

FROM BENCH TO BEDSIDE

Expansion and angiogenic potential of mesenchymal stem cells from patients with critical limb ischemia

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Background: Critical limb ischemia (CLI) is a life- and limb-threatening condition affecting 1% to 10% of the population with peripheral arterial disease. Traditional revascularization options are not possible for up to 50% of CLI patients, in which case, the use of cellular therapies, such as bone marrow-derived mesenchymal stem cells (MSCs), hold great promise as an alternative revascularization therapy. However, no randomized, controlled phase 3 trials to date have demonstrated an improvement in limb salvage with cellular therapies. This may be due to poor cell quality (ie, inability to generate a sufficient number of angiogenic MSCs) or to the inadequate retention and viability of MSCs after delivery, or both. Because concerns remain about the expansion and angiogenic potential of autologous MSCs in the CLI population, the objective of this study was to examine the effect of our novel culture media supplement, pooled human platelet lysate (PL), in lieu of the standard fetal bovine serum (FBS), to improve the expansion potential of MSCs from CLI patients. We also characterized the in vitro angiogenic activity of MSCs from the tibia of amputated CLI limbs compared with MSCs from healthy donors.

Methods: MSCs were obtained from the tibia of four CLI patients (ISC) and four ISC patients with diabetes mellitus (ISC+DM) undergoing major amputation. Healthy MSCs were aspirated from the iliac crest of four young and healthy donors. MSCs were isolated and expanded in culture with PL or FBS. MSCs from passage 3 to 6 were used for phenotypic marker expression and for adipogenic and osteogenic differentiation and were tested for their in vitro angiogenic activity on human microdermal endothelial cells. In parallel MSCs were cultured to passage 11 for population-doubling calculations.

Results: MSCs from ISC and ISC+DM patients and from healthy patients exhibited appropriate expression of cell surface markers and differentiation capacity. Population doublings were significantly greater for PL-stimulated compared with FBS-stimulated MSCs in all groups. Biologically active amounts of angiogens were identified in the secretome of all MSCs without consistent trends among groups. PL expansion did not adversely affect the angiogenic activity of MSCs compared with FBS. The ISC and ISC+DM MSCs demonstrated angiogenic effects on endothelial cells similar to those of healthy and ISC MSCs.

Conclusions: PL promotes the rapid expansion of MSCs from CLI and healthy persons. Importantly, MSCs expanded from CLI patients demonstrate the desired angiogenic activity compared with their healthy counterparts. We conclude that autologous MSCs from CLI patients can be sufficiently expanded with PL and be expected to deliver requisite angiogenic effects in vivo. We expect the improved expansion of ISC and ISC+DM with PL to be helpful in improving the successful delivery of autologous MSCs to patients with CLI. (J Vasc Surg 2017;65:826-38.)

Clinical Relevance: Despite the widely held belief that mesenchymal stem cells (MSCs) from patients with multiple vascular comorbidities (eg, diabetes, aged patient) are inferior in quality compared with MSCs from healthy patients, we demonstrate that MSCs from patients with critical limb ischemia have similar angiogenic function in vitro to that of

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healthy patients and that platelet lysate supplementation of culture media improves the expansion potential of these MSCs. These findings support the use of autologous MSCs to promote angiogenesis and our novel MSC expansion protocol for increasing the number of suitable MSCs from patients with critical limb ischemia for cellular therapy.

Critical limb ischemia (CLI) is the most severe form of peripheral arterial disease (PAD). CLI patients have inadequate perfusion, leading to rest pain, tissue loss, and amputation rates of ~30% at 1 year.¹ Given the increasing incidence of PAD worldwide,² the proportion of the population aged >70 years, and the longevity of cardiovascular patients, CLI is likely to increase substantially in the future.³ Because many patients with CLI do not have appropriate anatomy or conduits for traditional revascularization,^{4,5} major amputation, with its significant morbidity and 30-day mortality rate,⁶ can be the only definitive treatment option. Thus, novel treatments for CLI, such as cellular therapy, are critically needed.⁷

Cellular therapies are a promising technology that may be capable of improving limb salvage by preventing major amputation in CLI patients.⁸ Mesenchymal stem cells (MSCs) are particularly well suited for CLI because they promote angiogenesis and arteriogenesis through stromal and paracrine activity.^{9,10} To date, several early-phase clinical trials of MSC therapies have shown benefit,^{8,11} but there have been no phase 3 studies demonstrating improved limb salvage (ie, decreased amputation rate) in CLI patients.⁸ This has led to a call for deliberate testing and optimization of protocols, including MSC sourcing and expansion protocols.¹²

Despite the robust capacity of in vitro testing of MSC quality and expansion, there is an imbalance in the number of bench studies of human MSCs in CLI compared with that of small clinical series.¹³ Spurred by the inability to limit amputation rates in recent phase 3 clinical trials (JUVENTAS [Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation] and RESTORE-CLI [Use of Tissue Repair Cells (TRCs-Autologous Bone Marrow Cells) in Patients with Peripheral Arterial Disease to Treat Critical Limb Ischemia] (RESTORE-CLI^{11,14}), we believe further investigation into MSC quality and expansion protocols using clinically relevant MSCs is warranted (ie, moving from the bedside to the bench). In the study reported here, we used our access to MSCs from amputated limbs of CLI patients, as a readily available and clinically relevant human source of MSCs, to test their expansion and angiogenic capacity.

To improve the expansion capacity of MSCs in CLI patients, we have generated and validated a MSC manufacturing process that uses pooled human platelet lysate (PL) as a human serum supplement to stimulate rapid MSC expansion. PL avoids the xenogeneic effect of fetal bovine serum (FBS) on MSCs, which can even result in the host rejecting autologous cells.¹⁵ PL is generated following rigorous testing for contamination by the American Red Cross. Most importantly, we already have approval to use PL in a current MSC human trial (IND14825; NCT 01659762). Finally, because PL has been used to salvage MSCs from less robust cell sources,¹⁶

it may provide an advantage to the expansion of MSCs in CLI patients.

Similarly, autologous sourcing of MSCs expanded in human PL may be favored over allogeneic sourcing due to decreased concern for immunogenic rejection in CLI¹⁷ and with repeat dosing. However, there has been a persistent concern in the field of cellular therapy that autologous sourcing of cells in patients with cardiovascular disease may not provide the desired angiogenic effects. Heeschen et al¹⁸ first demonstrated this discrepancy using bone marrow-derived mononuclear cells (MNCs) from patients with chronic ischemic cardiomyopathy. This work led investigators to focus on the MSC component of the bone marrow MNCs,¹⁹ which are more angiogenic,²⁰ but Neef et al²¹ have recently demonstrated impaired quality of MSCs harvested from the sternum of coronary artery bypass grafting patients. Interestingly other investigators have not found these deficits in dialysis patients²² and other cardiovascular patients.²³ This is thus a topic of intense debate and experimental equipoise requiring greater investigation.

In this study, we used human MSCs from amputated limbs of CLI patients to test the hypotheses that PL promotes a more rapid expansion of MSCs than FBS and that autologous MSCs cultured from patients with CLI stimulate angiogenesis in vitro in a comparable fashion to that of MSCs from healthy donors. Our long-term aim is to develop improved pathways for cell expansion that enable expedited use of autologous MSCs from patients with CLI by using PL as a novel, human-based, blood product source.

METHODS

Cell harvest and culture. After Investigational Review Board approval and informed consent, healthy MSCs were isolated from 10 to 20 mL bone marrow aspirated from the iliac crest of four (three men, one woman) young healthy volunteer donors. Bone marrow aspirates were diluted 1:2 with phosphate-buffered saline (PBS) and layered by a Ficoll density gradient. The cells were centrifuged at 400*g* for 20 minutes, and thereafter, the MNCs were plated in complete human MSC medium (α -minimum essential medium [MEM; Corning Life Sciences, Lowell, Mass], 10% human platelet lysate [PL] or 10% FBS, 100 U/mL penicillin/streptomycin) at 100,000 to 300,000 cells/cm². Nonadherent hematopoietic cells were removed by changing the medium after 3 days of culture. MSCs were allowed to expand for 7 to 12 days; thereafter, the cells were passaged and reseeded in fresh MSC medium at 1000 cells/cm².

Ischemic (ISC) MSCs were harvested from the marrow of transected tibia after patient consent under an Investigational Review Board-approved protocol at Emory University Hospital. The transected bones were brought to the

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