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ORIGINAL ARTICLE

Safety and efficacy of sustained release of basic fibroblast growth factor using gelatin hydrogel in patients with critical limb ischemia

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Abstract As a form of therapeutic angiogenesis, we sought to investigate the safety and efficacy of a sustained-release system of basic fibroblast growth factor (bFGF) using biodegradable gelatin hydrogel in patients with critical limb ischemia (CLI). We conducted a phase I–IIa study that analyzed 10 CLI patients following a 200-μg intramuscular injection of bFGF-incorporated gelatin hydrogel microspheres into the ischemic limb. Primary endpoints were safety and transcutaneous oxygen pressure (TcO_2) at 4 and 24 weeks after treatment. During the follow-up, there was no death or serious procedure-related adverse event. After 24 weeks, TcO_2 (28.4 ± 8.4 vs. 46.2 ± 13.0 mmHg for pretreatment vs after 24 weeks, $p < 0.01$) showed significant improvement. Regarding secondary endpoints, the distance walked in 6 min (255 ± 105 vs. 318 ± 127 m, $p = 0.02$), the Rutherford classification (4.4 ± 0.5 vs. 3.1 ± 1.4, $p = 0.02$), the rest pain scale (1.7 ± 1.0 vs. 1.2 ± 1.3, $p = 0.03$), and the cyanotic scale (2.0 ± 1.1 vs. 0.9 ± 0.9, $p < 0.01$) also showed improvement. The blood levels of bFGF were within the normal range in all patients. A subanalysis of patients with arteriosclerosis obliterans ($n = 7$) or thromboangiitis obliterans (Buerger's disease) ($n = 3$) revealed that TcO_2 had significantly improved in both subgroups. TcO_2 did not differ between patients with or without chronic kidney disease. The sustained release of bFGF from biodegradable gelatin hydrogel may offer a safe and effective form of angiogenesis for patients with CLI.

Keywords Basic fibroblast growth factor · Angiogenesis · Critical limb ischemia · Drug delivery system

Introduction

Peripheral arterial disease (PAD), often caused by atherosclerosis obliterans (ASO) or thromboangiitis obliterans (TAO) (Buerger's disease), is a common circulatory problem in which narrowed arteries reduce blood flow to limbs.

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PAD can progress into critical limb ischemia (CLI), the most severe form. Among patients with CLI who are unreconstructible or in whom attempts at reconstruction fail, approximately 40 % will lose their leg within 6 months, and up to 20 % will die [1]. Although medications and surgical or endovascular revascularization help to alleviate CLI [2, 3], they have not become well-established treatment protocols for CLI patients.

Therapeutic angiogenesis (i.e., the administration of growth factor, gene therapy and cell transplantation) is a new treatment strategy designed to improve the perfusion of new blood flow to ischemic vascular beds that also attempts to reduce the damage and necrosis experienced by ischemic tissues. However, there are issues that still need to be resolved: the short half-life period of the angiogenic factors, the long-term safety [4], and the invasiveness involved in collecting the implanted cells [5]. For these reasons, no form of therapeutic angiogenesis has become a standard treatment option. Therefore, to overcome these issues, we developed a new drug delivery system (DDS) for basic fibroblast growth factor using a biodegradable acidic gelatin hydrogel.

Basic fibroblast growth factor (bFGF), first reported by Gospodarowicz [6, 7], is known to proliferate mesenchymal cells and induce neovascularization. Recombinant human bFGF has already been used clinically for the treatment of bedsores and ulcers in Japan. However, as the biological half-life of bFGF in the body is very short [3] because of rapid diffusion and enzymolysis, repeated administration is required for clinical use.

We developed a DDS for potent growth factors, such as bFGF, using biodegradable acidic gelatin hydrogel. Gelatin is a biopolymer used in many medical applications because of its nontoxic nature, as it is a water-soluble, denatured protein originating from collagen, which is the main protein component of connective tissues such as bone and skin. Because of these characteristics, our DDS is significant in terms of its simplicity and biosafety [8, 9]. We have also demonstrated the effectiveness of bFGF released from gelatin hydrogel in various animal models [10–18]. In addition, we reported the clinical application of this DDS for cases involving CLI [19].

In this study, unlike in the previous study [19], our gelatin hydrogel microspheres were made in accordance with standards of good manufacturing practice (GMP) in consideration of a future clinical trial, and this study was certified by the high-level medical care standards of the Japanese Ministry of Health, Labor and Welfare. Additionally, we previously reported the effectiveness of bFGF-incorporated gelatin hydrogel mainly in patients with TAO [19]. Unfortunately, most CLI patients suffer from severe or end-stage ASO. Thus, in the present study, we sought to investigate the safety and efficacy of bFGF-incorporated gelatin hydrogel, mainly in patients with CLI brought on by ASO, and we evaluated the efficacy for patients with ASO or TAO separately. Since renal dysfunction is a major cause of atherosclerosis, we also investigated the respective outcomes in patients with or without renal dysfunction.

Materials and methods

Study population

We included patients with CLI (ASO or TAO), defined by symptoms of rest pain and/or ischemic foot ulcers. The patients were not candidates for catheter-based angioplasty or surgical revascularization ("no options" patients). Patients were excluded if they had any of the following characteristics: history of cell transplantation or gene therapy for CLI, malignancy, proliferative diabetic retinopathy, hemodialysis, or severe infection. The study protocol was approved by The Kyoto University Graduate School and Faculty of Medicine, Ethics Committee in November 2009 as C-336. This study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN 000002671).

Preparation of bFGF-incorporated gelatin hydrogel microspheres

Human recombinant bFGF with an isoelectric point of 9.6 was purchased from Kaken Pharmaceutical Co (Tokyo, Japan). A gelatin sample with an isoelectric point of 5.0 was isolated from the porcine skin through the alkaline process (Nitta Gelatin Co, Osaka, Japan). Gelatin hydrogel microspheres were prepared in an aseptic room as previously described [20, 21]. Briefly, gelatin hydrogels were prepared through the glutaraldehyde cross-linking of gelatin in an aqueous solution. The resulting hydrogels were soaked in an aqueous solution of glycine for 3 h to block free aldehyde groups in the hydrogels; they were then washed with double distilled water. The aqueous solutions used here were sterilized by filtration through membrane filters with a pore size of 0.22 μm. Gelatin hydrogels were pulverized using a homogenizer. The homogenates were passed through sieves with different mesh sizes. The microspheres, with diameters ranging from 50 to 100 μm, were collected and freeze dried. After packing the microspheres in sterile glass vials, we confirmed that they were free of both residual glutaraldehyde and bacterial contamination. To incorporate bFGF into the gelatin microspheres, an aqueous solution of bFGF (200 μg) was applied to the freeze-dried microspheres (100 mg); they were then stored at an ambient temperature for 1 h. The microspheres slowly released bFGF for approximately 3 weeks. Gelatin

hydrogel microspheres were made in accordance with the GMP standards established by the Japanese Ministry of Health, Labor and Welfare.

Study design

This study used an open-label, single dose injection of bFGF-incorporated biodegradable gelatin hydrogel. The bFGF-incorporated gelatin hydrogel microspheres were injected into the gastrocnemius of the unilateral ischemic limb (single administration), and both the safety and feasibility of this method were evaluated (via the phase I–IIa study). We used a 200-μg dose of bFGF based on the safety standards adhered to in our previous animal studies [11–16] and the findings of other clinical reports [19]. We did not use a control group because the main purpose of the present study was to develop proof of concept for this particular DDS, and more importantly, the patients were not candidates for conventional treatments. Oral medications such as vasodilators or antiplatelet drugs remained unchanged during the study period. According to the protocol, intravenous administration of prostaglandin E1 and antithrombin was not given during the study period. Patients were followed up to 24 weeks after the treatment.

Endpoints

The primary endpoints were the safety and the efficacy of the treatment, as defined by the evaluation of adverse events, laboratory data, the blood level of bFGF, vital signs, physical findings, and the improvements in transcutaneous oxygen pressure [TcO_2 (mmHg)]. The improvements were evaluated by the changes from baseline to week 4 and 24. We checked for adverse events every day for 30 days, and at 8, 12, and 24 weeks after treatment on an outpatients basis, and blood tests were performed at 1, 2, and 3 days and 1, 2, 4, 8, 12, and 24 weeks after treatment. The blood concentrations of bFGF were measured 1, 3, 7, and 14 days after treatment.

The secondary endpoint was the efficacy of the treatment, as defined by the improvements in the ankle-brachial pressure index (ABI), the toe-brachial pressure index (TBI), the distance walked in 6 min [22], Rutherford classification [23], rest pain in the supine position, and the status of cyanosis and ulcer healing.

Transcutaneous oxygen pressure measurement

We measured the TcO_2 as previously described [19]. Briefly, after cleansing the measurement site with ethanol, we applied the probe to the toe web. When a steady-state temperature was achieved, a value expressed in mmHg was recorded. The measurements were performed in a room set at 28 °C and recorded after 30 min of continuous monitoring. TcO_2 has been shown to accurately predict the presence of significant vascular disease, to verify an appropriate correction by means of revascularization, and to confirm the success of major or minor amputations with or without revascularization [24].

Pressure measurements

The ABI and TBI were obtained at baseline and at 4 and 24 weeks after treatment. These data were each measured twice over a period of 24 h and an average value was used.

The distance walked in six minutes measurement

The six-minute walk test was performed at baseline and at 4 and 24 weeks after treatment. The walking distance was measured twice over a period of 24 h and an average value we used. In our protocol, patients were not allowed to sit on a chair during the six-minute walk test, but patients were permitted to walk with a cane or to rest.

Pain assessment

Pain assessment was evaluated by the rest pain scale: 0, no pain; +1, very slight pain which did not require non-steroidal anti-inflammatory drugs (NSAIDs); +2, slight pain which disappeared with NSAIDs; +3, moderate pain with NSAIDs; +4, severe pain unresolved with NSAIDs.

Cyanosis assessment

Cyanosis was assessed by the cyanotic scale: 0, no cyanosis; +1, localized on toes; +2, extensively on toes; +3, extended to dorsum of foot; +4, extended to ankle joint.

Ulcer healing

The status of all patients' ulcers was evaluated in terms of location and size and depth at pretreatment and at 4 and 24 weeks after treatment.

Treatment

The bFGF-incorporated gelatin hydrogel microspheres were dissolved into 40 ml of saline and intramuscularly injected into each injection site of the lower thigh (40 sites), in a 3 × 3-cm grid using a 22-gauge needle under spinal anesthesia. The average procedure time was approximately 15 min.

Statistical considerations

A sample size of 10 patients was determined to provide 90 % power to detect a minimum clinically

meaningful effect on change in TcO_2 (effect size: 1.04), at a one-sided significance level of $p = 0.05$. This trial was expected to have at least 90 % power to detect 10 mmHg changes in TcO_2 under an assumption that the SD of change in TcO_2 was 9.7 mmHg, which was derived from earlier clinical studies [19]. Changes in TcO_2, ABI, TBI, the distance walked in 6 min, and ulcer healing from baseline to week 4 or week 24 were examined with one-sided paired t tests. Changes in the category of the Rutherford classification, rest pain, and status of cyanosis were examined with one-sided Wilcoxon signed rank-sum tests. A p value <0.05 was taken to be significant without adjustment for multiplicity. Pre-specified subgroup analysis was planned for patients who had ASO, TAO, CKD, and for those without CKD individually. All analyses were performed according to the intention-to-treat principle and included all patients treated. All statistical analyses were performed by academic biostatisticians using SAS software version 9.2 (SAS Institute Inc, Cary, NC, USA).

Results

A total of 10 patients were entered into this study (66.9 ± 12.2 years old, seven males) (Table 1). Among them, seven patients were diagnosed with ASO and the others with Buerger's disease. Six patients had chronic kidney disease [estimate glomerular filtration rate (eGFR) of <60 ml/min/1.73 m^2].

Primary endpoints

There was no death or procedure-related adverse events attributable to topical use of sustained-release bFGF. The treatment did not induce focal inflammation or edema at the injection site. The post-procedural blood level of bFGF was undetectable or within the normal value in all patients.

There were two serious adverse events requiring hospitalization that occurred in the same patient (case 10) at 18 and 24 weeks after the treatment. These events were cellulitis (18 weeks after treatment) and intracranial hemorrhage (24 weeks after treatment). Neither of these severe adverse events was thought to be related to the treatment.

Concerning the adverse events, there were transient elevations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), C-reactive protein, creatine phosphokinase, and creatinine according to the Common Terminology Criteria for Adverse Events (CTCAE version 4.03, published by the U.S. National Cancer Institute, 14 June 2010). These values, however, returned to the normal range without further treatment (Table 2).

TcO_2 (28.4 ± 8.4 mmHg, pretreatment), which is the main analysis of validity, showed a significant improvement at both 4 weeks (42.8 ± 10.3 mmHg, $p < 0.01$) and 24 weeks (46.2 ± 13.0 mmHg, $p < 0.01$) after treatment (Table 3).

Secondary endpoints (Table 3)

The distance walked in 6 min increased significantly at both 4 weeks ($p < 0.01$) and 24 weeks ($p = 0.02$) compared

Table 1 Patient characteristics

Case	Age/sex	Diagnosis	DM	CKD	The location of occlusive lesion	TcO_2 (mmHg)	ABI	TBI	6 min (meter)	Pain[b]	Cyanosis[c]	Foot ulcer
1	78/M	ASO	No	Yes	Right SFA	37.5	0.53	(–)[a]	275	+1	+3	No
2	73/M	ASO	No	Yes	Left SFA	18.5	0.63	0.32	180	+4	+2	Yes (1100 mm^2)
3	79/F	ASO	No	Yes	Left SFA	21.5	(–)[a]	(–)[a]	103	+1	+3	No
4	64/F	ASO	Yes	Yes	Right Pop. A	18.5	0.49	(–)[a]	190	+2	+3	Yes (234 mm^2)
5	75/M	ASO	Yes	Yes	Right Pop. A	40.5	0.9	(–)[a]	305	+1	+3	No
6	51/M	Buerger	No	No	Right Pop. A	25.5	0.64	(–)[a]	368	+1	+1	Yes (15 mm^2)
7	42/M	Buerger	No	No	Left Pop. A	25.0	0.8	(–)[a]	365	+1	+1	No
8	60/M	Buerger	No	No	Left SFA	32.5	0.64	0.3	415	+1	+1	No
9	68/M	ASO	Yes	Yes	Left SFA	39.0	0.42	(–)[a]	163	+3	+3	No
10	77/F	ASO	No	No	Left Pop. A	25.0	(–)[a]	0.32	190	+2	0	Yes (6500 mm^2)

ASO arteriosclerosis obliterans, *Buerger* thromboangiitis obliterans (Buerger's disease), *DM* diabetes mellitus, *CKD* chronic kidney disease, *SFA* superficial femoral artery, *Pop. A* popliteal artery, *TcO_2* transcutaneous oxygen pressure, *ABI* ankle-brachial pressure index, *TBI* toe-brachial pressure index, *6 min* the distance walked in 6 min, *Pain* rest pain scale (see above), *Cyanosis* cyanotic scale (see above)

[a] Unmeasurable

[b] Rest pain scale: 0, no pain; +1, very slight pain which did not require non-steroidal anti-inflammatory drugs (NSAIDs); +2, slight pain which disappeared with NSAIDs; +3, moderate pain with NSAIDs; +4, severe pain unresolved with NSAIDs

[c] Cyanotic scale: 0, no cyanosis; +1, localized on toes; +2, extensively on toes; +3, extended to dorsum of foot; +4, extended to ankle joint

Table 2 Adverse events within 6 months according to CTCAE version 4.03

Case	Adverse events	The time of peak after treatment	Prognosis of event	Review result
3	↑ C-reactive protein (maximum: 2.4 mg/dl)	6 months	Recovered without treatment	No relation
6	↑ AST/ALT (maximum AST: 54 IU/l, ALT: 84 IU/l)	2 days	Recovered without treatment	Unknown
6	↑ Creatine phosphokinase (maximum: 342 IU/l)	3 months	Recovered without treatment	No relation
9	↑ AST/ALT (maximum AST: 107 IU/l, ALT: 75 IU/l)	7 days	Recovered without treatment	Unknown
9	↑ Creatinine (pretreatment: 1.7 mg/dl, maximum: 2.3 mg/dl)	2 months	Recovered without treatment	No relation
10	↑ AST/ALT (maximum AST: 98 IU/l, ALT: 96 IU/l)	3 days	Recovered without treatment	Unknown

The normal range: C-reactive protein <0.2 mg/dl, AST: 10–34 IU/l, ALT: 7–55 IU/l, creatine phosphokinase: 22–198 IU/l, creatinine: 0.6–1.3 mg/dl

AST aspartate aminotransferase, *ALT* alanine aminotransferase

Table 3 Changes in parameters (mean ± SD)

	Pretreatment	4 weeks	24 weeks
TcO_2 (mmHg)	28.4 ± 8.4	42.8 ± 10.3*	46.2 ± 13.0*
ABI	0.60 ± 0.15	0.59 ± 0.15	0.63 ± 0.18
TBI	0.31 ± 0.01	0.35 ± 0.11	0.28 ± 0.06
6 min (m)	255 ± 105	336 ± 93*	318 ± 127*
Rutherford classification	4.4 ± 0.5	3.4 ± 1.2*	3.1 ± 1.5*
Rest pain scale	1.7 ± 1.0	1.2 ± 1.3*	1.2 ± 1.3*
Cyanotic scale	2.0 ± 1.1	1.1 ± 0.7*	0.9 ± 0.9*
Foot ulcer (mm^2)	785 ± 2037	752 ± 2036	495 ± 1243

4 and 24 weeks after treatment

Abbreviations (see Table 1)

* $p < 0.05$ vs. pretreatment

with pretreatment results. Regarding the Rutherford classification, the parameter was improved significantly at both 4 weeks ($p = 0.02$) and 24 weeks ($p = 0.02$) after treatment. Six patients were changed for the better at 4 and 24 weeks, and no patients worsened. In terms of the rest pain scale, significant improvement was observed at both 4 weeks ($p = 0.03$) and 24 weeks ($p = 0.03$) after treatment, with five patients showing improvement.

Concerning the cyanotic scale, the parameter improved significantly at 4 weeks ($p = 0.03$) and 24 weeks ($p < 0.01$) after treatment. The area of the foot ulcer was found to have been reduced at both 4 weeks ($p = 0.13$) and 24 weeks ($p = 0.14$) when compared with the pretreatment area, but it did not result in any significant improvement. Among the improved patients, an intractable ulcer disappeared in 1 of the 4 patients, and ischemic rest pain disappeared in 4 of the 10 patients at 24 weeks after treatment.

The ankle-brachial pressure index did not increase at either 4 weeks ($p = 1.00$) or 24 weeks ($p = 0.22$) after treatment. Similarly, the toe-brachial pressure index did not increase at either 4 weeks ($p = 0.13$) or 24 weeks ($p = 1.00$).

Subgroup analyses (Tables 4 and 5)

We conducted subanalyses of ASO or TAO, and the presence or absence of chronic kidney disease (CKD). There were seven patients with ASO and six with CKD. Regarding ASO patients, TcO_2 significantly increased at both 4 weeks ($p < 0.01$) and 24 weeks ($p < 0.01$) after treatment. In terms of TAO patients, TcO_2 also significantly increased at 4 weeks ($p = 0.03$). Although a significant difference was not found at 24 weeks in TAO patients ($p = 0.053$) because of the small number of cases, TcO_2 did show an improvement tendency. Among patients with CKD, TcO_2 significantly improved at both 4 weeks ($p < 0.01$) and 24 weeks ($p < 0.01$) after treatment. In patients without CKD, TcO_2 also significantly improved at both 4 weeks ($p < 0.01$) and 24 weeks ($p = 0.01$).

Discussion

In the present study, we have shown the efficacy of the sustained release of bFGF using biodegradable gelatin hydrogel, as evaluated by TcO_2, the distance walked in 6 min, the rest pain scale, and the cyanotic scale. Regarding foot ulcers, the area of the ulcer also tended to shrink. These results indicate an increase in blood flow in the ischemic limb. In terms of safety, there was no death or major procedure-related adverse event. In the subclass analyses, the primary endpoint also improved significantly in patients with ASO, TAO, CKD, and in those without CKD. In addition, the mean ± SD value of TcO_2 before treatment was 28.4 ± 8.4 mmHg in this study, while the mean value of TcO_2 before treatment was 53.5 ± 5.2 mmHg in our previous study [19]. Although this study included more severe cases than did the previous one, we revealed that the therapeutic efficacy was equivalent to that of our previous study. We therefore believe this method offers a promising form of therapy, especially in terms of its safety and efficacy.

Table 4 Subanalysis of changes in patients with ASO or TAO (mean ± SD)

	ASO (7 patients)			Buerger (3 patients)		
	Pretreatment	4 weeks	24 weeks	Pretreatment	4 weeks	24 weeks
TcO_2 (mmHg)	28.6 ± 10.0	44.4 ± 12.1*	45.8 ± 14.3*	27.7 ± 4.2	39.0 ± 3.0*	47.2 ± 11.8
ABI	0.57 ± 0.16	0.57 ± 0.16	0.57 ± 0.18	0.69 ± 0.09	0.63 ± 0.12	0.75 ± 0.13
TBI	0.31 ± 0.01	0.38 ± 0.12	0.28 ± 0.05	0.30 ± 0.00	0.28 ± 0.00	0.28 ± 0.08
6 min (m)	201 ± 69	291 ± 67*	265 ± 114	383 ± 28	441 ± 42*	441 ± 34
Foot ulcer (mm^2)	1119 ± 2407	1074 ± 2411	706 ± 1463	5 ± 8.7	0 ± 0	0 ± 0

Abbreviations (see Table 1)

ASO arteriosclerosis obliterans, *Buerger* thromboangiitis obliterans (Buerger's disease)

* $p < 0.05$ vs. pretreatment

Table 5 Subanalysis of changes in patients with CKD or without CKD (mean ± SD)

	Patients with CKD (6 patients)			Patients without CKD (4 patients)		
	Pretreatment	4 weeks	24 weeks	Pretreatment	4 weeks	24 weeks
TcO_2 (mmHg)	29.3 ± 10.8	46.0 ± 12.3*	44.3 ± 15.0*	27.0 ± 3.7	37.9 ± 3.3*	49.1 ± 10.4*
ABI	0.59 ± 0.17	0.59 ± 0.16	0.60 ± 0.19	0.62 ± 0.16	0.58 ± 0.14	0.67 ± 0.19
TBI	0.30 ± 0.01	0.36 ± 0.13	0.27 ± 0.05	0.31 ± 0.01	0.33 ± 0.10	0.30 ± 0.07
6 min (m)	203 ± 75	302 ± 66*	300 ± 70*	334 ± 99	387 ± 113*	343 ± 197
Foot ulcer (mm^2)	222 ± 440	170 ± 328	162 ± 276	1629 ± 3248	1625 ± 3250	994 ± 1988

Abbreviations (see Table 1)

CKD chronic kidney disease

* $p < 0.05$ vs pretreatment

There are between 500 and 1000 new cases of CLI every year in a European or North American population of one million, and the incidence of major amputations from large population or nationwide data ranges from 120 to 500/million/year [1]. Although treatment of CLI is improving [1, 25] and surgical or endovascular revascularization helps to alleviate CLI [2, 3], they have not become well-established treatment protocols for CLI patients. Among CLI patients, about 20 % are not indicated for percutaneous transluminal angioplasty or surgical revascularization [26]. Therefore, efforts at improving regenerative medicine have been increasing. Recently, numerous clinical trials for CLI have been conducted based on advances in regenerative medicine, as have been done on myocardial regeneration therapy [27, 28], and although they may have shown good results and appropriate levels of safety, there are still concerns [5, 29–32]: the half-life period of angiogenic factors is short, gene therapy could not control the expression period and level of expression, and there are immune or inflammatory responses of genetic materials [33, 34].

For cell transplantation, the collection of cells requires general anesthesia or the systemic administration of G-CSF [5]. We were able to overcome these problems using bFGF and gelatin hydrogel microspheres as the DDS. Although the biological half-life of bFGF in its free form is very short [35], the half-life period of bFGF can be prolonged through its combination with gelatin hydrogel. In fact, since it has been shown that bFGF-incorporated gelatin hydrogel microspheres persist for a few weeks [8, 9], this therapy does not require high dosages or repeated administration. We have previously used bFGF with gelatin hydrogel microspheres and shown its efficacy in several animal [10–18] and clinical studies [19]. The basic fibroblast growth factor we used is recombinant human bFGF, which has already been clinically utilized for the treatment of bedsores and ulcers in Japan, and our gelatin hydrogel microspheres were made in accordance with GMP standards. Therefore, the advantages of this method are safety and efficacy.

In the present study, there was no death or procedure-related adverse event attributable to topical use of sustained-release bFGF, but some adverse drug reactions, such as the elevation of Cr, AST, and ALT, were observed. It is said that bFGF (FGF-2) is mitogenic for many renal cell types including glomerular endothelial and glomerular epithelial cells but this is also the case for mesangial and proximal tubule cells of the kidney [36]. In this study, only one patient's Cr increased from 1.7 mg/dl at pretreatment to 2.3 mg/dl at 12 weeks after treatment. At 24 weeks, however, this patient's Cr declined to 1.5 mg/dl without

further treatment. The effects of bFGF with gelatin hydrogel microsphere were thought to disappear after approximately 1 month [37, 38], so the possibility of a causal relationship with other treatments seemed to be very low.

In contrast to the improvement of other endpoints, ABI and TBI did not show significant improvement. Some studies of angiogenesis for CLI patients, which showed an efficacy of ulcer healing, an increase of collaterals, or an improvement of claudication walking distance, did not show any significant improvement in ABI [30, 32]. Shigematsu et al. [30] reported that the change in ABI was not associated with the ulcer healing, suggesting that HGF plasmid may act at the micro vascular level. Lee et al. [32] also reported that no change in ABI was noteworthy because this value primarily depends on the pressure of large arteries, and adipose tissue-derived mesenchymal stem cells form numerous small collateral arteries. Since TBI also depends on large arteries, the same could be said of our ABI and TBI results.

Therapeutic angiogenesis tended to be more effective in patients with TAO (Buerger's disease) than in patients with ASO [39, 40]. Concerning the long-term clinical outcome of angiogenic therapy using bone marrow mononuclear cells implantation, Matoba et al. [41] reported that the 3-year overall mortality rate was 0 % in the TAO group and 20 % in the ASO group, and that the 3-year amputation rate was 9 % in the TAO group and 40 % in the ASO group. Actually, PAD commonly results from ASO in the lower extremities [1], which was consistent with the findings of our study. Chronic kidney disease also exacerbates the condition of patients with CLI. The occurrence of CLI in patients with kidney insufficiency portends a strikingly high rate of subsequent major amputation and mortality, compared to those patients without kidney insufficiency [42]. In this study, TcO_2 significantly increased in not only TAO patients but also in ASO patients both at 4 and 24 weeks after treatment. Similarly, TcO_2 improved significantly in patients with CKD as well as in patients with normal kidney function. Since subset analyses based on a comparison of underlying disease were less convincing due to the underpowered size of the cohort, we did not perform a comparative analysis among subgroups. Therefore, a larger cohort of patients would be needed for a subsequent examination of this nature.

This study presents some challenges to subsequent studies. First, dialysis patients with CLI were excluded because most dialysis-dependent patients are in poor physical condition, which can be a strong confounding factor. Second, the number of the patients was not sufficient to conclude statistical significance. While the data of the subanalyses were not the validation results of the efficacy in our phase I–IIa study, and we understood the problem of sample size, we reported that the results of these subanalyses will provide important information for a future study. Third, because this study was a phase I (-IIa) open-label study, we did not include a control group, nor did we assess the dosage effect. There is an ethical concern related to having a control group whose patients had an increased possibility of lower leg amputation as a result of the disease. Additionally many reports on therapeutic angiogenesis [43–47] for CLI also did not have a control group because of these same concerns, so we felt it was the better ethical choice. Fourth, since the follow-up period was a mere 24 weeks, the long-term efficacy still needs to be evaluated. Therefore, we intend to address the above issues when investigating the benefits of this method in greater detail in further studies.

Conclusion

These data provide encouraging evidence of therapeutic angiogenesis via the sustained release of bFGF through the use of gelatin hydrogel. Additionally, more appropriately powered clinical investigations are warranted.

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Conflict of interest None declared.

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