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IV. 研究成果の刊行物・別冊

本研究と最も密接に関係する以下の総説を抜粋する

1. Tanaka M, Taketomi K, Yonemitsu Y.
Therapeutic angiogenesis: recent and future prospects of gene therapy in peripheral artery disease.
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2. 田中理子、米満吉和
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Therapeutic Angiogenesis: Recent and Future Prospects of Gene Therapy in Peripheral Artery Disease

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Abstract: Peripheral artery disease (PAD) is a highly prevalent disease, which still has unmet medical needs. Therapeutic angiogenesis for PAD, achieved by gene therapy, has achieved promising results in preclinical studies and early-phase clinical trials, yet few late-phase clinical trials have been conducted or have not shown efficacy. This article provides an overview of the progression of angiogenesis research in gene therapy field as it applies to PAD. The focus of angiogenic growth factors and clinical trials is introduced as a frontier of therapeutic angiogenesis. The article also includes insights into future directions from bench to bedside.

Keywords: Angiogenesis, clinical trials, gene therapy, growth factor, peripheral artery disease, plasmid.

1. INTRODUCTION

Peripheral artery disease (PAD) encompasses a range of conditions affecting the arteries in the limbs [1]. PAD is caused by stenosis or occlusive atherosclerosis in a vascular bed in the lower extremities [2]. Although the risk factors for PAD include smoking, diabetes mellitus, and hypertension, its prevalence increases with age, especially in people aged >70 years, in whom it increases to 15–20% [2-4]. PAD has become an important public health issue because it is a highly prevalent disease that affects up to 8 million patients in the US alone, which is 12% of the adult population [5, 6].

The clinical symptoms of PAD result from reduced blood flow to the legs. PAD is diagnosed by reduced ankle–brachial index (ABI), which is calculated as the ratio of the highest blood pressure in the right or left brachial artery and the highest blood pressure in the posterior tibial or dorsalis pedis [7]. Some patients with abnormally low ABI report no symptoms and 65–70% of patients with PAD are asymptomatic [8]. PAD is often asymptomatic or observed as numbness in the early stage; however, as PAD progresses, patients' quality of life (QOL) decreases because of limited walking ability (intermittent claudication [IC]), associated with pain in the lower legs during walking. If untreated, IC progresses to critical limb ischemia (CLI) in approximately one of four patients within 5 years [3], and induces pain at rest and ischemic ulcer/gangrene in the legs, resulting in substantial deterioration in QOL. This affects the prognosis of patients, who are forced to remain in bed for a long time. This issue has become a cause of medical economic pressure associated with long-term medical care.

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The prognosis of limb ischemia has greatly improved because of bypass surgery techniques, intra- and postoperative management, artificial blood vessel materials, endovascular treatment, and drug treatment. Based on previous studies showing that the 5-year survival rate is ~40% for CLI and 70–80% for IC, the most important objectives for the treatment of PAD are (1) avoiding amputation and (2) preventing progression from IC to CLI [2]. However, in patients with chronic arterial occlusion who have bypass surgery of the popliteal artery or the lower leg, the 5-year patency rate is ~40%. This suggests that such treatment is not yet sufficiently effective. In addition, there are many cases in which bypass surgery is not indicated because of the presence of severe peripheral lesions. Additionally, patients may not tolerate bypass surgery owing to a poor general condition associated with advanced arteriosclerosis in other organs. For CLI patients, mortality is as high as 20%, and major amputation is required within 1 year in 40% of patients [9]. There are many cases of complications with diabetes, which is an important risk factor of arteriosclerosis, because arteriosclerosis obliterans is a consequence of arteriosclerosis. Bypass surgery or endovascular treatment may not be indicated for patients with arteriosclerosis obliterans because they often have concurrent diabetic nephropathy that requires chronic dialysis. Additionally, they are often in poor health because of complications derived from diabetes, accompanied by diffuse vascular narrowing and advanced calcification. There is no effective treatment option available for such patients. Therefore, every year, tens of thousands of patients are estimated to progress gradually to CLI, resulting in amputation. The prognosis for patients who have had amputation is poor. According to the inter-society consensus for the management of PAD (TASC II), the mortality rate is twice as high as that for breast cancer, and is comparable to that of malignant tumors, such as colon cancer and Hodgkin's lymphoma [2].

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Table 1. Selected PAD gene therapy trials.

References	Patients	No. of subjects (treated/controls)	Angiogenic factor	Treatment	Vector	Route	Follow-up	Primary endpoints	Outcome*
Phase I									
Kim et al. [37]	CLI	9/-	VEGF	pCK-VEGF$_{165}$	Plasmid	Intramuscular	9 mo	Safety	Positive
Shyn et al. [38]	CLI	21/-	VEGF	phVEGF$_{165}$	Plasmid	Intramuscular	6 mo	Safety	Positive
Rajagopalan et al. [42]	IC	15/3	VEGF	adVEGF$_{121}$	Adenovirus	Intramuscular	12 mo	Safety	Equivocal
Comerota et al. [48]	CLI	51/-	FGF-1	NV1FGF	Plasmid	Intramuscular	6 mo	Safety	Positive
Yonemitsu et al. [67]	CLI	12/-	FGF-2	rSeV/df-hFGF-2	Sendai virus	Intramuscular	6 mo	safety and tolerability	Positive
Powell et al. [77]	CLI	78/26	HGF	pVAX1-HGF	Plasmid	Intramuscular	12 mo	Safety/TcPO2	Positive
Morishita et al. [82]	CLI	22/-	HGF	pVAX1-HGF	Plasmid	Intramuscular	6 mo	Safety/ABI, ulcer size	Positive
Gu et al. [85]	CLI	21/-	HGF	pCK-HGF-X7	Plasmid	Intramuscular	3 mo	Safety and tolerability	Positive
Rajagopalan et al. [101]	CLI	34/7	HIF-1	Ad2/HIF-1α/VP16	Adenovirus	Intramuscular	12 mo	Safety and efficacy	Positive
Phase II									
Kusumoto et al. [39]	Diabetes with CLI	27/27	VEGF	phVEGF$_{165}$	Plasmid	Intramuscular	100 d	Amputation	Negative
Rajagopalan et al. [43]	IC	72/33	VEGF	adVEGF$_{121}$	Adenovirus	Intramuscular	26 wk	Peak walking time	Negative
Makinen et al. [40]	IC/CLI	18/17/19	VEGF	adVEGF$_{165}$/ phVEGF$_{165}$	Adenovirus/ plasmid	Intraarterial	3 mo	Increased vascularity	Positive
Nikol et al. [50]	CLI	51/56	FGF-1	NV1FGF	Plasmid	Intramuscular	25 wk	Ulcer healing	Negative
Lederman et al. [55]	IC	127/63	FGF-2	rFGF2	Plasmid	Intraarterial	90 d	Peak walking time	Positive
Powell et al. [78]	CLI	21/6	HGF	VM202	Plasmid	Intramuscular	12 mo	Safety/TBI, VAS, ulcer	Positive
Creager et al. [102]	CLI	213/76	HIF-1	Ad2/HIF-1α/VP16	Adenovirus	Intramuscular	12 mo	Peak walking time	Negative
Grossman. [106]	IC	52/53	DEL-1	VLTS-589	Plasmid	Intramuscular	180 d	Peak walking time	Negative
Phase III									
Belch et al. [53]	CLI	259/266	FGF-1	NV1FGF	Plasmid	Intramuscular	12 mo	Amputation or death	Negative
Shigematsu et al. [83]	CLI	30/14	HGF	pVAX1-HGF	Plasmid	Intramuscular	15 mo	Rest pain, ulcer size	Positive

*Outcomes were evaluated for only primary endpoints, which did not include any improvement in secondary endpoints.

Currently, endovascular or surgical methods appear to be the best treatment option for PAD patients. Despite advanced techniques in surgical and endovascular treatment, a large number of patients are not suitable for revascularization [10]. Presently, there is no drug with proven efficacy for the treatment of CLI. Cilostazol and naftidrofuryl have been demonstrated as effective for the treatment of IC in several large clinical studies. Cilostazol is recommended as first-line treatment for IC, but there is no evidence to support the effectiveness of cilostazol in preventing the progression from IC to CLI [2]. Poor prognosis and increasing mobility and mortality of patients with PAD have created a growth need for new alternative therapies to induce angiogenesis, with most emphasis being placed on gene therapy [11].

Therapeutic angiogenesis alters the ischemic tissue to provide a proangiogenic environment through inducing formation of a capillary network [11]. It is based on the use of recombinant proteins or genes encoding angiogenic growth factors that enhance blood flow and expand the collateral circulation to ischemic tissue [1, 10]. Therapeutic angiogenesis has been proposed [9] as a novel therapy [12] and, since the end of the 1990s, it has become a clinically attractive potential alternative treatment option for chronic arterial occlusion [13, 14]. To date, the techniques of therapeutic angiogenesis using (1) recombinant protein (protein-based therapy), (2) gene delivery (gene therapy), and (3) bone-marrow/blood cells (cell therapy) have been clinically tested. Various growth factors exerting proangiogenic effects have been demonstrated to induce neovascularization in preclinical studies of therapeutic angiogenesis for PAD and coronary artery disease [15]. Proangiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), hypoxia-inducible factor (HIF)-1, and developmentally regulated endothelial locus (Del)-1 have demonstrated angiogenic effects in PAD. The important advantages of gene therapy are the transient local expression of proangiogenic protein without increase in systemic concentration, and the possibility of delivering two or more therapeutic genes [15]. An initial clinical trial was conducted using naked plasmid VEGF$_{165}$ in 1994 [16]. Since then, the proof of concept in preclinical studies has shown hopeful results, yet few clinical trials have been performed [17]. Gene therapy approaches from bench to bedside are not easy. The present article provides an overview of the progression of gene therapy, including protein-based therapy, for PAD as reported in clinical studies, and future directions.

2. GENE THERAPY

2.1. Vascular Endothelial Growth Factor

The VEGF family comprises seven major isoforms of VEGF-A–E and PLGF-1 and 2, and is the most widely studied endothelial growth factor [18, 19]. VEGF is a key regulator of endothelial cell migration, proliferation, vascular formation, and accelerated endothelial repair [20, 21]. Several preclinical *in vitro* as well as *in vivo* studies have shown effective angiogenesis in animal ischemic hindlimb models using VEGF, which is hypothesized as a potential therapy for PAD [22-25]. Also, zinc finger protein (ZFP) transcription factors are able to bind to DNA, found in the VEGF gene, and these engineered transcription factors can regulate gene expression *in vivo* [26, 27]. In particular, ZFP could activate VEGF-A, which is a mitogen that induces new blood vessel growth [28-30]. An engineered zinc finger, containing a transcription factor plasmid designed to activate the endogenous VEGF gene (ZFP-VEGF), was tested in a mouse and rabbit hindlimb ischemia model, and confirmed that ZEP-VEGF promotes angiogenesis, increases endogenous VEGF expression in striated skeletal muscle, improves perfusion, and limits tissue apoptosis [31-33].

Angiogenesis was also confirmed in a rabbit ischemic hindlimb model, which showed increased density of vessels in muscle after 1 week treatment with adenovirus-encoding

$VEGF_{165}$ [34]. Adenovirus-mediated $VEGF_{121}$ gene transfer preserved tissue perfusion after induction of rabbit and rat hindlimb ischemia models [35]. $VEGF_{121}$ and $VEGF_{165}$ have been recognized predominantly in clinical trials [1].

Initial clinical trials in CLI patients showed promising therapeutic results after intra-arterial and intramuscular administration of a plasmid mammalian expression vector carrying human VEGF-A_{165} cDNA (phVEGF-A_{165}) [14, 16]. Further phase I trials of phVEGF-A_{165} demonstrated improvements in ischemic peripheral neuropathy [36], ABI, vessel formation, new collateral vessels [37], ulcer healing, rest pain, and distal flow [38] in a group of CLI patients. VEGF was originally identified for its ability to increase vascular permeability [20], and leg edema was observed as the main complication. In a double-blinded placebo-controlled phase II study in diabetic patients with CLI, there was no significant reduction in amputation but there was improved ulcer healing [39]. There has only been one positive phase II trial of intra-arterial infusion of adenovirus VEGF-A_{165}, plasmid/liposome VEGF-A_{165} and Ringer's lactate to evaluate vascularity in patients with CLI and atherosclerotic infrainguinal occlusion or stenosis, who were suitable for percutaneous transluminal angioplasty [40]. Follow-up angiography revealed significant improvement of vascularity in the VEGF-treated patients.

An alternative for angiogenesis using a plasmid-based approach to deliver VEGF is CI-1023, a replication-deficient adenovirus encoding human VEGF isoform 121 ($Ad_{GV}VEGF_{121.10}$). In a phase I trial of $Ad_{GV}VEGF_{121.10}$ in CLI or IC, patients received $4\times10^{8.5}$ to 4×10^{10} particle units of the vector by intramuscular injection [41]. This resulted in increased acetylcholine and improved endothelial function but no improvement in basal blood flow [41]. Although CI-1023 was safe and well tolerated, no efficacy was found in a phase I trial using $Ad_{GV}VEGF_{121.10}$ in IC patients, who were administered 4×10^{8} to 4×10^{10} particle units or placebo via intramuscular injection. Thirty-three percent of the cohort receiving $Ad_{GV}VEGF_{121.10}$ reported edema in the extremity that received the injection [42, 43]. The Regional Angiogenesis with Vascular Endothelial Growth Factor (RAVE) study was a double-blind, placebo-controlled, phase II clinical study that tested the efficacy and safety of intramuscular $AdVEGF_{121}$. Patients with IC and unilateral PAD were stratified on the basis of diabetic status and randomized to placebo, low-dose (4×10^{9} particle units), or high-dose (4×10^{10} particle units) of $AdVEGF_{121}$ administered as 20 intramuscular injections in a single session. There was no difference in walking function, ABI, or QOL between the placebo and treated groups, and administration of AdVEGF-A_{121} was associated with increased peripheral edema [43].

2.2. Fibroblast Growth Factor

FGF belongs to a family of over 20 proteins. It was recognized as an angiogenic factor based on its induction of endothelial cell proliferation, migration, and morphogenesis, extracellular matrix degradation, and vessel maturation [44]. FGF has been extensively studied in the context of PAD. The bulk of experimental data related to the prototypic FGF-1 and FGF-2 have been obtained *in vivo* to establish the potential for gene therapy trials in PAD [45, 46]. Intramuscular administration of human naked plasmid DNA encoding FGF-1 (NV1FGF) to a hamster model of PAD fed a cholesterol-rich diet promoted vascular growth in ischemic muscles [47]. This provides a potential way to overcome perfusion defects in patients with PAD.

NV1FGF was administered intramuscularly into the ischemic thigh and calf for patients with unreconstructible PAD in a phase I clinical trial. NV1FGF was well tolerated and improved perfusion, although there was no evidence in a dose-dependent manner [48]. Later, NV1FGF plasmid distribution and transgene expression were tested to support the concept of multiple-site injection for therapeutic use [49]. The randomized double-blind phase II TALISMAN 201 trial was conducted to administer eight intramuscular injections of placebo or NV1FGF in CLI patients [50]. Although there was no difference in ulcer healing between the placebo and NV1FGF groups, there was a significant reduction in amputation risk at 12 months after administration of NV1FGF. The long-term evaluation of patients with CLI treated with NV1FGF or placebo was reported but the study was too small to evaluate safety and efficacy [51]. In the phase III, randomized, double-blind, placebo-controlled TAMARIS study, 525 CLI patients were enrolled at 171 sites in 30 countries [52]. The patients received eight intramuscular injections in the index leg of 0.2 mg/mL NV1FGF or placebo. There was no evidence of a reduction in amputation or death in the NV1FGF group [53].

The safety and efficacy of intra-arterial injections of FGF-2 was tested in patients with mild IC, which revealed that FGF-2 was safe, well tolerated, and improved lower extremity blood flow [54]. Recombinant (r)FGF-2 was infused intra-arterially in the phase II, randomized, double-blind, placebo-controlled TRAFFIC study of 190 patients with moderate to severe IC caused by infrainguinal atherosclerosis. This resulted in a significant increase in peak walking time at 90 days [55]. However, the TRAFFIC study was not powered to detect differences at 180 days with type II errors [56]. The limited clinical outcome and moderate adverse events owing to the systemic leakage of FGF-2 caused the TRAFFIC study to be terminated.

Various synergistic effects have been reported with FGF-2 [21, 57-59]. The optimum carrier for FGF-2 to induce pronounced angiogenesis has also been studied [60-62], but using recombinant Sendai virus (SeV) seems efficient [63-65]. Intramuscular injection of SeV strongly boosted FGF-2, with the levels being as much as 300-fold higher than at baseline [66]. A phase I/IIa, open-label, four-dose-escalation clinical trial was conducted to administer a new gene transfer vector based on a nontransmissible rSeV expressing the human FGF-2 gene (rSeV/df-hFGF-2) intramuscularly in 12 patients with CLI. rSeV/df-hFGF-2 was administered at 30 sites with varying ischemic conditions, up to 5×10^{9} CIU/60 kg, to one limb per patient. The vector was safe, well-tolerated, and significantly improved walking function [67]. Consequently, a phase IIb, randomized, double-blinded clinical trial is planned to start this year with IC patients [68].

2.3. Hepatocyte Growth Factor

HGF is a multifunctional cytokine that is a powerful mitogen and motility factor in various cell types, acting through

the receptor tyrosine kinase encoded by the c-*Met* proto-oncogene [69, 70]. HGF stimulates the growth of endothelial cells, morphogenesis, and angiogenesis [71, 72]. HGF administered as a recombinant protein or naked plasmid, and its synergistic effects with other proteins induce therapeutic angiogenesis in animal ischemic hindlimb models [73-76].

Several clinical trials have been performed to assess the safety and potential efficacy of HGF in patients with PAD. In the phase I/II HGF-STAT trial, HGF-plasmid (AMG001) was evaluated by in a double-blind, randomized, placebo-controlled study in 104 patients with CLI. Eight intramuscular injections of placebo or AMG001 was administered at 0.4 mg on days 0, 14, and 28 (low dose); 4.0 mg on days 0 and 28 (middle dose); and 4.0 mg on days 0, 14, and 28 (high dose). Injections were administered at two anterior and two posterior locations above the knee joint and four posterior locations below the knee. The plasmid was safe and well tolerated. However, there was no indication of effectiveness in ABI, toe brachial index (TBI), pain relief, ulcer healing, or amputation in either group, but there was a significant improvement in transcutaneous partial pressure of oxygen ($TcPO_2$) in the high-dose AMG001 group at 6 months [77]. The same research group conducted the HGF-0205 trial to evaluate duplex-ultrasound-guided injections of AMG001 in 27 patients with CLI. Limb perfusion was maintained and rest pain decreased, although there was no difference in major amputation of the treated limb or mortality at 12 months in the placebo group [78]. A phase III clinical trial is now in progress with 560 patients with CLI [79].

Another phase I/IIa, open-label clinical trial using naked plasmid DNA encoding HGF was conducted in CLI patients. HGF inserted into a 3.0-kb plasmid vector (pVAX1) was injected intramuscularly, either at 2 mg at four sites or 4 mg at eight sites, twice, into the calf or distal thigh of the ischemic limb under echosonographic guidance. The study demonstrated the safety and feasibility of HGF gene therapy, and improvements in ABI, pain relief, and ulcer size at 6 months [80, 81]. Despite the short duration of gene expression, the long-term efficacy of HGF gene therapy was reported, showing functional improvement in blood flow up to 2 years after administration [82]. A phase III, randomized, double-blind, placebo-controlled clinical trial was conducted in CLI patients in Japan. There was significant improvement of ischemic ulcers but no improvement in ABI and pain relief at 12 weeks in patients receiving plasmid HGF. No serious adverse events were noted [83]. HGF gene transfer did not induce limb edema as VEGF therapy did.

Recently, two isoforms of HGF (pCK-HGF-X7) were tested in phase I clinical trials for safety, tolerability, and preliminary efficacy in CLI patients. pCK-HGF-X7 is a 7377-base pair nonviral plasmid DNA expressing two isoforms of HGF consisting of the mammalian expression vector, pCK, and a genomic cDNA hybrid of human HGF gene, HGF-X7 [84, 85]. In preclinical studies, two human HGF isoforms, HGF_{728} and HGF_{723}, were effective for myoblast cell migration and stimulated umbilical vein endothelial cells. pCK-HGF-X7 also improved anterior wall thickness and capillary density and inhibited myocardial fibrosis an *in vivo* ischemic heart disease model [86, 87]. Both phase I clinical trials were open-label, dose-escalation studies in which 2–16 mg pCK-HGF-X7 was injected intramuscularly into the calf and/or thigh on days 1 and 15. Both trials demonstrated improved pain relief, $TcPO_2$, and ulcer healing without any serious adverse events, which supported conducting phase II clinical trials [84, 85]. A phase II clinical trial was designed as a double-blind, randomized, placebo-controlled study to administer pCK-HGF-X7, 16 injections four times, to CLI patients. This study will have been completed by now but no reports of the results are available at present [88].

2.4. Hypoxia Inducible Factor-1α

HIF-1α is a regulator of cellular response to hypoxia. HIF-1α plays a key role in the initiation of rapid gene expression in response to low oxygen levels [89-95]. In a preclinical study in a limb ischemic model, an adenovirus encoding a constitutively active form of HIF-1α (AdCA5) improved tissue perfusion and enhanced neovascularization [96-99]. A naked DNA encoding HIF-1α/VP16 hybrid transcription factor was constructed by truncating HIF-1α at amino acid 390 joining the transactivation domain of herpes simplex virus VP-16 protein. This naked DNA enhanced arteriogenesis and angiogenesis responses by improving calf blood pressure ratio, angiographic score, regional blood flow, and capillary density in a rabbit model of hindlimb ischemia [100]. In a phase I, dose-escalation clinical trial, a constitutively active HIF-1α with adenovirus (Ad2/HIF-1α/VP16) was injected into the lower extremity of 34 no-option patients with CLI. Ad2/HIF-1α/VP16 is a recombinant, replication-deficient adenovirus type 2 with an insert containing the DNA-binding and dimerization domains from the HIF-1α subunit and VP16 transactivation domain to facilitate constitutive activation. A single treatment of 10 intramuscular injections of Ad2/HIF-1α/VP16 (100 μL for a total dose of 0.1 mL) in a single limb was demonstrated to be safe and improved ulcer healing and rest pain in some patients, thus supporting further trials [101]. In the phase II, randomized, double-blind, placebo-controlled WALK study, 289 patients with IC were treated intramuscularly with Ad2/HIF-1α/VP16 [102]. Ad2/HIF-1α/VP16 (2×10^9, 1×10^{10}, or 2×10^{11} viral particles) or placebo was administered by 20 injections to each leg. However, Ad2/HIF-1α/VP16 did not improve peak walking time, claudication onset time, ABI, or perceived walking distance. They concluded that gene therapy with HIF-1α is not effective for patients with IC secondary to PAD.

2.5. Developmentally Regulated Endothelial Locus

Del-1 is an extracellular matrix protein expressed in ischemic tissue, which stimulates angiogenesis in the absence of exogenous growth factors, and is considered to be a novel therapeutic agent for patients with PAD [103, 104]. In a preclinical study, a plasmid encoding human Del-1 protein formulated with poloxamer 188 promoted new blood vessel formation and restored muscle function in mice and rabbits [105]. The phase IIa DELTA trial compared the plasmid encoding the angiomatrix protein Del-1 in conjunction with poloxamer 188 (VLTS-589) with poloxamer 188 alone as a control. A total of 105 IC patients received 21 intramuscular injections (2 mL each) into each leg, which amounted to 42 mg VLTS-589 or the control agent. Both VLTS-589 and the control agent significantly improved peak walking time,

claudication onset time, ABI, and QOL, but there was no difference in outcome measures associated with Del-1 plasmid [106]. A major placebo effect was observed in this study, which underlines the importance of having a placebo group [12]. A subsequent phase IIb clinical trial was negative [18].

3. FUTURE DIRECTIONS

In the past two decades, experimental studies and clinical trials have established that gene therapy can be regarded as an alternative method of treatment for PAD [15]. A meta-analysis from six randomized controlled trials has shown a significant clinical improvement compared with placebo in patients with PAD (odds ratio = 1.427; 95% confidence interval = 1.03–2.0; $P = 0.033$), and no differences were found in mortality from any cause [107, 108]. However, the marked efficacy of angiogenic gene therapy in preclinical studies was not demonstrated in clinical trials. There are many boundaries in gene therapy approaches such as safety, trial design, type of vector, duration of treatment, administration route, and dose [109].

3.1. Study Design

Although recombinant protein approaches may also achieve the desired response, several phase I–III clinical trials showed superior results by using gene constructs rather than protein [10]. Pharmacokinetics and pharmacodynamics of various types of gene therapy, however, are not all sophisticated. Moreover, many clinical trials have not been adequately powered to evaluate clinical efficacy. A large randomized controlled study is needed to overcome this and to reduce placebo effects.

Selection of PAD patients for gene therapy has been complicated because the optimal treatment of patients with PAD, IC and CLI differs considerably [12]. CLI patients have limited life expectancy with severe comorbidity, and thus a high percentage may experience adverse events including vascular events. Therefore, gene therapy within this population may be biased toward failure [78]. Clinical trials of patients with CLI have often enrolled "no-option" patients with wounds that vary from extensive gangrene to mild skin erosions, which requires a large number of this heterogeneous group of patients to establish efficacy. TASC II recommends that amputation-free survival remains the gold standard endpoint for trials in patients with CLI [2].

Furthermore, biomarkers for the endpoints are difficult to establish in PAD. X-ray angiography has a spatial resolution of 200 μm, whereas the caliber of new arterioles and small arteries may be in the range of 20–200 μm [56]. Therefore, blood flow is tested by limb functional tests such as the treadmill, ABI and TBI, and TcO_2 tension for CLI or near-infrared spectroscopy for IC. Those parameters are used in a variety of assessment methods that are of uneven quality. The optimized walking function test in the setting of multicenter clinical trials remains uncertain [110].

3.2. Gene Delivery Vectors

Appropriate vector selection is one of the most critical factors in successful gene therapy. Gene delivery vectors can be classified as nonviral and viral. Nonviral methods use naked plasmid DNA to transfer the gene encoding the targeted angiogenic protein to the tissue [10]. Plasmids are usually expressed in the target cells for 1–2 weeks [111]. Plasmid-mediated gene therapy of human tissues has low efficiency if compare using viral vectors, which tend to be efficient tools for induction of angiogenesis [112], although no trials have been conducted to compare directly the efficacy of plasmids versus viruses [1]. Viral vectors are well adapted to escape the host immune system and carry transgenes into the genome [1]. The major advantages of using adenoviral, retroviral, lentiviral, and Sendai-viral vectors have been revealed in most preclinical and clinical studies of gene therapy for PAD and cardiovascular disease [113]. With regard to therapy of angiogenesis, the important factors are local concentration and duration of expression of angiogenic factors, and thus cell targeting is not a critical factor. However, successful clinical studies have not been conducted; therefore, optimal expression level should be determined. Although viral vectors are efficient, there are limiting factors for their clinical use. Their immunogenicity limits the dilation of gene expression as well as repeat administration, particularly in the case of adeno-associated virus (AAV). Long-term gene expression can be achieved by AAV or lentivirus; however, that is controversy issue of inefficient gene transfer such as case of forming malignancies due to long lasting expression of angiogenic factors.

The nonoptimal delivery method of proangiogenic genes into tissue is also one of the disappointing results of clinical trials of therapeutic angiogenesis [114]. Delivery methods for bioactive endothelial proteins and genes encoding growth factors have several barriers, including: (1) complex process; (2) host immune system that neutralizes virus-based gene delivery, degrades naked plasmid DNA, and ability of the host to integrate and express the cloned gene; (3) lack of standardized dosage of growth factors and genes; (4) short half-life of growth factors; and (5) technical difficulties to deliver growth factors and genes to target sites noninvasively [18].

3.3. Delivery Route, Dose, and Duration of Therapy

Genes are commonly administered by intra-arterial or intramuscular injections of a plasmid or viral vector. Angiogenic factors administered by intra-arterial injection have short half-lives and less focal delivery to the target site, but may be more relevant for treating ischemic tissue in skin rather than muscle [1]. Intramuscular injections, however, have the advantages of prolonged therapeutic effects and repeated administration [10]. Optimization of the delivery route depends on the characteristics of the angiogenic factor, but stimulating angiogenesis is required for improvement of muscle perfusion as well [111]. In addition, a major challenge for therapeutic angiogenesis is to determine a sufficient amount of angiogenic factor at the ischemic site to promote actively neovascularization [10]. At present, no evidence is available for the optimal dose of angiogenic gene therapy. Investigators seem to decide the dose from basic preclinical data as well as consideration of safety issues, which tends to reduce the dose administered in clinical trials. Moreover, how to determine the injection sites is also a vital consideration in gene delivery. To optimize proangiogenic

effects of genes at the most needed sites, detection of the presence of donor arteries is essential.

3.4. Safety

Particular theoretical concerns related to angiogenesis are hemorrhage from new vessels, tumor growth, retinal neovascularization, and enhanced hypertension, inflammatory responses, and atherogenesis [111]. At this point, there is no evidence of adverse effects in target and non-targeted organs, which is clearly related to angiogenic gene therapy. Long-term follow-up of 2 years was reported in a phase I/IIa clinical trial using naked plasmid DNA-encoding HGF, and there were no severe complications and adverse effects caused by gene transfer [82]. However, one patient who died from adenoviral infection has heightened awareness of the potential danger [115]. Overall, in several clinical trials with angiogenic gene therapy, adverse effects, such as death or amputation, have generally been consistent with the baseline rate in the population of patients studied and no differences were found in the placebo groups. However, safety monitoring is still required until long-term safety data from large trials are available [116]. All gene therapy for PAD seems to be safe and well tolerated, although peripheral edema is a frequent side effect in treatment using recombinant VEGF.

CONCLUSION

Despite noticeable success of proangiogenic gene therapy in numerous preclinical studies and early-phase clinical trials, clinical benefits still do not fulfill expectations. There are still many limitations in gene therapy approaches and techniques. To overcome failure from bench to bedside, gene therapy is still waiting for large, randomized, placebo-controlled, double-blind, phase III clinical trials and long-term safety evaluation. The efficacy of cell therapies at the preclinical level has not yet reached that of gene therapy to promote angiogenesis; therefore, combination of gene and cell therapies could have potential for angiogenic therapy. New angiogenic mechanisms, the effects of administering multiple angiogenic growth factors, and combinations of factors, such as bionanotechnology and cell therapy, may enhance synergistic effects. With further studies providing insight into the angiogenesis factors, we hope to gain a benefit and limit the suffering for PAD patients.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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PATIENT CONSENT

Declared none.

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特集 心血管再生医療はどこまで来たか

FGF-2による遺伝子治療の臨床*

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Key Words：FGF-2, peripheral artery disease (PAD), Sendai virus vector

はじめに

閉塞性動脈硬化症に起因する下肢慢性動脈閉塞症(peripheral artery disease：PAD)は，わが国において近年急速に罹患者数が増加している疾患の一つである．薬物療法や血行再建術を行っても十分な治療効果が望めず，下肢切断やQOLが低下していく治療抵抗性症例に対し，遺伝子治療によって虚血組織に新たな血管を誘導することで，虚血状態改善を目指す「血管新生遺伝子治療」が新たな治療戦略として注目されている(図1)．本稿ではPADにおける*FGF-2*遺伝子治療の臨床について概説する．

FGF血管新生遺伝子治療

線維芽細胞増殖因子(*fibroblast growth factor*：*FGF*)は線維芽細胞をはじめ，血管内皮細胞，血管平滑筋細胞など多種の細胞増殖を促進するため，血管新生効果が期待できる．FGFの優れている点は血管内皮細胞増殖作用のみならず，プラスミノーゲンやコラゲナーゼの活性促進に寄与し，管腔形成を刺激する点である．*FGF*を用いた血管新生遺伝子治療はPADあるいは冠動脈疾患を対象に実際に臨床応用がされているが，

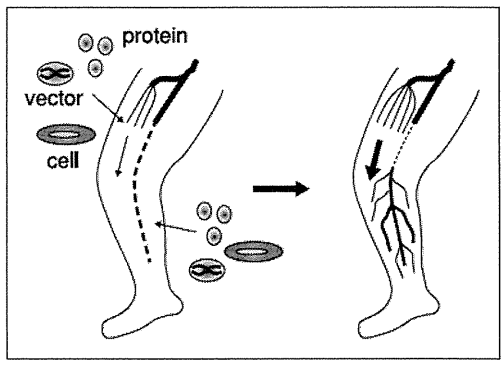

図1 血管新生遺伝子治療の概念図

PADを対象にその研究が進んでいる*FGF*ファミリーは，*FGF-1*：酸性線維芽細胞増殖因子(*acidic fibroblast growth factor*：*aFGF*)と*FGF-2*：塩基性線維芽細胞増殖因子(*basic fibroblast growth factor*：*bFGF*)である．サノフィ社の実施してきたヒト*FGF-1*遺伝子を発現するプラスミドNV1FGFの第I～III相試験では，基礎・前臨床試験で得られた有用性が続く後期相試験で再現できず開発は中止された[1)~3)]．アデノウイルスベクターを用いた*FGF-4*(*Ad5-FGF-4*)遺伝子治療は冠動脈疾患を対象に研究が進んでいるものの，PADに対しては開発がすでに中止されている[4)~6)]．これらの開発中止例とは一線を画し，*FGF-2*遺伝子治療はその効果が期待されている(表1)．

*FGF-2*を用いた血管新生遺伝子治療の可能性は

* Clinical experience of FGF-2 gene therapy.

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表1 FGF遺伝子を用いた代表的なPAD臨床研究の開発状況

学術誌 発表年 試験名	研究相	対象	症例数 (実薬/ プラセボ)	遺伝子	ベクター	投与経路	フォロー アップ 期間	主要 評価項目	結果*	文献 番号
<FGF-1>										
2002年	I	CLI	51/—	NV1FGF	Plasmid	筋肉注射	6か月	安全性	Positive	1
2008年 TALISMAN	II	CLI	51/56	NV1FGF	Plasmid	筋肉注射	25週間	潰瘍治癒	Negative	2
2011年 TAMARIS	III	CLI	259/266	NV1FGF	Plasmid	筋肉注射	12か月	下肢切断回避率・死亡率	Negative	3
<FGF-2>										
2000年	I	IC	13/6	rFGF2	Plasmid	動脈注射	6か月	安全性	Positive	7
2002年 TRAFFIC	II	IC	127/63	rFGF2	Plasmid	動脈注射	90日	最大歩行時間	Positive	8
2013年	I/IIa	CLI	12/—	rSeV/df-hFGF-2	Sendai virus	筋肉注射	6か月	安全性	Positive	9
<FGF-4>										
2005年	I	CLI	10/3	Ad5FGF-4	Adenovirus	筋肉注射	12週間	安全性	Positive	4

*結果は主要評価項目に対する評価のみで，副次的評価項目である血流改善効果などの評価は反映していない．
CLI：重症虚血肢，IC：間歇性跛行

多くの動物実験ですでに立証されている．最初の臨床研究では，2000年にLazarousら[7]が実施した間歇性跛行を対象にした第I相試験において，*FGF-2*の動脈注射の投与における安全性が示唆された．その後，間歇性跛行症状を呈するPAD患者190例を対象として，*FGF-2*遺伝子を用いた第II相無作為二重盲検試験(TRAFFIC試験)が行われた．本試験はプラセミドを使用しており，動脈注射による*rFGF-2*(*recombinant FGF-2*)を単一投与(30 ug/kg)群，重複投与群，プラセボ群で比較した結果，投与後90日目に単一投与群において最大歩行時間とABIで有意な差がみられた[8]．プラスミドはエンドソームで細胞膜内に取り込まれ，さらに核膜を通過するがゲノムには取り込まれずに遺伝子を発現する．安全性は高いが，取り込まれる細胞が限られており，ウイルスベクターを用いる遺伝子治療に比べると遺伝子導入率はかなり低くなる．世界ではじめてウイルスベクターを用いたFGF-2遺伝子治療は日本で実施された．本試験はセンダイウイルスベクターを使用し，重症虚血肢を呈する12例に筋肉注射による投与が行われ，安全性と有用性が示唆されている[9]．本試験の詳細はFGF-2遺伝子治療臨床試験の実際として，後に詳しく述べる．

その他の血管新生因子を用いたPAD遺伝子治療研究の現状として，先行したプラセミドやアデノウイルスベクターを用いた血管内皮増殖因子(*vascular endothelial growth factor*：*VEGF*)遺伝子治療では，前臨床試験で得られた有用性が示せずに開発が中止された．プラセミドを用いた肝細胞増殖因子(*hepatocyte growth factor*：*HGF*)遺伝子治療は現在もその開発が進んでいる状況である．

高性能ウイルスベクターを用いたFGF-2遺伝子治療臨床試験の実際

九州大学で現在進めている「塩基性線維芽細胞増殖因子(*bFGF/FGF-2*)搭載センダイウイルスベクターによるPADに対する臨床研究」は，圧倒的に高い治療効果を示す血管新生遺伝子治療である．

1．センダイウイルスベクターの特徴と骨格

遺伝子治療に用いるウイルスベクターの中で，国産ベクターとしてはセンダイウイルスベクターが応用されている．センダイウイルスはパラミクソウイルスに属するRNAウイルスである．1999年に米国ペンシルベニア大学でのアデノウイルスベクターでの死亡事故[10]や，2001年のフランスでのレトロウイルスベクターによる白血病の発症報告[11]があったことから，より安全性の高い新たなベクターの開発が望まれている中，われわれが独自に開発を進めている非伝搬型組み換えセンダイウイルスベクター(*recombinant Sendai virus*：*rSeV/dF*)は，導入細胞質内でRNAのまま転写・複製を行うことより宿主細胞染色体に影

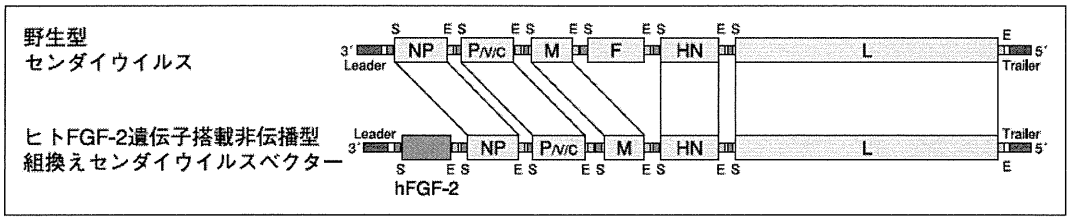

図2　センダイウイルスベクターの構造

響を与えることはない．またF遺伝子を欠損させることにより，感染二次粒子を放出しないこともわかっている．センダイウイルスは既存のベクターと比べ，以下のような利点があげられる[12]．

①安全性が高い：細胞質RNAベクターとして，治療遺伝子を細胞質で疑似ウイルス遺伝子として発現するため，宿主細胞染色体への障害の危険性がない．また，一般の低分子薬剤と異なり，生体内で免疫系により排除されるため，重篤な免疫不全でない限り胆道・腎機能不全においても適応可能である．

②遺伝子導入率が高い：哺乳動物の細胞膜上に存在するシアル酸と*HN*(ヘマグルチニン/ノイラミニダーゼ)を介して結合するため，多くの細胞に感染可能である．また，非分裂細胞においても同様に多くの細胞腫，組織に広く遺伝子が導入される．

③遺伝子発現率が高い：センダイウイルスは細胞に感染するとただちにゲノムの転写・複製を開始し，遺伝子発現は対数的に増加するため，既存のベクターと比較してきわめて高い遺伝子発現を示す．そのため，治療遺伝子に要する有効量の粒子数がほかのベクターに比べ1/1,000以下で済む．

④病原性がない：センダイウイルスの人への病原性は報告されていない．

2．FGF-2遺伝子を発現する遺伝子治療製剤(DVC1-0101)の開発

われわれはPAD治療薬として，組み換えセンダイウイルスを基本骨格とし，*FGF-2*の生理活性を利用したRNA製剤DVC1-0101の開発を進めている．センダイウイルスベクターを用いた実験的検証において，既知の血管新生因子を下肢虚血局所で発現させた場合の治療効果について比較した結果，*FGF-2*が血管新生因子として最も再現性よく高い治療効果を示し，ほかの血管新生因子と異なる特徴的なシグナル系を活性化しながら，*VEGF*および*HGF*など内因性血管新生因子群を強力に誘導することが明らかとなった[13)~15)]．*FGF-2*は間葉系細胞を利用してarteriogenesis誘導因子である*MCP-1*(*monocyte chemoattractant protein-1*)および*VEGF*の両シグナルを独立して作動させることにより，還流効率の高い機能性血管誘導を促進すると考えられた[16]．そこで，*FGF-2*遺伝子を搭載したF遺伝子欠損非伝搬型組み換えセンダイウイルスベクター(*rSeV/dF-hFGF2*)を新規治療薬候補として選択した(図2)．

3．FGF-2血管新生遺伝子治療臨床試験(第I/IIa相)の概要

九州大学では2006年4月～2010年8月まで，*rSeV/dF-hFGF2*を用いた遺伝子治療臨床研究(第I/IIa相)をGMP/GCP準拠で実施した．本臨床研究は，これまでに国際的に人体投与実績のないF遺伝子欠損非伝搬型組み換えセンダイウイルスベクター(*rSeV/dF*)を基本とする遺伝子治療製剤(開発コード：DVC1-0101)を投与することをかんがみ，安全性を主要評価項目とした4段階の用量漸増オープンラベル方式のデザインで実施された(図3)．DVC1-0101は，ヒト線維芽細胞増殖因子(*hFGF-2*)遺伝子を下肢骨格筋に投与することで強制的に発現させ，下肢骨格筋内局所におけるhFGF-2の濃度を一時的に上昇させることで新しい血管を再生させる．この作用により虚血に陥った下肢を救済し，安静時疼痛の緩和と歩行機能を改善させることにより，患者のQOLを向上させることが期待されている．対象疾患は，閉塞性動脈硬化症あるいはバージャー病(Fontaine III度あるいはIV度)の重症虚血肢で，人工血管あるいは自家静脈による大腿動脈以下の血行再建術の適応がなく，2週間の継続した薬

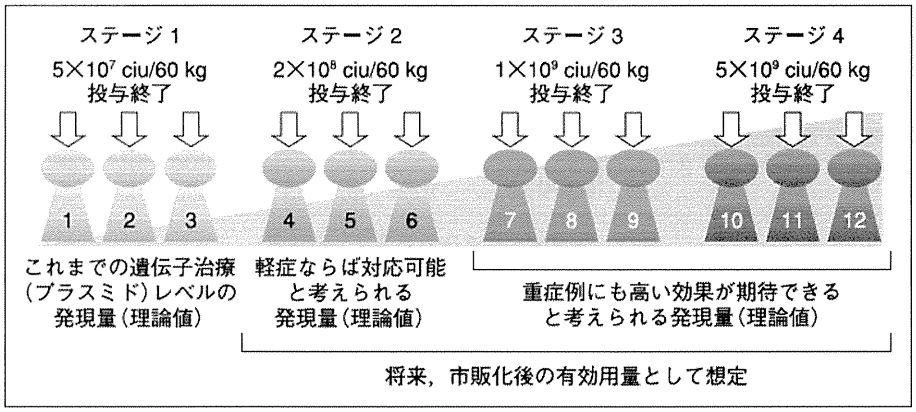

図3　FGF-2遺伝子治療臨床試験(第 I/IIa相)のデザイン

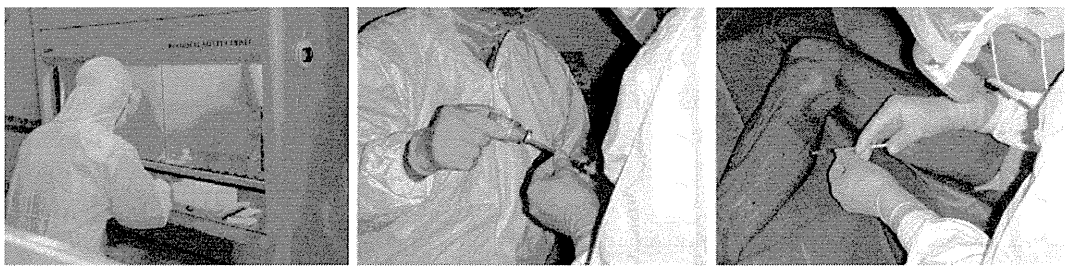

図4　FGF-2遺伝子投与の実際

物療法で改善がみられない症例であった．DVC1-0101投与量は，動物実験において最大の効果を示した投与量(5×10^9 ciu/60 kg：ciu＝cell infectious unit遺伝子導入効率)の100分の1の5×10^7 ciu/60 kgを第1ステージとし，以降第2ステージ2×10^8 ciu/60 kg，第3ステージ1×10^9 ciu/60 kg，第4ステージ5×10^9 ciu/60 kgと設定し，各ステージ3症例，合計12症例への投与を実施した(図3)．カルタヘナ法(遺伝子組み換え生物等の使用等の規制による生物の多様性の確保に関する法律)に基づき，試験薬の投与は遺伝子治療室(バイオセーフティーレベル2)で実施され，投与後のウイルス残存検査で陰転化が認められた後に隔離解除を行った(図4)．観察期間は投与後6か月とし，2011年3月に厚生労働省へ研究終了報告がなされているが，現在も投与後5年間の追跡調査期間中である．

4．FGF-2血管新生遺伝子治療臨床試験(第 I/IIa相)の結果

対象は平均年齢65歳(48～82歳)であった．有害事象は，投与後12例120件発生し，2014年8月末日現在，遺伝子治療臨床研究実施計画書において定義された「重篤な有害事象」に該当したものは6症例10件に発生している．いずれの事象も，用量依存性は認められず，臨床研究薬との因果関係が明確である有害事象は認められていない．また，尿中・血中ウイルス残存検査のうち，血中ベクターゲノム検出検査では，12例中3件に陽性所見が検出されたが，最長で15日目に陰転化がみられた．血中炎症性サイトカイン動態と血中血管新生因子動態(*FGF-2*, *VEGF*, *HGF*)については，用量依存性は認められていない．これらの結果から，本臨床研究において使用されるレベルの投与用量において，特定の併存疾患を有しない重症虚血肢患者に対するDVC1-0101の認容性は高く，安全に使用できると考えられた．

臨床効能については，臨床病期評価として，安静時疼痛，潰瘍，ABI，TPI，PVRを測定した．歩行機能評価としては，最大歩行可能距離，跛行出現距離を測定した．その他の参考評価として，サーモグラフィー，レーザードップラー血流，血管造影検査などの検査を実施した．その

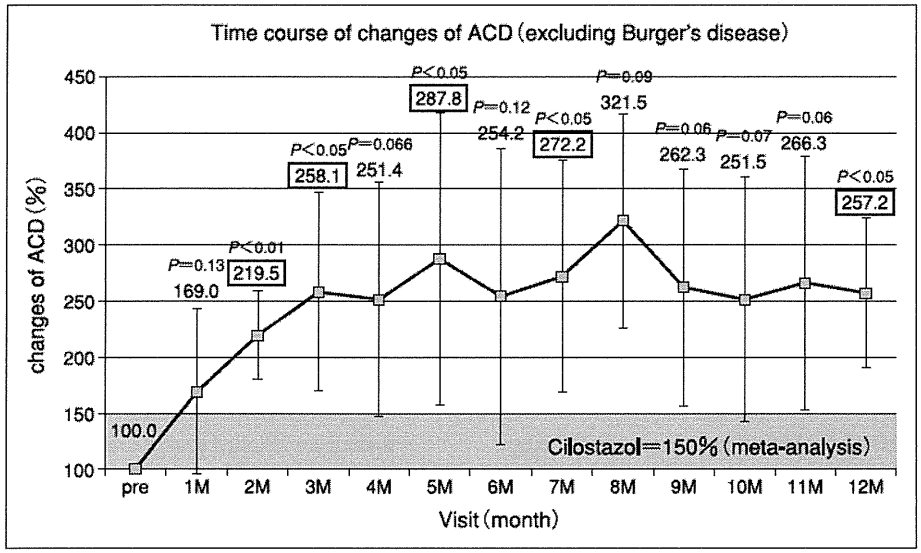

図5　DVC1-0101試験における最大歩行距離増加率

結果，安静時疼痛の消失と歩行機能改善が持続的に認められるなど，DVC1-0101の有効性を示唆するデータが複数の評価項目で得られた．特に最大歩行距離増加率の推移については，基準治療薬シロスタゾールで約150%へ改善するのに対し，DVC1-0101では投与後1年後においても300%弱程度の延長がみられている(図5)．

5．今後の課題と展望

先行研究の結果からDVC1-0101は特に臨床的に歩行機能の持続的な改善に寄与する可能性が考えられるため，続く医師主導型治験(第IIb相用量反応試験)では，高度間歇性跛行肢(最大歩行距離200 m以下)を対象とし，プラセボ対照，並行群間用量探索試験のデザインとした．すでに治験薬(DVC1-0101)の国内GMP製造が完了し，治験届の提出も終えており，2014年秋の第一症例登録を目前に控えている．

また，米国NIH-RAC会議においてDVC1-0101の米国治験計画が了承されており，米国FDA/CBERで開催されたINDミーティングでは日本での先行研究の結果が高く評価され，米国での第II相からの臨床研究開始とその試験デザインについて基本合意が得られた．

PADに対する血管新生遺伝子治療研究を実施する上で，課題となるのは血行動態パラメータのreliabilityである．現行ではパラメータとして，トレッドミルによる歩行機能が第1選択肢にあげられるが，測定者や施設間におけるデータのばらつきが大きいことや，プラセボ効果が問題視されている[17][18]．われわれは下肢閉塞性動脈硬化症の診断・治療指針の国際的取扱規約TASC IIのチーフエディターであり，同疾患治療薬臨床試験の世界的第一人者である米国コロラド大学心臓血管内科William Hiatt教授の全面協力のもと，同氏がトップを努めるコロラド大学CPC Clinical Researchが開発したEQuIP(Endpoint Quality and Intervention Program)を日本ではじめて導入した．このEQuIPは，下肢虚血に特異的な検査方法を標準化することで，確実な主要評価項目の測定と測定誤差の軽減による測定データの質向上を実現している．将来の第III相国際共同治験へ向けたEQuIPによる日米のシームレスな効能評価システム構築により，当該分野でのドラッグラグの解消に邁進している．

おわりに

血管新生療法が臨床応用され始めてから20年が経過したが，基礎・前臨床試験で得られた有用性が，続く後期相試験で再現されることは難しい．PADの病態の複雑さから，対象の選択(間歇性跛行もしくは重症虚血肢)や投与部位の検討が困難であることと，遺伝子導入の安全性と効

率性など遺伝子治療全般が抱える問題点があり，今後の臨床応用での大規模かつ長期的な検討がbench to bedsideには必要である．対象疾患は違えども，リポ蛋白質リパーゼ欠損症を抱えた再発性急性膵炎を煩う患者を対象に，2012年にはGlybera®(一般名：alipogene tiparvovec)が遺伝子治療としてははじめて欧州医薬品審査庁の認可を獲得しており，今後の血管新生遺伝子治療にも期待が寄せられているところである．

文　献

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