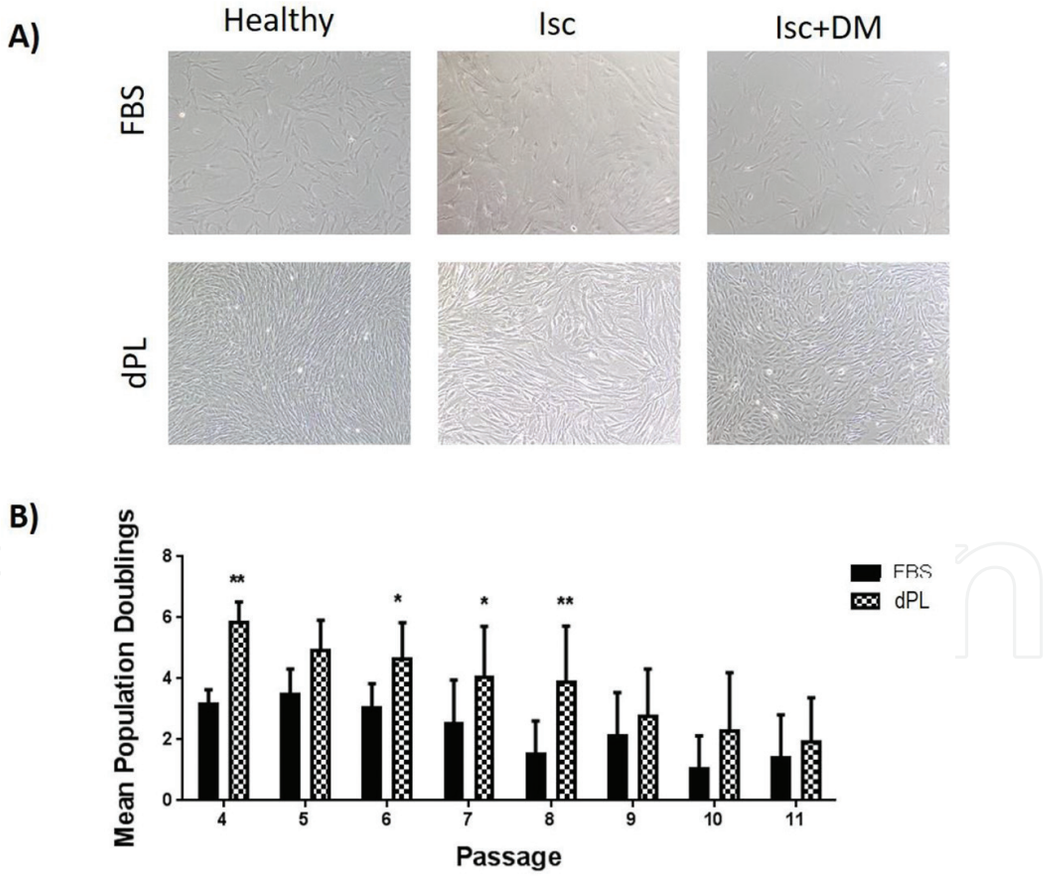


Figure 2. Cell culture with dPL leads to robust proliferation of human MSCs. In (A), MSCs from healthy controls are shown in comparison to MSCs isolated from the bone marrow of patients with CLI or CLI with concomitant diabetes mellitus. In (B), quantification of population doublings in MSCs across all patient groups was higher when grown in dPL than FBS at early passages, but by later passages, there was no difference between culture conditions. Reprinted from Brewster et al. [40] with permission from Elsevier.

We also characterized the functional capacity of MSCs across the different patient groups in vitro, in order to predict potential efficacy of MSCs for therapeutic angiogenesis in future human trials. A colony-forming unit (CFU) assay was performed on MSCs from each patient group. The number of CFUs in MSCS from patients with critical limb ischemia was similar to healthy control donors, suggesting that clonal capacity was independent of patient characteristics. A robust secretome analysis was also performed for all patient groups on MSCs that were cultured in either FBS or dPL. We specifically examined MSC production of bFGF, VEGF, HGF, and MCP-1. The secretome profiles were similar across all patient groups and culture conditions, with only minor differences noted. Furthermore, there were no obvious functional differences when conditioned media from the different treatment and patient groups were used to stimulate endothelial cell migration or proliferation. In order to assess the proangiogenic function of MSCs from patients with fibrinogen-rich platelet lysate (frPL), a coculture assay was used where MSCs from each patient mixed with a human endothelial cell line and embedded as cell pellets in 3D fibrin hydrogels. In this model the two cell types form sprouts which invade the fibrin hydrogel. Sprout length was quantified as a surrogate for angiogenic potential and was noted to be similar among all patient groups when MSCs were precultured in dPL. When MSCs were precultured in FBS, sprout length in the CLI group (without diabetes) was significantly lower than the CLI group with diabetes and the health controls. Despite any residual impairment within MSCs from patients with CLI that may result from epigenetic changes to the cells, the functional quality of MSCs from patients with CLI cultured with dPL appears to be equivalent of that healthy controls. These data support the use of dPL for expansion of MSCs and suggest that autologous cell therapy with MSCs for augmentation of neovascularization is a viable treatment strategy in patients with CLI.

Our study was the first in vitro testing of the proangiogenic characteristics of the bone marrow from amputated limbs in patients with CLI, but it is notably limited by the small sample size for each group. However, two important conclusions can be drawn from this study. First, we provide compelling data that, despite numerous reports that progenitor cell populations are impaired in patients with cardiovascular disease or associated risk factors, at least early-passage MSCs from patients with CLI are similar in quality to healthy controls when expanded ex vivo. Diabetes specifically has been shown to impair mesenchymal stem cell (MSC) function [41], at least partially due the effect of oxidative stress from Nox4 upregulation. Diabetes has also been shown to induce epigenetic changes in the promoter of IL-12 in bone marrow cells, such that cell fate is predisposed toward a pro-inflammatory phenotype. This raises the possibility that MSCs in patients with diabetes mellitus may have irreversible changes to the epigenetic signature. However, our study is consistent with other reports that demonstrate functional capacity of MSCs in patients with CLI is equivalent to healthy controls after short-term culture [42] and that ex vivo expansion in either FBS or PL may at least transiently overcome the epigenetic changes induced by patient comorbidities.

2.2. Platelet lysate as a scaffold for MSC delivery

2.2.1. Generation of fibrinogen-rich platelet lysate

Poor cell retention and viability at targeted sites of delivery have impaired advancement of cell therapies, as the number of functioning cells in the desired tissue appears to be critical

for therapeutic efficacy [31, 43, 44]. Numerous biologic materials have been developed to promote cell retention and viability including encapsulation with alginate [45, 46], prefabrication of a tissue engineered patch [47, 48], and seeding of elongated fibrin strands [49]. While these approaches demonstrate that scaffold-mediated cell delivery improves the functional impact of cell therapy, the scaffold designs fail to incorporate any nutritive support for MSCs. In contrast to the fibrinogen-depleted form of platelet lysate used as a cell culture supplement for MSC growth, modifying the production process to retain clotting factors within platelet lysate (fibrinogen-rich platelet lysate or frPL) permits thrombin-induced self-assembly of a hydrogel with incorporated growth factors.

In order to generate platelet lysate that can spontaneously polymerize into 3D hydrogels, we modified the production process of dPL to maximize fibrinogen content of the platelet lysate. This was achieved by eliminating the step in which fibrin clot is formed and extracted from the solution. Our protocol again involves obtaining human platelets from Emory University blood bank in collaboration with EPIC. The platelets are then pooled and exposed to two sequential freeze-thaw cycles [freezing at -80°C for 48 h, then rapidly thawing at 37°C for 8 h] followed by centrifugation at 1500×g for 10 min. The rapid thawing phase is essential to prevent the formation of cryoprecipitate, which will deplete the solution of soluble clotting factors. The supernatant is then collected and stored at -20°C until ready for use. For hydrogel formation, the frPL is rapidly thawed at 37°C immediately prior to use and then centrifuged at 10,000×g for 10 min in 1.5 mL microcentrifuge tubes and sequentially filtered through 0.45 and 0.2 μm syringe tip filters.

Using this processing technique, we generated frPL with a fibrinogen concentration of 450 μg/mL which rapidly self-assembled into a 3D hydrogel with the addition of thrombin [50]. The hydrogel production process was refined for optimal durability and seeding with MSCs. For most applications, a 50% frPL hydrogel has preferential mechanical properties and provides appropriate MSC support. For hydrogel formation, an activating solution is prepared containing αMEM media with calcium chloride and thrombin so that the final concentrations are 5 mM and 2 U/mL, respectively, in a 50% PL gel. MSCs are then added to the activating solution at the desired cell density. The complete activating solution is then quickly mixed in a 1:1 ratio with frPL and cast in a cell culture plate and stored at 37°C for 1 h.

2.2.2. Structural composition of frPL

Microstructural analysis of [50] frPL indicates that fibrin is an essential component of frPL hydrogels. To evaluate the contribution to the frPL scaffold, frPL hydrogels and fibrin controls were loaded with 5% fluorescein isothiocyanate (FITC)-labeled fibrinogen and imaged with confocal microscopy to visualize the fibrin microstructure. The resulting images reveal an organized fibrin network within the frPL hydrogels that is more dense than control fibrin hydrogels but lacks clear elongated fibers (**Figure 3**). Additional imaging with scanning electron microscopy shows that the morphology of the frPL consists of thin, highly interconnected branched networks that are distinct from the fibrin hydrogels, which formed more distinct elongated fibrils (**Figure 3**) [50]. This was surprising since the 50% frPL contained only 225 μg/mL of fibrinogen, compared to the 1 mg/mL concentration of the fibrin-only controls. The proteolytic activity of thrombin rapidly initiates the polymerization for liquid frPL

into a 3D scaffold. The conversion of fibrinogen to fibrin clearly plays an important role in hydrogel formation, but other structural components are also likely. At present, it is unclear which specific proteins contribute to the mesh network visualized with microscopy, although we speculate that there are additional clotting factors and retained membrane and cytoskeletal elements from platelets that incorporate into the scaffold.

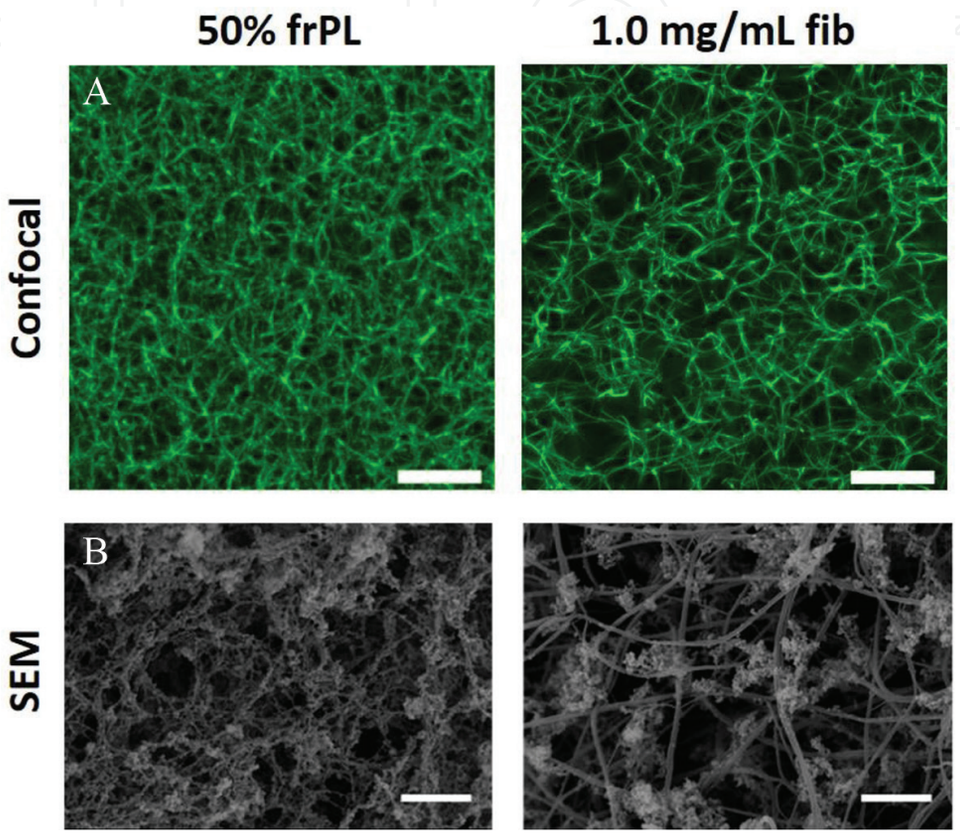


Figure 3. Microstructure of frPL and fibrin hydrogels is visualized in (A) with confocal microscopy after spiking with 5% FITC-labeled fibrinogen (scale bar equal to 20 μm) and (B) with scanning electron microscopy (SEM, scale bar equal to 1.0 μm). Reprinted from Robinson et al. [50], with permission from Elsevier.

The functional attributes of frPL hydrogels are also unique when compared to gels containing only fibrin. Mechanical testing on frPL revealed that these hydrogels behave as a viscoelastic solid with a storage and loss modulus equivalent to fibrin hydrogels with four times greater fibrin content [50]. The specific etiology of this property is unclear, but it may be the result of enhanced fibrin crosslinking due to the presence of additional components of the coagulation cascade, including Factor XIII. Additionally, numerous extracellular proteins (i.e., fibronectin, collagen), proteoglycans, and adhesion proteins such as Von Willebrand factor within the PL may reinforce the underlying fibrin network. Under experimental conditions, composite hydrogels containing additional elements such as collagen have improved mechanical strength over their homogenous control hydrogels without a change in total protein content [51, 52]. Therefore, the presence of these additional proteins may essentially

act to form composite fibrin hydrogels with improved mechanical properties. The improved mechanical properties of frPL may also result from thrombin-induced polymerization of alternative macromolecules within the frPL that either alter fibrin binding sites or function as molecular crowders, thereby enhancing the viscoelastic behavior of the gel at relatively low fibrin concentrations [53, 54].

The microstructural differences between frPL and standard fibrin hydrogels extend beyond the microscopic appearance of the scaffold. Diffusion of FITC-labeled dextran molecules from 20 to 150 kDa that were embedded in hydrogels and diffusion of labeled dextran were significantly decreased. However, these differences between diffusion rates within hydrogels were due, at least in part, to the resistance of frPL hydrogels to proteolytic degradation [50]. This was supported by the fact that inclusion of aprotinin (a protease inhibitor) in culture media abrogated the differences between frPL and fibrin hydrogels. Additionally, frPL hydrogels containing 5% FITC-labeled fibrinogen retained labeled fibrin for up to 7 days, while fibrin-only gels rapidly degraded. Again, degradation rates were significantly decreased in fibrin gels with the addition of aprotinin, indicating that frPL is highly resistant to protease degradation compared to pure fibrin hydrogels [50]. Although the specific mechanism by which frPL hydrogels are stabilized has not been explored, it is likely due to the presence of serine protease inhibitors within frPL such as α2-antiplasmin or plasminogen activator inhibitor-1 (PAI-1), which are abundant in platelets and plasma and significantly impair fibrinolysis.

During hydrogel polymerization, the abundant growth factors present in soluble PL become entrapped within the frPL scaffold. We found that frPL hydrogels are enriched in PDGF-BB, which is released over 20 days in vitro, and ~45% of PDGF-BB persisted within the frPL gel at completion of that time course [50]. The retention of PDGF-BB in frPL hydrogels is superior to that seen in optimized formulations of fibrin-only hydrogels in vitro [55, 56], where greater than 90% of PDGF-BB is released after 7 days [57]. In addition to serving as a proangiogenic growth factor [58], PDGF-BB is also a critical mediator of MSC engraftment into tissue [59]; therefore, frPL can serve to both enhance engraftment of MSCs delivered within the scaffold and also exert a proangiogenic effect on native endothelial cells. The unique microstructure of frPL and resistance to degradation permit sustained release of these growth factors, such that the therapeutic window of MSCs embedded within the frPL scaffold is prolonged.

2.2.3. Impact of frPL on MSC function

The enriched milieu of growth factors and cytokines contained within frPL hydrogel has numerous beneficial effects on both MSCs and endothelial cells in vitro. Seeding of MSCs in frPL hydrogels leads to extensive proliferation of MSCs when quantified with MTS assay. In fact, cell number was higher in an frPL gel than fibrin-only gels when quantified over 7 days and also higher than in cells grown in a monolayer with dPL supplemented media. The frPL does not appear to have the same mitogenic effect on endothelial cells, as HUVECs grown in frPL hydrogels showed very little proliferative activity compared to controls [50].

The frPL hydrogel also has a significant impact on cell invasion. Sprout length from MSC/EC coculture pellets embedded in hydrogels was significantly longer than sprout length in fibrin controls (**Figure 4**) [50]. When MSCs or ECs alone were embedded in frPL, there was a notable difference in effect between the two different cell types. MSC sprouting appeared to be dependent on fibrin content, as sprout invasion was greater in low concentration fibrin hydrogels in addition to frPL. In contrast to its effect on MSCs, the frPL scaffold led to superior invasion of HUVECs compared to both high and low fibrin controls. Cells with the frPL scaffold receive both biochemical and biomechanical cues, which have a variable effect on different cell types. The frPL induces endothelial migration through biochemical signaling, but does not impact proliferation. On the other hand, growth factor signaling within the frPL causes substantial proliferation in MSCs, while the soft substrate of the scaffold provides mechanical cues to stimulate cell migration of MSCs. Based on this in vitro data, we can infer that frPL hydrogels embedded with MSCs have the ability to recruit remote endothelial cells, as demonstrated in the transwell migration assay. These data support the proposed clinical treatment strategy, whereby PL gel embedded with MSCs recruits host ECs for neovascularization following implantation in ischemic tissues.

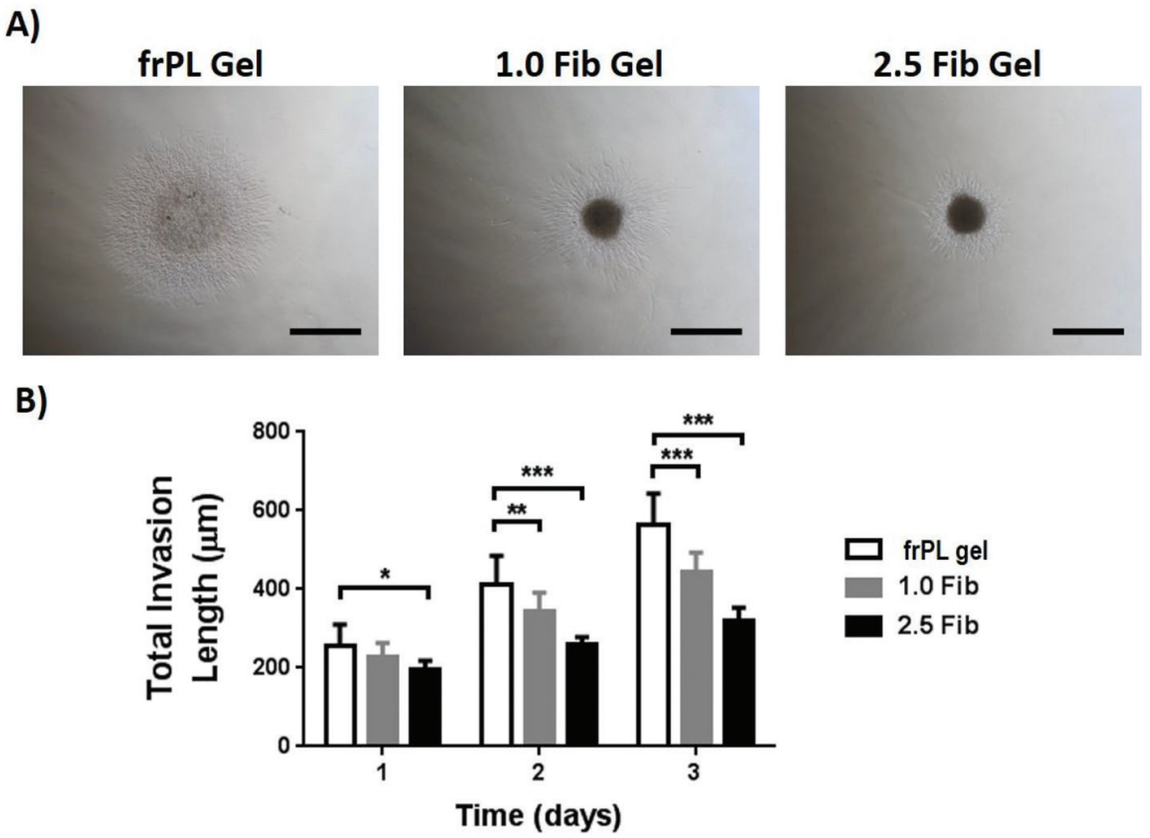


Figure 4. Cell sprouting of hydrogels. Human MSCs and HUVECs were mixed in a 1:1 ratio and embedded in frPL, 1.0 mg/mL fibrin, or 2.4 mg/mL fibrin hydrogels. Sprout length was assessed over 3 days in culture. Representative bright field images of each group at day 3 are shown in (A). Sprout length is quantified in (B). Reprinted from Robinson et al. [50], with permission from Elsevier.

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Preliminary testing in a mouse model of hind limb ischemia supports this treatment strategy. Implantation of MSCs embedded in PL into ischemic limbs in a mouse model of HLI led to rapid neovascularization of ischemic tissues by 8 days when assessed with LDPI. Rapid and complete neovascularization of gastrocnemius muscle occurred in 8 days, which was increased significantly when compared to PL gel alone and MSCs alone (**Figure 5**) [50].

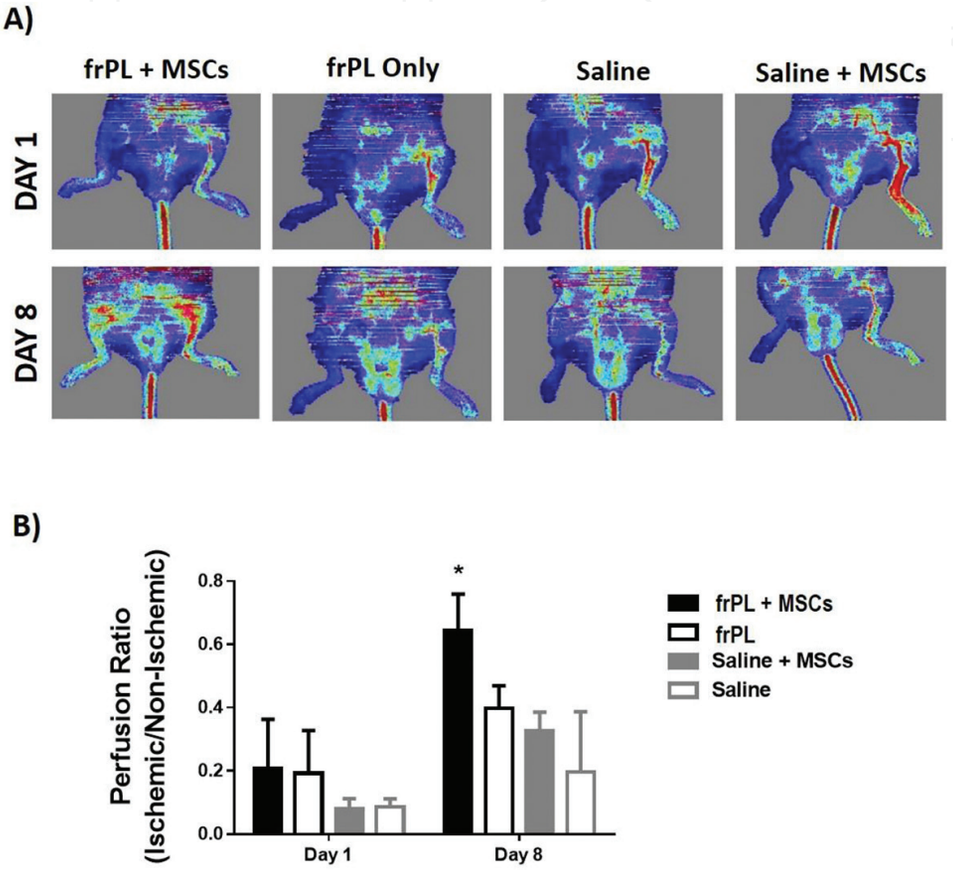


Figure 5. MSCs in saline or frPL were injected into the ischemic hind limb of mice, and perfusion with LDPI was assessed after 8 days. (A) Representative LDPI images are shown of the different groups. (B) Perfusion ratio of the ischemic to nonischemic limb is quantified for each group. Reprinted from Robinson et al. [50] with permission from Elsevier.

2.2.4. Encapsulation of MSCs with frPL

More recently, we have identified an additional novel application of frPL that results from its unique effect on MSCs. For most experiments hydrogels are cast in sterile, tissue culture treated polystyrene wells. In most cases the hydrogel will adhere to the walls and floor of the dish. However, when tissue culture plates are preincubated with a solution containing 2% albumin, hydrogels are no longer tethered to the plastic. When MSCs are embedded in fibrin gels, they exert a modest contractile effect on the scaffold that results in ~75% reduction in gel volume. However, when MSCs are embedded in an untethered frPL, hydrogel gel volume is reduced to 1–2% of the initial volume over 3 days (**Figure 6**). Cells were stained with CellTracker Red, and labeled fibrinogen was added to the frPL, and the reorganization

of fibrin strands can clearly be seen with rounding of the cell bodies with loss of extending processes (**Figure 6**). Cell viability is preserved in cells encapsulated within frPL pellets. The pellets can be degraded and MSCs can be released with dispase treatment. Viability of MSCs within the frPL pellets is preserved for up to 3 days in vitro, as determined with a live/dead assay. The ability of frPL to form dense cell spheroids containing MSCs provides yet another practical application of platelet lysate. The frPL MSC spheroids could serve as an additional mechanism of cell delivery, by encapsulating MSCs in a thin fibrin shell.

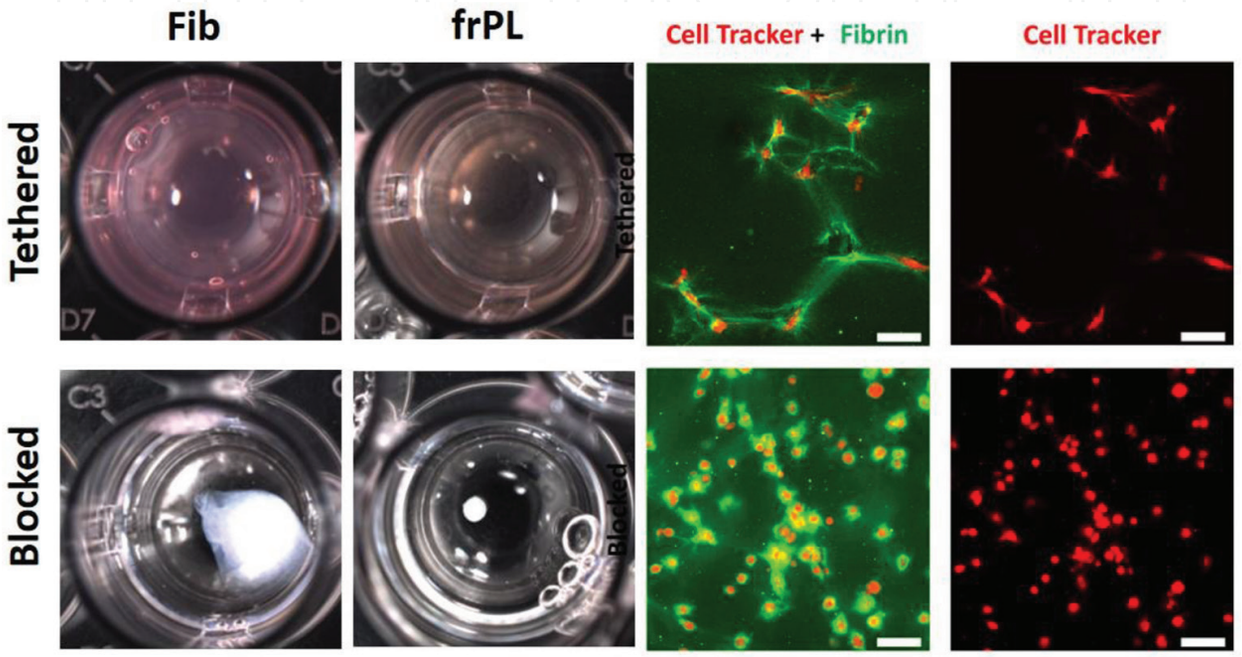


Figure 6. MSCs embedded in untethered frPL form cell spheroids. 2×10^5 MSCs/mL were embedded in frPL or fibrin hydrogels in six well tissue culture dishes with (untethered) and without (tethered) preblocking the plate with 2% albumin solution. MSCs within the frPL formed dense spheroids. Hydrogels were labeled with 5% FITC-dextran and seeded with CellTracker Red-labeled MSCs in tethered and untethered culture conditions. Scale bars represent 20 μm (original figure).

3. Conclusion

Innovative strategies are needed for enhancing quality of MSCs in patients with CLI while also improving delivery and retention of MSCs for cell-based therapy. Here we discuss the dual use of human platelet lysate as both a cell culture supplement and a scaffold for cell delivery. When bereft of clotting factors, depleted form of platelet lysate (dPL) supplemented media enables rapid expansion of MSCs without diminishing their angiogenic activity. In contrast, with preservation of clotting factors, frPL forms a rapidly assembling hydrogel with desirable structural properties and biological activity on MSC and ECs. In both soluble and hydrogel form, PL augments the proangiogenic qualities of MSCs and is readily derived from human source materials that have been tested for safe delivery to patients. As a result of these

unique traits, PL hydrogel is ideally suited to serve as a cell culture supplement for MSC growth and as a vector for delivery of MSCs to ischemic tissues.

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