A Phase I Study of Human Cord Blood-Derived Mesenchymal Stem Cell Therapy in Patients with Peripheral Arterial Occlusive Disease

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Background and Objectives: Half of patients with critical limb ischemia (CLI) are ineligible for revascularization at diagnosis. The aim of this study was to assess the safety and feasibility of intramuscular human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) therapy in patients with CLI due to atherosclerosis obliterans (ASO) or thromboangiitis obliterans (TAO).

Methods and Results: A total of eight patients (all male, median age 52 years, range 31~77) with CLI were enrolled in this phase I trial. All patients were considered ineligible for further revascularization to improve CLI. We injected 1×10^7 hUCB-MSCs per single dose intramuscularly into the affected limb. The primary end points of safety were occurrence of adverse events (procedure-related complication, allergic reaction to hUCB-MSCs, graft-versus-host disease, cardiovascular and cerebrovascular events) and improvement of symptoms/clinical parameters (healing of foot ulcer, ankle-brachial index, and pain-free walking distance). Angiogenesis was measured with conventional angiography and scored by an independent reviewer. There were four adverse events in three patients. One patient, developed whole body urticaria after injection on treatment day, which disappeared after one day of antihistamine treatment. The other adverse events included diarrhea, oral ulceration, and elevation of serum creatinine level; all conditions improved without treatment. Abnormal results of laboratory parameters were not detected in any patients. Three of four ulcerations (75%) healed completely. Angiographic scores increased in three of eight patients.

Conclusions: This phase I study demonstrates that intramuscular hUCB-MSC injection is a safe and well tolerated treatment for patients with end-stage CLI due to ASO and TAO.

Keywords: Stem cell, Cord blood, PAOD, Mesenchymal

Introduction

For patients with peripheral arterial occlusive disease

(PAOD), including atherosclerosis obliterans (ASO) or thromboangiitis obliterans (TAO), revascularization of the lower extremity arterial tree is the treatment of choice for limb salvage in patients with critical limb ischemia (1). However, some of these patients are not candidates for surgical or endovascular revascularization and, therefore, face the possibility of limb loss (2). For these patients, therapeutic angiogenesis by gene therapy and stem cell therapy consistently have been studied to improve limb perfusion through the growth of new vessels from preexisting blood vessels (3).

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In previous studies, we assessed the feasibility and safety of autologous whole bone marrow stem cell (WBMSC) therapy to treat patients with TAO (4). These results supported the findings of previous studies that demonstrated the safety and effectiveness of therapeutic angiogenesis using autologous implantation of bone marrow mononuclear cells (5). However, using autologous whole bone marrow as the source of MSC has disadvantages: harvesting these stem cells requires an invasive procedure and it is difficult to mass produce the stem cells as a commercial product. Therefore, we recently reported on the safety and efficacy of human umbilical cord blood-derived mononuclear cells (hUCB-MSCs) for inducing angiogenesis in the ischemic limbs of an animal model and in patients with TAO (6, 7).

Based on the results of previous studies, we undertook a phase I clinical trial in patients with PAOD due to ASO or TAO to assess the feasibility and safety of intramuscular human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSCs) injection and to test the hypothesis that this therapy would be effective at improving limb ischemia and healing foot ulcerations.

Materials and Methods

Patient selection

Patients were considered for recruitment in this study if they were not suitable for surgical and/or endovascular intervention to improve limb perfusion for CLI due to ASO or TAO, as determined by an independent interventional cardiologist, radiologist and vascular surgeon.

Enrolled patients' CLIs were defined as category 3, 4, 5 or 6 on the Rutherford scale, presented for a minimum of four weeks with resting ankle-brachial index <0.9 , and had arterial occlusion/stenosis of the lower extremities confirmed by diagnostic angiography. The patients with category 3 CLI on Rutherford scale were only enrolled if symptoms did not improve after lifestyle modification and medical treatment for three months. According to Shionoya's suggestions (8), we diagnosed TAO using the following criteria: (a) smoking history; (b) onset before the age of 50; (c) infrapopliteal arterial occlusions; (d) involvement of the upper limb or phlebitis migrans; and (e) absence of atherosclerotic risk factors other than smoking.

Exclusion criteria included age less than 18 years, history of leg bypass graft and/or angioplasty within the past two months, prior gene or cell therapy for limb ischemia, unstable cardiovascular disease (angina, myocardial infarction, brain infarction within the past month, heart failure of classification III~IV based on the New York Heart Association system), autoimmune disease, and anaphylac-

tic shock and/or allergy. Patients were also excluded if they had an estimated life expectancy of less than 12 months, were on immunosuppressants, chemotherapy or radiation therapy, or had evidence of malignancy. Finally, patients with the following abnormal laboratory findings were excluded: white blood cell count $<4,000$ or $>15,000 \mu\text{l}$; hemoglobin $<10 \text{ dl}$; platelet count $<100,000 \mu\text{l}$; hemoglobin A1c $>8\%$; serum creatinine $>2.5 \text{ mg/dl}$; aspartate transaminase and/or alanine transaminase $>100 \text{ U/L}$.

Study design

This phase I study was an open-label, single-center, prospective study designed to investigate the safety and efficacy of intramuscular direct injection of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) in patients with CLI due to ASO and TAO. A total of eight patients were enrolled to undergo injection of hUCB-MSCs. This study was approved by the Institutional Review Board of the Samsung Medical Center, and we obtained written consent for participation from all of the enrolled patients. Based on previous preclinical safety and bioactivity analyses, 1×10^7 hUCB-MSCs were used in a single dose of a local intramuscular injection in the unilateral ischemic limb. A control group was not established in this study because there was no alternative treatment modality that was appropriate for all of the enrolled patients. Furthermore, the patients participating in this study were not candidates for conventional treatments, and thus, none were suitable to be in a control group. If not contraindicated, all medical therapies, including antihypertensives, oral hypoglycemic agents, antihyperlipidemia and antiplatelet drugs remained unchanged throughout the course of the study period. In order to investigate the safety and efficacy of hUCB-MSC injections on enrolled patients, we evaluated medical records, current medications, electrocardiogram (ECG) results, blood chemistry and hematologic results, vital signs, physical examination findings and digital photos of wounds at baseline and throughout follow-up on day seven and at one, three, and six months. Any adverse events were recorded throughout the six-month follow-up period (Fig. 1).

Materials and procedures

Preparation and manufacturing of hUCB-MSCs: The UCB samples were obtained from umbilical veins immediately after delivery with the informed consent from the mother. All procedures for cell therapy drug processing were carried out according to Good Manufacturing Practice (GMP) protocols from the Korean Food Drug Administration-accredited institutional GMP facility, NKBIO

Fig. 1. Study protocol for intramuscular hUCB-MSCs trial.

Co. (Sungnam, Korea). Briefly, the hUCB samples were mixed with HetaSep solution (Stemcell Technologies, Vancouver, BC, Canada) at a ratio of 5 : 1, and then incubated at room temperature to deplete erythrocyte counts. The supernatant was carefully collected and mononuclear cells were obtained using Ficoll density-gradient centrifugation at 2,500 rpm for 20 minutes. The cells were washed once or twice in phosphate-buffered saline (PBS) and seeded at a density of 2×10^5 to 2×10^6 cells/cm² on plates in a humidified atmosphere of 5% CO₂ at 37°C. Growth media consisted of endothelial basal medium-2 (EBM-2, product code CC-3156, Lonza, Walkersville, MD, USA), endothelial growth medium-2 (EGM-2, product code CC-4176, Lonza, Walkersville, MD, USA), and 20% fetal bovine serum (catalog No. 16000, GIBCO, Grand Island, NY, USA). After three days, non-adherent cells were removed. The adherent cells formed colonies and grew rapidly, exhibiting a spindle-shaped morphology.

MSC release criteria for clinical use were as follows: absence of pathogen contamination (bacterial, fungal, viral, or mycoplasma) at the end of the harvest and before injection; viability >90% as evaluated by trypan blue staining at the end of the harvest and before injection; immune phenotype, showing the expression of CD73 and CD105 (Src homology-2) surface molecules (>90%); the absence of CD34 and CD45 markers (<10%). For therapeutic purposes, cultured MSCs were trypsinized, washed in normal saline, and suspended in saline at a concentration of 1.0×10^7 cells/ml in a sterile vial for injection.

Procedure

Under intravenous sedation with propofol (monitored anesthesia care, MAC), direct intramuscular injection of hUCB-MSCs was performed in the affected limb. One ml

Fig. 2. Injection sites for stem cell therapy.

containing 1×10^7 hUCB-MSCs was diluted into a final volume of 20 ml of saline and injected as twenty aliquots of 1 ml (5×10^5) into each injection site on the limb using 23-gauge needles. Injection sites were selected below the knee at 20 different sites of ischemic calf muscle along the tibial and peroneal arteries (Fig. 2).

Endpoints

As a primary endpoint of this phase-I clinical trial, we evaluated the safety related with the procedures of this therapy. As a secondary endpoint, we evaluated the efficacy for the improvement of ischemic symptoms.

Primary endpoint: safety

The safety variables including adverse events, chemistry and physical exam were obtained at baseline and through-

out the six-month follow-up. Adverse events included procedure-related complications and/or death, anaphylactic shock and/or allergic reaction to hUCB-MSCs, acute or chronic graft-versus-host disease, and cardiovascular (acute coronary syndrome, myocardial infarction) or cerebrovascular events (transient ischemic attack and/or stroke). Based on clinical severity, adverse events were categorized as mild (the patient barely feels any discomfort and there is no interference with normal activity or need for treatment), moderate (the patient feels discomfort that interferes with normal activity, and treatment is needed for continuation of the study), and severe (the patient cannot continue with normal activities and cannot continue with the study or requires hospitalization for treatment). All patients who experienced adverse events were reported to and assessed by the Data, Safety, and Adverse Events Monitoring Committee of the Samsung Medical Center.

Secondary endpoint: efficacy

The endpoints for efficacy included improvements in the following parameters at baseline compared to one week, one month, three months and six months after injection of MSCs in the affected limb: status of limb wound, standardized treadmill test of total walking and pain-free walking distance, and ankle-brachial index (ABI) at each visit. Additionally, conventional angiography was performed before and six months after injection of hUCB-MSCs to detect an increase in the number of visible vessels. An independent reviewer, who was blinded to pa-

tient treatment assignment, scored the digital subtraction angiography according to predefined specifications (7).

Results

Patient characteristics

Between May 2011 and July 2012, a total of eight patients (three with ASO and five with TAO) were evaluated for safety and efficacy data at baseline, one week after MSC injection, and one-, three- and six-month points after MSC injection. Table 1 shows baseline demographics and clinical characteristics of the patients. Participant median age was 52 years (range 31~77) and all patients were male. Five (63%) of these patients underwent a previous endovascular and/or surgical intervention in the index limb.

Safety

Intramuscular injection of hUCB-MSCs was well tolerated by all patients. No patients developed procedure-related complications such as hematoma, cramping pain, or local inflammatory/allergic reaction. No death or serious adverse events were observed during the six-month study period. There were four adverse events in three patients. On the day of treatment, one patient suffered a mild oral ulceration and diarrhea. A second patient experienced a moderate degree of whole body urticaria. The oral ulceration and diarrhea improved without any treatment and the urticaria disappeared after one day of antihistamine treatment. Six months after injection of hUCB-MSCs, ele-

Table 1. Demographics and clinical features (n=8)

Case	Age/sex	Diagnosis	Risk factors & comorbidities	Clinical symptoms ^a	Rutherford scale	Past history
1	31/M	TAO	Ex-smoker	Ulceration (G2)	5	1 thrombectomy
2	46/M	TAO	Ex-smoker	Claudication	3	No
3	72/M	ASO	Ex-smoker HTN DM CAD	Claudication	3	2 bypass
4	56/M	ASO	Ex-smoker	Rest pain	4	1 bypass 1 amputation
5	56/M	ASO	Smoker	Ulceration (G2)	5	3 thrombectomy 2 PTA 3 bypass 1 amputation
6	77/M	TAO	Ex-smoker	Ulceration (G2)	6	No
7	48/M	TAO	Ex-smoker	Claudication	3	1 thrombectomy
8	44/M	TAO	Smoker	Ulceration (G2)	5	No

ASO: atherosclerosis obliterans; TAO: thromboangiitis obliterans; HTN: hypertension; DM: diabetes mellitus; CAD: coronary artery disease; PTA: percutaneous transluminal angioplasty.

^aG1: loss of epidermis; G2: loss of subcutaneous tissue.

vation of serum creatinine level (1.28 mg/dl at baseline to 1.75 mg/dl) was detected in one patient with a history of hypertension and diabetes mellitus. Serum creatinine level was normalized after hydration and conservative management. This patient was suspected to have progressive, chronic kidney disease. All of these adverse events were considered to be unrelated to the stem cell therapy by specialist review and by the Data, Safety, and Adverse Events Monitoring Committee. Adverse events are summarized in Table 2.

Fig. 3. Mean values of pain-free walking distance (PFWD)^a and ankle-brachial index (ABI). ^aMeasurement of pain-free walking distance was available in only 4/8 patients.

Table 2. Adverse events (n=3)

Case	Events	Onset	Grade ^a	Manage	Result
2	Oral ulcer, diarrhea	Treatment day	Mild	No	Improved
3	Elevation of sCr level	6 months	Mild	No	Improved
8	Urticaria, whole body	Treatment day	Moderate	Anti-H ₁	Improved

sCr: serum creatinine.

^aMild: the patient barely feels any discomfort and there is no interference with normal activity or need for treatment; Moderate: the patient feels discomfort that interferes with normal activity, and treatment is needed for continuation with the study; Severe: the patient cannot continue with normal activities and cannot continue with the study or requires hospital admission for treatment.

Efficacy

Ankle-brachial index and pain-free walking distance

The mean ABI index did not improve after intramuscular injection (0.51 at baseline to 0.57 at 6 months, $p>0.05$). Although the mean value of pain-free walking distance increased in patients who took a treadmill test ($n=5$, 76.3 m at baseline to 189.4 m at six months), but this difference was not statistically significant ($p>0.05$) (Fig. 3).

Ulcer healing and amputation

Among the participants, four entered the study with non-healing ulcerations in the index limb. Complete healing of the ulcerations was achieved in three of these patients by six months (Fig. 4). There were no amputations during the six-month follow-up period.

Angiography

Results of angiography at six months after injection showed increasing scores compared with those at baseline in three of eight patients according to the predefined scoring of run-off vessels, angiogenesis, and arteriogenesis. Representative angiograms are shown in Fig. 5.

Discussion

This Phase I trial of intramuscular injection of hUCB-MSKs was designed to assess the safety and feasibility of

Fig. 4. Complete healing of ulcer over 6-month follow-up period (case No. 8). (A) Non-healing ulcer in 5th toe at baseline. (B) Complete ulcer healing at 6 months after treatment.

Fig. 5. Representative angiography of index limb before and after injection of hUCB-MSCs (case No. 8). Follow-up angiography after 6 months (right) shows increased formation of collateral vessels (black arrows) compared to baseline angiography (left).

this potential treatment in patients with CLI due to ASO or TAO. The present study has demonstrated that patients with CLI did not experience any serious adverse events after the injection of hUCB-MSC in the ischemic limb.

CLI is a more aggressive form of PAOD, in which reduction of distal tissue perfusion induces insufficiency of metabolic requirements, causing pain while resting and ischemic ulceration or gangrene in the foot or toe (9). The incidence of CLI is approximately 500 to 1,000 per million per year, according to a North American study (10). Although endovascular and/or surgical revascularizations can often improve symptoms of limb-threatening ischemia, it is not appropriate for all CLI patients (11). Previous studies reported a rate for major amputation in the range of 9~46.4% in patients with CLI due to ASO (12, 13). Surgical revascularization for patients with TAO is also usually not possible because of the diffuse and segmental nature of the distal anastomotic donor site. Often the distal target vessel is unsatisfactory for bypass surgery (14). In our present study, a total of 12 revascularizations (two endovascular and 10 surgical) were performed on five of the enrolled patients before this clinical trial. Additionally, two enrolled patients underwent amputation prior to this study after insufficient response to treatment of the ischemic toe, foot or limb. There was no endovascular or surgical option for revascularization to treat CLI in any of the study patients.

Although therapeutic angiogenesis (using angiogenic

growth factors and cell components) has been studied for patients with PAOD who were unsuitable candidates for endovascular and surgical interventions, the therapeutic results were unfavorable. Several angiogenic growth factors, such as vascular endothelial growth factor (VEGF) isoforms and fibroblast growth factor-1 (FGF-1), have been shown to achieve safe therapeutic angiogenesis through gene transfer to target cells in some phase I studies (15, 16). However, results from the phase II randomized clinical trial of VEGF-A gene therapy showed no differences in limb salvage/restenosis rates, peak walking time/ABI, or amputation rate compared with the control group (17-19). Additionally, in the recent phase III Therapeutic Angiogenesis for the Management of Arteriosclerosis in a Randomized International Study (TAMARIS), there were no statistical differences in major amputation and death rate between the group receiving intramuscular FGF-1-encoding plasmid (NV1FGF) and the placebo group (20).

The first reported trial of therapeutic angiogenesis using stem cells was the Therapeutic Angiogenesis by Cell Transplantation (TACT) trial published in 2002 (21). Since then, there have been several published studies regarding this therapeutic strategy (22-24). Stem cells have some advantages for stimulation of angiogenesis compared with angiogenic growth factors in gene therapy. Transplanted stem cells can release multiple growth factors and cytokines as a renewable source. Delivery systems to carry angiogenic genes, such as plasmid or viral vectors, are not needed in stem cell therapy for angiogenesis. Additionally, stem cells can be established from various tissue sources, such as bone marrow, peripheral blood, and umbilical cord blood. Recent studies have demonstrated the safety of intramuscular injection of autologous stem cells or combined cells, including endothelial progenitors and mesenchymal stem cells, in patients with critical limb ischemia (25, 26). However, the invasive procedure for harvesting autologous bone marrow stem cells before intramuscular injection was a disadvantage. In this respect, cell therapy using hUCB-MSCs have the merit of being less invasive and a reduced procedure time. Therefore, in the present study, hUCB-MSCs were utilized as therapeutic stimulators of angiogenesis in patients with CLI or TAO.

Our phase I trial of intramuscular injection using hUCB-MSCs indicates good immediate and short-term safety. The intramuscular injections of hUCB-MSCs were well tolerated, without serious adverse events. There was no evidence of significant complications related to the intramuscular injection procedure. Moreover, no patients who underwent intramuscular injections of hUCB-MSCs showed mortality related to the procedure during the fol-

low-up period. These observations are consistent with the assessment that intramuscular injection of hUCB-MSCs into ischemic limbs for therapeutic angiogenesis is a safe procedure. In addition, although this study was a phase I trial to assess safety, the results included improvement in ulcer healing and an increase in pain-free walking distance, which shows the potential for a therapeutic effect of intramuscular injection of hUCB-MSCs.

Quantitative analysis of angiographic findings is essential to assess the therapeutic efficacy of intramuscular injections of hUCB-MSCs in patients with CLI. In a recent study regarding the safety and efficacy of intra-arterial administration of autologous bone-marrow mononuclear cells in diabetic patients with CLI, neovasculogenesis was assessed using digital subtraction angiography quantified by MetaMorph[®] software (Molecular Devices, Sunnyvale, CA, USA) to evaluate the degree of vascularization (27). In a previous study, we similarly assessed digital subtraction angiography in patients who underwent intramuscular injection of whole bone-marrow stem cells for limb ischemia due to TAO (7). In this study, neovascularizations were also demonstrated using follow-up angiography of recanalization of run-off vessels, arteriogenesis, and angiogenesis. Although all patients who underwent intramuscular injection of hUCB-MSCs showed increasing scores of digital subtraction angiography, we were able to observe the development of collateral vessels in the injected limbs of three patients. These findings indicate that MSCs have a angiogenic potential and vascular remodeling (28).

In conclusion, this Phase I trial has demonstrated that intramuscular injection of hUCB-MSCs in ischemic limbs for patients with CLI due to ASO or TAO is safe and well-tolerated, with no serious adverse events. Based on these results, a phase II randomized, double-blind, placebo-controlled trial in patients with CLI using hUCB-MSCs is warranted.

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Potential conflict of interest

The authors have no conflicting financial interest.

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