Review

Regenerative Medicine in Critical Limb Ischemia

—Current and Future Directions

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Critical limb ischemia (CLI) is commonly caused by atherosclerotic arterial obstruction or stenosis in the leg, as demonstrated by rest pain, skin ulcers and gangrene (Fontaine III or IV), often fails to respond to conservative treatments, and carries a high risk for limb amputation, with a particularly dismal prognosis. Although surgical revascularization techniques may be used for certain CLI patients, such techniques are not indicated for most CLI patients due to the diffuse nature of the responsible lesions, distal location of the obstruction, or coexisting systemic comorbidities. For such CLI patients with no alternative treatments, the potential utility of cell therapies has been investigated. Indeed many clinical trials are being carried out by academic sectors, and their achievements will facilitate clinical development by pharmaceutical companies.

In order to understand the situation regarding competitive international R&D of revascularization seeds for CLI, we surveyed the status of clinical trials. As a result, we identified 58 clinical trials on revascularization for CLI, with the majority in the early phase (<phase II: 82.7%). Revascularization seeds for CLI are in the development and competition phase, and promising seeds are expected to appear in the near future.

In this review, we discuss how to develop optimal regenerative medicine concerning the selection of cell origin, cell type, combination with growth factor, and the influence of concomitant disease.

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Key words; Critical limb ischemia (CLI), Regenerative medicine, Stem cell, Growth factor

Innovative Therapeutic Approaches for CLI

Atherosclerosis is an inflammatory disease and endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis. Endothelial dysfunction is an important early event in the pathogenesis, contributing to plaque initiation and progression^{1, 2}.

Chronic ischemia in the lower extremities is commonly caused by arterial obstruction or stenosis in the leg. It is well known that atherosclerotic peripheral arterial disease (PAD) is the most common cause of chronic limb ischemia. The clinical consequences of

critical limb ischemia (CLI) manifest as rest pain, skin ulcers and gangrene (Fontaine III or IV), which often fail to respond to conservative treatments, and lead to a high risk for limb amputation. In order to maintain blood flow at the ischemic site, surgical revascularization therapy may be used. These techniques, such as endovascular treatment (stent implantation, laser revascularization) or bypass surgery, can in some cases restore blood flow, preventing gangrene formation and amputation. Nevertheless, such treatment may cause restenosis due to phenotypic redifferentiation of neointimal smooth muscle cells³, and these techniques are not applicable due to the diffuse nature of the responsible lesions, distal location of the obstruction, or coexisting systemic comorbidities.

For such no-option patients, alternatives have been proposed, mainly focused on the modulation of apoptosis, such as the p53-mediated pathway⁴ and induction of revascularization by angiogenic gene or protein delivery or by stem cell regenerative medicine.

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Stem cells are known to secrete many cytokines containing angiogenic factors, and under strenuous hypoxic and serum starvation the expression of several of these factors is upregulated. This has prompted the isolation of mesenchymal stem cell (MSC)-conditioned medium (MSC-CM), to be concentrated and used therapeutically. Kinnaird *et al.* reported the presence of cytokines such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2 or bFGF), interleukin (IL)-6, placental growth factor (PIGF), and monocyte chemotactic protein-1 (MCP-1), and that intramyocardial injection of MSC-CM improved collateral blood flow recovery and limb function and reduced muscle atrophy. Because all of these cytokines are known to promote angiogenesis and vascularization, the conclusion was that paracrine signaling rather than direct cell incorporation may be responsible for the effects of bone marrow cell therapy in ischemic injury⁵. In addition, MSC has anti-inflammatory properties which is said to be suppressed through secretion of anti-inflammatory cytokines such as IL-10, transforming growth factor (TGF)- β , and IL-1 receptor antagonist⁶. The successful effect of MSC has been used to treat animal models in cases of inflammatory pathologies such as colitis, rheumatoid arthritis, and ischemic injury⁷. Furthermore, adipose tissue-derived stem cells (ADSC) are also known to secrete a number of cytokines such as hepatocyte growth factor (HGF), VEGF, and TGF- β ⁸.

Unfortunately, clinical trials based on growth factor delivery alone have not yielded the expected results, thereby demonstrating the need for alternatives^{9, 10}. On the other hand, evidence indicating that angiogenesis from endothelial progenitor cells (EPC) is a process that contributes to therapeutic vascularization¹¹ has fueled interest in the use of this alternative paradigm for therapeutic revascularization. Recombinant human granulocyte colony-stimulating factor (G-CSF) has been extensively used to mobilize stem cells, including EPC from bone marrow. In addition to revascularization, G-CSF reduces atherosclerotic plaque formation and contributes to maintaining the vessel wall¹². Different stem cell populations have been tested in animals for the treatment of hind limb ischemia: unselected bone marrow mononuclear cells (MNC)¹³, ADSC¹⁴, MSC¹⁵, and EPC^{16, 17}. Many clinical trials using autologous or allogeneic bone-marrow or adipose tissue-derived somatic stem cells are now being carried out and some of these trials have already led to commercial clinical development.

Regenerative Medicine for CLI in Japan

Regenerative medicine for CLI, using bone marrow mononuclear cells (MNC)¹⁸, peripheral origin stem cells, peripheral bone marrow MNC, and G-CSF-mobilized bone marrow MNC^{19, 20}, has been approved as “advanced medicine” treatment by a regulatory agency of the Japanese government, the Ministry of Health, Labour and Welfare. Furthermore, as research and development (R&D) into regenerative medicine has been accelerated, a development portfolio consisting of peptides and genes of various growth factors in addition to cell therapy, including autologous ADSC and allogeneic bone marrow-derived stem cells, has been constructed, and it is expected that CLI will be overcome.

However, because of the number of regulatory requirements and the high cost of capital investment required for R&D of regenerative medicine, pharmaceutical companies are unwilling to enter this area in earnest. Because of this situation, and in order to put regenerative medicine to practical use in our country, the role of academia is growing.

The Coordination, Support and Training Program for Translational Research (TR program) was initiated by MEXT (Ministry of Education, Culture, Sports, Science and Technology) in 2007²¹. The aim of this program is to accelerate the application of basic research to patient care, through enabling organizations consisted of nine universities and one research institute to establish translational research centers (TR centers). TR centers would not only increase the interaction between basic and clinical scientists, but also accelerate the translational development of new pharmaceuticals, medical devices, and treatment strategies from the laboratory bench to hospital bed. During the four years since the start of this TR program, 85 TR projects (seeds) have been aggressively developed in TR centers. In particular, the seeds related to regenerative medicine account for about 40% of the whole and are being developed in all TR centers. Four seeds are targeted toward CLI (Table 1).

International R&D Status of Regenerative Medicine for CLI

In order to understand the situation regarding competitive international R&D of revascularization seeds for CLI, we surveyed the status of the clinical trials. Using databases such as Thomson Pharma, ClinicalTrials.gov, and UMIN from August 17 to 18, 2011, we detected 58 clinical trials (seeds) on revascularization for CLI as a result of this investigation

Table 1. Revascularization seeds for CLI in the TR program

TR center	Compound/Medical Device	Status
Kyoto Univ.	bFGF + gelatine-hydrogel	Patient recruiting (under “advanced medical evaluation track”)
FBRI	Isolex: CD34-positive cell isolation system	Phase I completed
Kyushu Univ.	rSeV/dF-hFGF2 (viral vector)	Preparing for IND (Phase IIb)
	Pitavastatin-loaded PLGA nanoparticles	Non-clinical phase

Abbreviations: bFGF, basic fibroblast growth factor, same as FGF2; FBRI, Foundation for Biomedical Research and Innovation; IND, investigational new drug; PLGA, poly(lactic-co-glycolic) acid; TR, translational research, rSeV/dF, F-gene-deleted recombinant Sendai virus

Table 2. Summary: Registered clinical trials of regenerative medicine for CLI

Area	No.	Type						Phase						Sponsor	
		Auto SC	Allo SC	Gene	Biologics	Device	Other	I	I-II	II	II-III	III	III-IV	Academia	Industry
US	20	7	1	4	4	3	1	5	4	9	0	2	0	8	12
Japan	9	6	0	1	2	0	0	0	3	5	0	1	0	9	0
Germany	5	5	0	0	0	0	0	1	2	1	1	0	0	4	1
India	4	3	1	0	0	0	0	0	3	0	0	0	1	2	2
International	3	0	1	2	0	0	0	1	0	1	0	1	0	0	3
France	3	2	1	0	0	0	0	0	2	0	0	1	0	2	1
China	2	1	1	0	0	0	0	1	1	0	0	0	0	2	0
Israel	2	1	0	0	0	0	1	1	1	0	0	0	0	0	2
Netherlands	2	2	0	0	0	0	0	0	1	0	1	0	0	2	0
Spain	2	2	0	0	0	0	0	0	2	0	0	0	0	2	0
Brazil	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0
Czech Republic	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0
Iran	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0
Italy	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0
Korea	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0
Russia	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0
Total	58	35	5	7	6	3	2	11	21	18	2	5	1	37	21
(%)	(100)	(60.3)	(8.6)	(12.1)	(10.3)	(5.2)	(3.5)	(19.0)	(36.2)	(31.0)	(3.5)	(8.6)	(1.7)	(63.8)	(36.2)

Abbreviations: Auto SC, autologous somatic stem cell; Allo SC, allogeneic somatic stem cell

(excluding duplicate seeds). When the results were classified by country, the United States had 20 seeds, Japan, 9, Germany, 5, India, 4, and France, 3 (out of those with 3 seeds or more; **Table 2**). Furthermore, when the results were classified by seed type, the largest group of 35 (60.3%), used autologous somatic stem cells; 7 (12.1%) used growth factor genes; 6 (10.3%), growth factor peptides; 5 (8.6%), allogeneic somatic stem cells; 3 (5.2%) used devices; and 2 (3.5%) were other types of seed (**Table 2**). Because early-phase (<phase II) seeds are in the majority (43 seeds; 82.7%), revascularization seeds for CLI are still in the competitive development phase and promising seeds can be expected to appear in the near future

(**Table 2**).

In particular, a non-viral plasmid vector with fibroblast growth factor 1, riferminogen pecaplastid (NV1FGF), developed by Sanofi-Aventis Co. Ltd. is the most advanced seed and has completed a Phase III study; however, it was not able to meet the primary endpoint (time to major amputation of the treated leg or death from any cause, whichever comes first) (NCT00566657)²². On the other hand, Marui A. *et al.* reported the results of a phase I-IIa study, in which 7 patients had critical limb ischemia. The patients were intramuscularly injected with 200 µg of bFGF-incorporated gelatin hydrogel microspheres into the gastrocnemius of the ischemic limb and the authors

Table 3. Ongoing randomized controlled regenerative medicine trials for CLI (as of August 31, 2011)

ID/Acronym	Sponsors/Location	Comparison	Phases	Pt.	Completion
NCT00797056/ STEMPAD	Washington University School of Medicine/US	Biologics: G-CSF vs. Placebo	III	60	12/2013
UMIN000002280/ IMPACT study	Hokuyu Hospital/JP	auto·BMMNC vs. Standard Therapy	III	144	01/2014
NCT01245335/-	Harvest Technologies/US	Device: BMAC (Auto·BMMNC) vs. Placebo	III	210	06/2014
NCT00539266/ABC	Leiden University Medical Center/NL	auto·BMMNC vs. Placebo	II/III	108	10/2012
NCT01064440/-	ViroMed Co., Ltd./US	gene: VM202 (Low & High HGF-X7) vs. Placebo	II	50	12/2012
NCT01410331/-	Juventas Therapeutics, Inc./US	biologics: JVS-100 (SDF-1) vs. Placebo	II	48	06/2013
NCT01408901/ PROPEL	NHLBI/US	biologics: GM-CSF vs. Placebo	II	267	12/2016
NCT01079403/ CeTMAd/ICPD200	Carlos III Health Institute/ES	auto·ATMSC (Low, Medium or High dose)	I/II	36	12/2011
NCT00883870/-	Stempeutics Research Pvt Ltd/IN	allo·BMMSC vs Placebo (Plasmalyte A)	I/II	20	12/2011
NCT00987363/-	Fundacion Progreso y Salud/ES	auto·BMMNC (Low, Medium or High dose)	I/II	60	12/2011
CTRI/2010/091/ 000565/-	Indian Gov./IN	auto·BM vs. Placebo	I/II	80	01/2013
NCT01351610/-	Apceth GmbH & Co. KG/DE	allo·BMMNC + PTA vs. PTA	I/II	30	03/2013
NCT01216865/-	Qingdao University/CN	allo·UCMSC vs. Placebo	I/II	50	07/2013
NCT01049919/-	Biomet Biologics, LLC/ US	Device (Auto·BMMNC) vs. Placebo	I/II	152	05/2014
NCT00913900/ SCRIPT-CLI	University of Wisconsin, Madison/US	auto·BM (CD133+) vs. Placebo	I	24	09/2012

Abbreviations: allo, allogeneic; AT, adipose tissue-derived; auto, autologous; BM, bone marrow; CN, China; DE, Germany; ES, Spain; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IN, India; JP, Japan; MNC, mononuclear cell; MSC, mesenchymal stem cell; NHLBI, National Heart, Lung, and Blood Institute; NL, Netherlands; Pt, patients; PTA, percutaneous transluminal angioplasty; SDF-1, stromal cell-derived factor-1; UC, umbilical cord.

demonstrated the safety and effectiveness of this novel approach²³). Apart from the phase or endpoint, the main difference between trials is whether they allow the inclusion of patients with gangrene. The Sanofi-Aventis NCT00566657 trial allowed patients with gangrene to be included, but the trial reported by Marui *et al.* did not, although patients with ulcers were included. This difference indicated that trial NCT00566657 targeted more severe CLI and this may be the most likely reason why it was not able to

meet the primary endpoint. Patient selection is crucial because the efficacy shown in a phase II trial is not always replicated in a phase III in which broader inclusion criteria are used. These reports suggest that if a phase III trial is not designed based on the results of the earlier phase trial which has been carried out to evaluate the potential of a drug candidate, the usefulness of the candidate is unverifiable.

As some clinical studies using stem cells have so far demonstrated an extension of walking distance,

and the improvement and disappearance of ulcers, this may be a promising therapy; however, it is necessary to execute large, randomized controlled trials before drawing definite conclusions about the effectiveness and safety of these regenerative medicines for CLI. A number of currently ongoing randomized controlled trials for CLI will further define the role of regenerative medicine (**Table 3**). These include the IMPACT study (UMIN000002280), CeTMAd/ICPD200 (NCT01079403) and “Mesenchymal Stem Cells in Critical Limb Ischemia” (NCT00883870).

The IMPACT study evaluates the therapeutic value of the transplantation of G-CSF-mobilized autologous peripheral blood mononuclear cells for no-option CLI patients with or without diabetes mellitus. It is an investigator-initiated, 1:1 randomized, multicenter study in Japan, including 144 patients with no option for revascularization or after failed revascularization. Patients are randomized either to G-CSF-mobilized peripheral blood mononuclear cell transplantation, or to a standard therapy based on the guideline “TASC2: Teaching and Assessing Science Core Concepts.” The primary endpoint is progression-free survival after one year. Secondary endpoints are changes in the Fontaine classification or Rutherford classification, overall survival, time to amputation, amputation-free survival, ulcer/gangrene size, severity of ischemic pain in the lower limbs, ankle brachial pressure index (ABPI), and the initial claudication distance (ICD) and absolute claudication distance (ACD) from baseline.

CeTMAd/ICPD200 is a randomized, single-site study in Spain to evaluate the safety and feasibility of regenerative therapy with intra-arterial infusion of adipose tissue-derived autologous mesenchymal stem cells (ATMSC), in diabetic patients with CLI without revascularization options. It will include 36 patients, 1:1 randomized to a low, medium or high dose of ATMSC, or standard therapy only. The primary endpoints are safety assessment (number of adverse events), angiographic evaluation of angiogenesis at the target limb for 6 months, and evaluation of angiogenesis by magnetic resonance angiography at the target limb for one year.

“Mesenchymal Stem Cells in Critical Limb Ischemia” is a randomized, double-blind controlled multicenter, commercial trial by Stempeutics Research Pvt Ltd in India to assess the safety and efficacy of intramuscularly administered allogeneic mesenchymal stem cells in patients with CLI. It will include 20 patients, randomized either to allogeneic mesenchymal stem cells or to Plasmalyte A as a placebo. The primary endpoint is safety assessment (number of adverse

events) and secondary endpoints are the increase in transcutaneous partial pressure of oxygen (TcPO₂) and ABPI for 6 months.

The invasiveness and the cost of therapy in addition to effectiveness and safety are becoming important factors in determining the market share. Furthermore, R&D of regenerative medicine intended for stroke and ischemic heart disease is being promoted, and the treatment policy is changing radically because many regenerative medicines are being put to practical use. This will have a great impact on clinical practice.

Directions in Regenerative Medicine

Regenerative medicine using somatic stem cells is being put to practical use, and many clinical studies are ongoing. In order to accelerate the utilization of regenerative medicine, standardized methods which include the origin of the cells, source, cell type, cultivation, purity, administration route, etc. should be developed. The standard methods should be judged by the degree of invasiveness of the extraction process, the technical difficulty, and the cost of cell processing to separate the required cells, because there are many possible sources, such as bone marrow, adipose tissue, and the umbilical cord, for example. Furthermore, the standard method will depend on the target disease. For example, for a disease which requires urgent treatment, the extraction of stem cells of personal origin (autologous) is not suitable. In this case, adaptation using allogeneic stem cells will be considered, despite the adverse effects and the increased medical expense of immunosuppressants, which are used to avoid rejection.

Several investigators have reported that the proliferative, migratory, and vasculogenic functions of CD34-positive cells including EPC are impaired in patients with coronary risk factors such as diabetes, hypertension, smoking, and aging^{24, 25}. Such interpatient differences in stem cell potential and the variation in patient characteristics in each treatment group may be critical for the evaluation of cell-based therapy. Ferraro *et al.* reported that diabetes induces changes in bone marrow physiology and microanatomy, and pointed to a potential intervention to overcome poor hematopoietic stem and progenitor cell mobilization, using AMD3100 to interrupt the interaction of CXCR12 (a molecule known to mediate hematopoietic stem cells) with its receptor CXCR4 in diabetic patients²⁶. Furthermore, Kawatomo *et al.* reported that patients with Buerger's disease were younger and had fewer coronary risk factors than those with PAD and that this difference in clinical background may

explain the result that the CD34-positive cell yield was significantly greater in patients with Buerger's disease than in those with PAD²⁷⁾. These results suggest that concomitant disease, including diabetes, may affect the function and mobilization of autologous stem cells for the target disease therapy, and in these patients investigators should consider the use of allogeneic stem cells prepared from healthy human volunteers. Furthermore, in patients in whom angiogenesis therapy using stem cells or growth factor delivery alone for CLI with gangrene and/or coronary risk factors such as diabetes has not yielded the expected effect, additional therapy such as a stem cell-growth factor combination and repetitive administration of each of these or a combination can be considered. However, in order to confirm the proof of concept concerning either combination or repetitive administration, investigators need to carry out well designed non-clinical trials.

In order to establish optimal regenerative medicine, investigators have to examine various possibilities before they start clinical trials. When two or more cell therapies exist, clinical trials are needed to compare them and to determine the most useful therapy, although it is good news for patients that the options for medical treatment are increasing. For this purpose, clinical trials designed on the basis of past evidence have to be carried out to prove the usefulness of the therapy.

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