Cell Therapy and Critical Limb Ischemia: Evidence and Window of Opportunity in Obesity

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Critical Limb Ischemia

Critical Limb Ischemia (CLI) is the most advanced clinical stage of advanced Peripheral Artery Disease (PAD). CLI occurs in approximately 12% of American adults [1]. CLI is a chronic disease process that occurs when an atherosclerotic blockage in the arteries markedly reduces blood flow and oxygenation to the extremities. It is defined as rest pain or impending limb loss secondary to proven arterial occlusion for more than two weeks [2]. This arterial insufficiency can manifest as ischemic rest pain, slow-healing ulcers, and gangrene. CLI is associated with high morbidity and mortality. If left untreated, CLI can lead to amputation of a limb and significant disability of an individual [3]. Indeed, 30% of patients with CLI undergo limb amputation, and the five-year mortality rate is 60% [1]. So far, there is no pharmacotherapeutic agent available for the treatment or prevention of CLI. The current treatment options aim at improving distal arterial perfusion by endovascular or surgical approaches or combination of both. However, amputation is often inevitable in the majority of patients [4].

Mechanisms of Physiological Vascular Repair

Ischemia is defined as a state in which blood flow is insufficient to meet metabolic demands. A functional response to increased metabolic demand is dilation of arteries along with decreased vascular resistance. Endothelium-dependent vasodilation can be impaired in presence of cardiovascular risk factors such as hyperlipidemia, diabetes mellitus, hypertension and tobacco use [5], which could be attributed in part to decreased production and increased degradation of nitric oxide [6]. Patients with PAD experience impaired vaso-reactivity [7], which renders intra-arterial infusions of vasodilators ineffective [8]. Functional response to ischemia is followed by structural alterations in the vasculature. These alterations include angiogenesis, arteriogenesis and vasculogenesis. Angiogenesis was first described by Judah Folkman in 1971 [9] and is defined as the expansion of microvasculature, because of sprouting of

Endothelial Cells (ECs) from pre-existing capillaries, followed by their proliferation, migration, and capillary formation [10]. By contrast, arteriogenesis describes the remodeling of existing collateral channels, so that they can deliver more blood flow to the limb [11]. Finally, adult vasculogenesis involves the recruitment of Endothelial Progenitor Cells (EPCs), which migrate from the bone marrow to the area of ischemia and differentiate to form new blood vessels [12].

On the other hand, muscle ischemia is associated with an increase in oxidative stress [13]. Oxidative stress occurs when the cell or tissue fails to detoxify the free radicals produced during metabolic activity. Free radicals damage proteins, lipids and nucleic acids. The excessive production of free radicals may be a unifying mechanism of vascular and skeletal muscle injury in PAD. Inflammatory process was also shown to play a key role in response to vascular injury, initiating recovery. Inflammatory mediators including cell adhesion molecules, cytokines, chemokines and growth factors direct the recruitment of inflammatory cells including monocytes/macrophages, neutrophils, and T-lymphocytes to the site of injury. Platelets are also important for the elaboration of inflammatory mediators [14]. Recruitment of EPCs can also be initiated through inflammation to induce repair [15]. While oxidative stress and inflammation are involved in the repair and initially activated as an adaptive mechanism, excessive oxidative stress or persistent release of inflammatory mediators can inhibit angiogenesis.

Cell therapy in CLI: window of opportunity

Under normal physiological conditions, arterial wall injury triggers an inflammatory response to induce repair. Nevertheless, the reparative processes seem to be diminished in both diabetes and metabolic syndromes. Currently, lifestyle modification and surgical procedures are the only intervention available to prevent ischemia. To fill this gap in therapy, researchers are continuing to investigate possible molecular targets that could lead to the development of pharmaceuticals to not only treat but also prevent critical limb ischemia. To date, over 50 clinical studies have been reported on the treatment of peripheral artery disease with progenitor stem cells [16] with the first pilot clinical study reported in 2002 [17]. The next section will review and evaluate

the prevalence of CLI in metabolic disorders including diabetes and obesity and whether cell therapy can offer a window of opportunity to effectively treat CLI. We are shedding light into studies performed in the period from 2000 to present. The key words used in the search were Critical Limb Ischemia (CLI) alone or in conjunction with diabetes, obesity, metabolic syndrome, stem cells, Endothelial Progenitor Cells (EPCs), Mononuclear Cells (MNCs) or Mesenchymal Stem Cells (MSCs).

Stem cells commonly used in CLI

A stem cell is defined by its capacity for both self-renewal and directed differentiation. Two broad categories of stem cells have been recognized; the Embryonic Stem Cells (ESCs) and the so-called adult stem cells. ESCs are derived from the inner cell mass of the fetal blastula and they are pluripotent. They have the ability to differentiate into any cell type or any organ found in the adult body including; endodermal, mesodermal or ectodermal lineage [18]. On the other hand, adult stem cells exist in the bone-marrow and the circulation or as residents within a specific tissue [19]. In contrast to ESCs, adult stem cells are multipotent. They can give rise to cells of a given germ layer, in other words, they are lineage-committed [20].

The therapeutic application of adult stem cells in vascular regeneration is farther along in clinical development than any of the other stem cell approaches [21]. There are number of traits that make adult stem cells appealing in the field of cell-based vascular regeneration. Most of the time, they are isolated from the patient in whom they are ultimately to be injected and there is no need to overcome an immunologic barrier. Moreover, their use is not burdened by the ethical concerns that surround the use of human embryos [21]. In the following section, we will overview the common types of adult stem cells that have been shown to be of therapeutic potential in CLI.

Bone Marrow Derived Mono-Nuclear Cells (BM-MNCs)

BM-MNCs contains mixed population of cells that have not been completely characterized [22], yet includes hematopoietic cells, fibroblasts, osteoblasts, and myogenic cells as well as endothelial lineage [23]. Within this population of cells, there are true Endothelial Progenitor Cells (EPCs) that can incorporate into the vascular network. These cells constitute 1-2 per 100 million MNCs [24].

Endothelial Progenitor Cells (EPCs)

EPCs were first identified from the bloodstream and recognized for their regenerative potential in 1997 [25]. EPCs are a subgroup of peripheral blood monocytes that express stem cell-like antigenic determinants including CD34+, VEGFR2, CD31+, CD133+, CD117+, P1H12+, cKit+, Sca1+ and CXCR4+ [26]. Yet at present, there are no surface markers that clearly distinguish early endothelial progenitors. The best approach currently is to define endothelial lineage functionally, in other words, by the ability of true EPCs to inosculate into a new or pre-existing vascular network [27]. Enhancement of EPCs is considered very

promising therapeutic alternative for cardiovascular disease [28]. EPCs mobilization was successfully achieved by erythropoietin and other growth factors [29,30]. EPCs regeneration was found to increase by physical exercise in a nitric oxide-dependent way [31]. Statin therapy is an extensively studied pharmacological pathway known to increase EPCs mobilization [32-34]. Besides their lipid lowering and anti-inflammatory effect, statins work by direct stimulation of EPCs synthesis and release into the circulation, leading to an overall reduced aggression to the vascular wall and eventually passivate the vascular system [35].

Mesenchymal Stem Cells (MSCs)

Mesenchymal Stem Cells (MSCs) are multipotent non hematopoietic, fibroblast-like cells that can be isolated from various tissues, including Bone Marrow (BM), adipose tissue, placenta, and umbilical cord blood [36]. In addition to their differentiation capabilities into bone, fat ... etc., MSCs exert vascular therapeutic effects via secretion of paracrine factors that may have anti-inflammatory and immunomodulatory effects [37,38]. Of note, immune-modulation is a unique feature for MSCs, making their combination with other stem cells subtypes very appealing to enhance allogeneic injection for inducing repair [39-41].

Clinical evidence of CLI in diabetes

Clinically, CLI is somewhat regarded as a condition almost restricted to patients with diabetes mellitus, as it has been estimated that nearly 25% of patients with diabetes have evidence of CLI [42]. Recent reports showed that, diabetes can increase the risk of PAD by two- to four folds [42]. Moreover, the risk for PAD is aggravated by poor glycemic control in diabetic patients [43]. Since CLI is the last stage of arterial occlusive disease, atherosclerosis in association with hypertension, hypercholesterolemia, cigarette smoking and diabetes are the main risk factors. Less frequent causes of CLI are Buerger's disease, arteritis or thromboangiitis obliterans [44]. While, CLI does not occur solely in the diabetic patient population [45], data on CLI in non diabetic subjects are relatively scarce. Experimentally, the murine hind-limb ischemia model is the most commonly used as a preclinical model for PAD. In this model, the superficial femoral artery is ligated proximally and distally, and the segment in between the ligatures is excised. The common femoral artery is also ligated, to obstruct flow to the deep femoral artery. Typically, C57Bl6 mice are used over the age of 12 weeks, as younger animals recover so quickly and completely that angiogenic agents cannot be easily tested [27]. Majority of CLI preclinical data are generated using type 1 diabetic model induced by the toxin streptozotocin injection [46-48].

Alteration of EPCs number and function in diabetes

The number of circulating EPCs was found to be 44% lower in patients with diabetes [49] and in patients with PAD [50]. Diabetes can impair the function of EPCs as assessed by their ability to form colonies and to incorporate into pre-existing vascular networks ex vivo [51]. Type 2 diabetic patients were reported to have lower level of EPCs. Moreover, EPCs number

extensively decreases in type 2 diabetic patients who develop peripheral vascular complications [52]. In support, Fadini GP, et al. [53] reported that EPCs could be novel biological marker of diabetic vasculopathy where diabetic patients with PAD displayed a significant 53% reduction in circulating EPCs versus patients without PAD. Type 1 diabetes is associated with reduced vascular repair as indicated by impaired wound healing and reduced collateral formation. These vascular complications in type1 diabetes were attributed to EPCs dysfunction [49].

Clinical evidence of CLI in obesity

Although diabetes is the most well-defined risk factor for the development of CLI, additional risk factors include the components of metabolic syndrome. Metabolic syndrome is a cluster of several cardiovascular risk factors, with insulin resistance as a major characteristic in addition to obesity, abdominal obesity, glucose intolerance, high blood pressure, and high cholesterol. Obesity is defined as a body weight that is greater than what would be considered a healthy weight for a given height. More than one third of adults in the United States are obese [54]. Obesity is more than just a cosmetic issue; the American Medical Association officially recognized obesity as a disease state in 2013 [42]. Obesity increases the risk of many health problems including but not limited to coronary heart disease, hypertension, stroke, type 2 diabetes, dyslipidemia, cancer, osteoarthritis, sleep apnea, gallstones, reproductive problems, and metabolic syndrome [55]. Metabolic syndrome is an accumulation of hypertriglyceridemia, hyperinsulinemia, abdominal adiposity, hypertension, and reduced HDL cholesterol, all of which occur due to its hallmark clinical features of central obesity and insulin resistance [56]. Insulin resistance increases the risk for a patient to develop ischemic cardiovascular diseases even in non-diabetic milieu [57]. The clinical manifestations of metabolic syndrome all contribute to the development of atherosclerotic blockages, so it is important to recognize the risk of CLI not only in the diabetic population, but also in the growing patient populations with obesity and/or metabolic syndrome.

Experimental evidence of CLI in obesity

Using hind limb ischemia model, Albadawi, et al. [58] has shown that diet-induced obesity in mice demonstrated defective functional recovery during the regenerative phase of limb ischemia-reperfusion injury. This finding could be of strong relevance to perfusion of lower extremity in patients with metabolic syndrome and insulin resistance. Our group has shown that, high fat diet-induced obesity can compromise vascular recovery in response to ischemic insult evident by impaired blood flow and significant reduction in vascular density compared to mice kept on normal diet. The mechanisms involve increasing oxidative and nitrative stress that impaired the signal of the Vascular Endothelial Growth Factor (VEGF) to its receptor (VEGFR2) in response to high fat diet [59]. Furthermore, Tsai, et al. [60] demonstrated that diet-induced obesity impaired recovery of the ischemic areas compared to normal diet. The mechanism was attributed to a decrease in number and function of circulating cells involved in endogenous repair.

Interestingly, when comparing the vascular recovery and neovascularization following hind limb ischemia in type 1 and type 2 diabetic mice, blood flow recovery was markedly reduced in both diabetic mice groups. Moreover, blood flow recovery was significantly less within type 2 diabetic mice compared to type 1 diabetic group [61]. These findings lend further support that other components including, hyperlipidemia and obesity, contribute to the observed impaired blood flow in type 2 diabetes compared to type-1.

Impairment of EPCs number and function in obesity

In addition to proven diabetes, patients with metabolic syndrome only were shown to have significantly lower number of EPCs as well as decreased function, demonstrated by decreased colony-forming capacity [62]. Furthermore, type 2 diabetes coupled with obesity seemed to induce intrinsic EC progenitor dysfunction [63]. Many studies reported the association of obesity with impaired EPCs number and function. Westerweel PE, et al. [64], reported that level of circulating EPCs was reduced by nearly 40% in obese men with metabolic syndrome, which was reversed by combination therapy of low-dose Statin with ezetimibe or high-dose Statin monotherapy. Obesity is associated with decreased numbers of circulating progenitor cells and increased carotid intima media thickness, which were reversed by weight loss [65]. MacEneaney OJ, et al. [66] showed that, number of circulating EPCs was lower in adult obese subjects compared to over-weight and normal weight adults. They also reported that, EPCs colony formation was significantly less in the obese and overweight compared with normal weight adults. Another study by Tobler K, et al. [67] reported decreased number and premature senescence of EPCs in obese volunteers which could function as early contributors to the development and progression of vascular dysfunction in obesity. In overweight adolescents, the number of CD34-negative EPCs, but not CD34-positive EPCs was reported to be higher which could indicate higher risk for future cardiovascular disease in obese teenagers [68]. About the underlying mechanism, Heida NM, et al. [42] reported that EPCs isolated from obese subjects showed impaired angiogenic capacity, which was associated with increased basal activation of the stress marker; p38 MAPK. Inhibition of p38 MAPK was able to restore angiogenic properties of EPCs. Persons who achieved significant weight reduction revealed normalization of p38 MAPK and improved EPCs function.

Therapeutic utility of EPCs in CLI

Teraa M, et al. [69] reported increased inflammation and reduced levels of BM and circulating EPCs in CLI patients. The patients experienced attenuated neovascularization, which was attributed to inflammation-induced BM exhaustion and a disturbed progenitor cell mobilization response. The therapeutic potential of EPCs was identified as early as 2003 in a study done by Kudo FA and colleagues, [70] where autologous transplantation of peripheral blood EPCs (CD34+) was reported to induce satisfactory clinical improvement in patients with CLI. Longer-term clinical benefits were also reported in a randomized, double-blinded, controlled study in which 28 patients with CLI

received CD34+ cells intramuscularly. Patients showed decreased amputation rates and improved amputation-free survival [71].

Therapeutic utility of BM-MNCs in CLI

The first report of stem cell therapy for treatment of ischemic limbs was conducted by Tateishi-Yuyama, E et al. [17] using unselected MNCs from both the bone marrow and peripheral blood for the treatment of CLI. The safety and feasibility of BM-MNCs in ischemic limb treatment was investigated thereafter in different studies [16,72]. As reported in many studies, intramuscular administration of BM-MNCs in ischemic limb improved clinical outcomes including improved ankle brachial index, pain-free walking distance/time, wound healing and limb salvage [73-76]. In support, recent studies confirmed the clinical improvement in patients with severe limb ischemia following intramuscular administration of BM-MNCs in terms of lower amputation rates and improved ankle-brachial index [77-79]. In contrast, a small randomized trial reported non-significant trends toward improved ankle brachial index, and wound healing in patients treated with BM-MNC [80].

Therapeutic utility of MSCs in CLI

The protective potential of MSC against hind limb ischemia was reported in many studies. Hypoxic preconditioning of MSCs was reported to improve their tissue regeneration potential in a murine model of hind limb ischemia, whereas, cells cultured under normoxic conditions exerted therapeutic potential but to lesser extent [81]. Preconditioning improved MSCs therapeutic potential owing to Hepatocyte Growth Factor (HGF)/c-Met signaling pathway as demonstrated by a subsequent study by the same group [82]. In support of this finding, Hoffmann, et al. [83] reported that transplantation of hypoxic MSCs into murine ischemic limbs resulted in increased vessel density. Optimal timing for MSCs injection is crucial as well where administration of MSCs or conditioned media immediately after induction of hind-limb ischemia did not improve revascularization but did so when administered one day later as reported by Kinnaird T, et al. [84].

In a mouse model of CLI, placental-derived Adherent Stromal Cells (ASCs) were found to rescue limb function. These cells share the adherence and marker expression of BM-derived MSCs but lack their differentiation potential [85]. In addition, priming human MSCs chemically to enhance the angiogenic potential by increasing the levels of VEGF and hepatocyte growth factor without genetic modification of MSC potentiated therapeutic angiogenesis and cell survival in the same mouse model [86].

Clinically, human umbilical cord blood-derived MSCs relieved ischemic rest pain in patients with Buerger's disease and in an animal model of hind limb ischemia as well [87]. Intravenous administration of autologous BM-derived MSCs was shown to improve revascularization in a patient suffering from acute gangrene of upper and lower limbs secondary to systemic sclerosis [88]. Couple of studies showed that intramuscular injection of a combination of MSCs and BM-MNC improved walking time, ankle brachial pressure and quality of life in patients with diabetes

mellitus and moderate to severe PAD [89,90]. Meanwhile, Lu D, et al. [91] reported that BM-MSCs could be better tolerated and more effective than BM-MNCs in increasing lower limb perfusion and promoting foot ulcer healing in their study done on 41 CLI patients with diabetes mellitus.

Future and challenges in cell therapy

So far, the development of stem cell therapies to treat PAD has proven difficult for multiple reasons including the complex nature and multiplicity of cell types that coordinate revascularization as well as limitation of the regenerative potential of transplanted human bone marrow derived cells [92]. Challenges facing stem cell-based vascular recovery start with isolation of a particular subtype. Isolation of MSCs or EPCs is relatively easy as they can be collected from bone marrow or peripheral blood. Meanwhile, organ-originated stem cells are difficult to collect because of their unique location and limited number [93]. Ex vivo expansion and purification of specific subpopulation is another challenge, with administration of exogenous cytokines as well as flow cytometry being applicable [94]. Moreover, cells can be dysfunctional with impaired regenerative ability owing to genetics, disease, injury or aging. Integration among native body cells and immunological rejection are also debatable. Additional efforts to improve selection of BM-MNCs with higher regenerative ability are in progress. For instance, human BM-MNCs was isolated and enriched for high Aldehyde Dehydrogenase (ALDH), which is an oxidizing enzyme highly expressed in both embryonic and adult stem cells. The regenerative capacity of human ALDH cells was assessed by intravenous transplantation into immune-deficient mice with limb ischemia. Compared with recipients injected with unpurified nucleated cells containing the equivalent of 2- to 4-fold more ALDH cells, mice transplanted with purified ALDH cells showed augmented recovery of perfusion and increased blood vessel density in ischemic limbs [95]. The majority of studies on cell therapy for CLI have used whole MNC fractions and at this moment it is unclear whether administration of more selected cell populations or ex-vivo culture toward an endothelial phenotype would be more effective [96].

In Summary, there is clinical and experimental evidence that obesity and insulin resistance without incidence of frank diabetes can be associated with impaired perfusion to lower extremities that can progress into critical limb ischemia. Over the past 15 years, there is general agreement that stem cells are useful for vascular diseases. Yet, cell therapy is faced with multiple challenges starting with identification and isolation of a particular stem cell subtype through targeted differentiation. Researches have to overcome these challenges before stem cell therapy can be a successful reality for vascular diseases.

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