A Novel Approach to Therapeutic Angiogenesis for Patients With Critical Limb Ischemia by Sustained Release of Basic Fibroblast Growth Factor Using Biodegradable Gelatin Hydrogel

--- An Initial Report of the Phase I-IIa Study ---

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Background  Limb ischemia remains a challenge. To overcome shortcomings or limitations of gene therapy or cell transplantation, a sustained release system of basic fibroblast growth factor (bFGF) using biodegradable gelatin hydrogel has been developed.

Methods and Results  A phase I-IIa study was performed, in which 7 patients had critical limb ischemia. They were intramuscularly injected with 200 μg of bFGF-incorporated gelatin hydrogel microspheres into the gastrocnemius of the ischemic limb. End-points were safety and feasibility of treatment after 4 and 24 weeks. One patient was excluded from the study for social reasons, but only after symptomatic improvements. In the evaluation of the other 6 patients, significant improvements were observed in the distance walked in 6 min (295±42 m vs 491±85 m for pretreatment vs after 24 weeks, p=0.023) and in transcutaneous oxygen pressure (53.5±5.2 mmHg vs 65.5±4.0 mmHg, p=0.03). The rest pain scale also improved (3.5±0.2 vs 1.0±0.6, p=0.022). The ankle-brachial pressure index improved at 4 weeks but not at 24 weeks. Among 5 patients who had a non-healing foot ulcer, the ulcer was completely healed in 3 patients, reduced in 1, and there was no change in 1 patient at 24 weeks. The blood levels of bFGF were undetected or within the normal level in all patients.

Conclusions  The sustained release of bFGF from gelatin hydrogel might be simple, safe, and effective to achieve therapeutic angiogenesis because it did not need genetic materials or collection of implanted cells, and because it did not have any general effects, which was supported by there being no elevation of the bFGF serum level. (Circ J 2007; 71: 1181 – 1186)

Key Words: Angiogenesis; Basic fibroblast growth factor; Limb ischemia

Gene therapy or cell transplantation might have shown encouraging results in various clinical studies in the cardiovascular field.6,8 However, there are still unignorable concerns for effectiveness, immune or inflammatory responses of genetic materials,9–11 and invasiveness, which stems from the collection of implanted cells such as general anesthesia or granulocyte-stimulating factor (G-CSF) administration.6–8,12–14

As a novel approach, we have developed a drug delivery system of potent growth factors such as basic fibroblast growth factor (bFGF), using biodegradable acidic gelatin hydrogel.15,16 We have demonstrated the effectiveness of bFGF protein released from gelatin hydrogel in various animal models (ie, either non-diabetic or diabetic) for acute myocardial infarction, prevascularization for cardiomyocyte transplantation to the ischemic heart, limb ischemia, and bone regeneration of the sternum.17–24 One of the most important advantages of the system is that it uses biodegradable gelatin hydrogel instead of genetic materials as a sustained release carrier for angiogenic growth factors.

Based on the results in animals, we started a clinical trial to test the safety and feasibility of the sustained release system of bFGF from gelatin hydrogel in patients with critical limb ischemia, who had no option of medical or surgical treatment.

Methods

Study Population

Patients qualified to participate in the study if they had chronic limb ischemia [atherosclerosis obliterans (ASO) or thromboangiitis obliterans (Buerger’s disease)], including...
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 bovine bone through the alkaline process (Niita Gelatin Co, Osaka, Japan). Gelatin hydrogel microspheres were prepared in an aseptic room as previously described. 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. Gelatin hydrogels were pulverized by using a homogenizer. The homogenates were passed through sieves with different mesh sizes. The microspheres with a diameter ranging from 50 to 100 μm were collected and freeze-dried. After sterilization of the microspheres, we confirmed that there were no residual glutaraldehyde and bacterial contamination in the microspheres. To incorporate bFGF into gelatin microspheres, an aqueous solution of bFGF (200 μg) was applied to freeze-dried microspheres (100 mg); they were then left at an ambient temperature for 1 h. The microspheres slowly released bFGF for approximately 3 weeks.

Study Design

The bFGF-incorporated gelatin hydrogel microspheres were injected into the gastrocnemius of the unilateral ischemic limb (single administration), and its safety and feasibility were evaluated (ie, the phase I-IIa study). We used the dose of bFGF (200 μg) in view of safety standards according to our previous animal studies and other clinical reports. We did not have a control group in this study because that was only required in the phase I trial and, more importantly, the patients who participated were ill (ie, they were not candidates for conventional treatments). Oral medications such as vasodilators or anti-platelet drugs remained unchanged during the study period. No intravenous drugs such as prostaglandins were used during the study period. Patients were followed up to 24 weeks after the treatment.

End-Points

The primary end-point was the safety of the treatment, as evaluated by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) Ver 3.0. The secondary end-point was feasibility of the treatment, as defined by the improvements in rest pain, the distance walked in 6 min (m), the ankle-brachial pressure index (ABI), transcutaneous oxygen pressure (TcO2; mmHg), laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) (the mean value of the blood perfusion in the back of the ischemic foot: relative unit), thermography (mean temperature of the ischemic foot: °C), and the status of ulcer-healing. We measured the TcO2 as follows: after cleansing the measurement site with ethanol, we applied the probe, and heated the skin surface to 43.5°C. When a steady-state temperature was achieved, a value expressed in mmHg was recorded. The measurement was performed when patients were breathing room air in a supine position.

The improvements were evaluated by the changes from baseline to week 4 and 24. Rest pain was scaled as previously reported. The blood level of bFGF was measured before treatment, and 1, 2, 7 and 28 days after the treatment.

Procedure

The bFGF-incorporated gelatin hydrogel microspheres were dissolved into 40 ml of saline and intramuscularly injected into each injection site (40 sites), with a 3 × 3 cm grid and by using a 23-gauge needle under spinal anesthesia.
Table 1  Results of the Study

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>DM</th>
<th>HD</th>
<th>Past history</th>
<th>ABI</th>
<th>6 min</th>
<th>Pain***</th>
<th>TcO2</th>
<th>LDPI</th>
<th>Therm</th>
<th>Foot ulcer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24/M</td>
<td>Buerger</td>
<td>No</td>
<td>No</td>
<td>Bypass (occluded)</td>
<td>+15%</td>
<td>+87%</td>
<td>0</td>
<td>+89%</td>
<td>+105%</td>
<td>+1.1°C</td>
<td>Healed</td>
</tr>
<tr>
<td>2</td>
<td>64/M</td>
<td>ASO</td>
<td>Yes</td>
<td>Yes</td>
<td>BMCT</td>
<td>-12%</td>
<td>-26%</td>
<td>+4</td>
<td>-10%</td>
<td>+1%</td>
<td>+0.9°C</td>
<td>No change**</td>
</tr>
<tr>
<td>3</td>
<td>40/M</td>
<td>Buerger</td>
<td>No</td>
<td>No</td>
<td>Bypass (occluded)</td>
<td>+9%</td>
<td>+26%</td>
<td>+1</td>
<td>+10%</td>
<td>+71%</td>
<td>-0.6°C</td>
<td>Healed</td>
</tr>
<tr>
<td>4</td>
<td>33/M</td>
<td>Buerger</td>
<td>No</td>
<td>No</td>
<td>Splenomegaly</td>
<td>+50%</td>
<td>+124%</td>
<td>0</td>
<td>+19%</td>
<td>+18%</td>
<td>+0.3°C</td>
<td>Healed</td>
</tr>
<tr>
<td>5</td>
<td>53/M</td>
<td>Buerger</td>
<td>No</td>
<td>No</td>
<td>Bypass (occluded)</td>
<td>-10%</td>
<td>+69%</td>
<td>0</td>
<td>+15%</td>
<td>+43%</td>
<td>+1°C</td>
<td>Healed</td>
</tr>
<tr>
<td>6</td>
<td>62/F</td>
<td>ASO</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(Reduced at 4 weeks)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>69/M</td>
<td>ASO</td>
<td>Yes</td>
<td>Yes</td>
<td>PTA (repeated)</td>
<td>+28%</td>
<td>+83%</td>
<td>+1</td>
<td>+56%</td>
<td>+64%</td>
<td>+1.2°C</td>
<td>Reduced</td>
</tr>
</tbody>
</table>

Values of ABI, 6 min, TcO2, and LDPI are expressed as % increase (decrease) from the baseline.
Case 6 was excluded from this study because of social reasons 4 weeks after the treatment.
**Superficial femoral artery showed new stenosis during the study period.
***Rest pain scale: +4, severe pain unresolved with non-steroidal anti-inflammatory drugs (NSAID); +3, moderate pain NSAID necessary; +2, slight pain NSAID unnecessary; +1, very slight pain; 0, completely resolved.
DM, diabetes mellitus; HD, hemodialysis; ABI, ankle-brachial pressure index; 6 min, the distance walked in 6 min; Pain, rest pain scale (see above); TcO2, transcutaneous oxygen pressure; LDPI, laser Doppler perfusion image; Therm, thermography; ASO, atherosclerosis obliterans; BMCT, bone marrow cell transplantation; PTA, percutaneous transluminal angioplasty.

Table 2  Changes in Parameters of Limb Ischemia (Mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Pretreatment</th>
<th>4 weeks</th>
<th>24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI</td>
<td>0.62±0.12</td>
<td>0.73±0.14*</td>
<td>0.68±0.11</td>
</tr>
<tr>
<td>6-min walk (mm)</td>
<td>295±42</td>
<td>488±81*</td>
<td>491±85*</td>
</tr>
<tr>
<td>Rest pain scale</td>
<td>3.5±0.2</td>
<td>1.3±0.4*</td>
<td>1.0±0.6*</td>
</tr>
<tr>
<td>TcO2 (mmHg)</td>
<td>53.5±5.2</td>
<td>66.5±5.0*</td>
<td>65.5±4.0*</td>
</tr>
<tr>
<td>LDPI (relative unit)</td>
<td>436±66</td>
<td>520±80*</td>
<td>642±61*</td>
</tr>
<tr>
<td>Thermography (*°C)</td>
<td>27.2±0.54</td>
<td>27.9±0.44*</td>
<td>27.4±0.48</td>
</tr>
</tbody>
</table>

*p<0.05 vs pretreatment.
Four and 24 weeks, 4 and 24 weeks after the treatment.
Abbreviations see in Table 1.

Fig 2. Changes in parameters of limb ischemia in each patient. ABI, ankle-brachial pressure index; 6-min walk, distance walked in 6 min; TcO2, transcutaneous oxygen pressure; LDPI, laser Doppler perfusion image; Therm, thermography; ASO, atherosclerosis obliterans; BMCT, bone marrow cell transplantation; PTA, percutaneous transluminal angioplasty.

Statistical Analysis
All values are expressed as mean±SD. Changes in variables from baseline to week 4 or week 24 were analyzed with the Wilcoxon t-test. All statistical analyses were performed with Statview software (SAS Institute Inc NC, USA). A p value <0.05 was considered to be significant.

Results
Seven patients were entered into the study (49.3±17.2
years old, 6 males) (Table 1). Among them, 4 patients (Case 1, 3, 4, and 5) were diagnosed as having Buerger’s disease and the other 3 had ASO. Cases 2 and 7 were on chronic hemodialysis. Case 4 had splenomegaly of an unknown origin, and was referred to our institute because he could not have G-CSF for peripheral blood mononuclear cells transplantation for fear of splenic rupture.28,29

**Primary End-Point**

There were no deaths or events that were against the NCI CTCAE Ver 3.0 during the whole study period. The treatment did not induce focal inflammation or edema at the injected site. In blood analysis, WBC and CRP transiently elevated, but did not sustain, and normalized within 2 weeks in all patients except 1 patient whose foot ulcer did not heal at 24 weeks (Case 2). Blood levels of bFGF were undetected or within the normal value in all patients.

**Secondary End-Point (Tables 1, 2; Fig 2)**

**Subjective Parameters**  The distance walked in 6 min (295±42 m, pretreatment) increased both at 4 weeks (448±81 m, p=0.023) and 24 weeks (491±85 m, p=0.023). Similarly, the rest pain scale (3.5±0.2, pretreatment) improved both at 4 weeks (1.3±0.4, p=0.015) and 24 weeks (1.0±0.6, p=0.022). Three patients (Cases 1, 4, and 5) became free from rest pain completely.

**Objective Parameters**  ABI (0.62±0.12, pretreatment) improved at 4 weeks (0.73±0.14, p=0.024), but not at 24 weeks (0.68±0.11). However, at 24 weeks, 4 patients showed an increase in ABI from 9% to 50% from the baseline. TcO2 (53.5±5.2 mmHg) increased both at 4 weeks (66.5±5.0 mmHg, p=0.015) and 24 weeks (65.5±4.0 mmHg, p=0.03). LDPI (436±66 relative unit) also increased both at 4 weeks (520±80 relative unit, p=0.024) and 24 weeks (614±61 relative unit, p=0.015) (Fig 3).

Five of the 6 patients had a non-healing foot ulcer; the
The gene transfer of angiogenic growth factors might have shown good results and safety in phase I-IIa clinical trials, although there are still concerns about the unpredicta-
dible duration and level of gene expression, or immune or inflammatory responses of genetic materials.1–4,9–11 In addition, autologous bone marrow cell transplantation needs the aspiration of cells under general anesthesia.3–4 Peripheral mononuclear cell transplantation needs the systemic admin-
istration of G-CSF; they might induce serious complications such as myocardial infarction, particularly in patients with systemic atherosclerosis,2,11 or splenic rupture, although it has been reported to be rare.28,29 The sustained release of bFGF from gelatin hydrogel does not require gene therapy, general anesthesia, or G-CSF and therefore might solve these problems.

As a carrier biomaterial, gelatin hydrogel might be suitable for clinical use in terms of ease processability and the versatile controlled release of various growth factors.15,16 Gelatin hydrogel is easily processed to microsphere, sheet, or disk. Microspheres are easily dispersed in the water and can be injected into various organs, while sheets could be placed on the heart, bone, and other tissues.17–24 Furthermore, by changing the cross-linking extent, each growth factor could be released at a desirable rate and duration for tissue regeneration.15,16 They enable a sustained release by a single administration across various fields of regenerative medicine.

We used a sustained release of bFGF because bFGF shows not only potential for angiogenesis and arteriogene-
sis, but also synergistic effects with other angiogenic agents such as VEGF30 HGF31 or PDGF-BB.32 In addition, we have shown that a combination of bFGF and sarpogrelate, a serotonin blocker22 or heparin,23 enhanced collateral vessel flow effectively. Therefore, bFGF might induce more mature vessels and promote more collateral vessel development than other angiogenic agents, which is important to improve long-term results. This advantage is prominent particularly in high-risk patients who have severe diabetes mellitus and hypercholesterolemia, or who are on chronic hemodialysis.

Although this is an initial report of the project of sus-
tained release of bFGF from biodegradable gelatin hydrogel microspheres for patients with severe limb ischemia, the results are promising in its effectiveness to increase blood flow and relieve signs and symptoms. Moreover, the serum level of the bFGF did not increase at any time after the treatment, which suggests no systemic effects of bFGF such as hypotension or proteinuria.2–3 If no systemic effects are confirmed, the method will be a purely local treatment that is very safe and suitable for patients with carcinoma or proliferative retinopathy or cerebral/cardiac arterial dis-
 ease.10

Cases 5 and 7 showed a high ABI in spite of their severe ischemic symptoms. In Case 5, an ABI measurement of the dorsalis pedis artery was high, however, a pulse of the pos-
terior tibial artery was undetectable. The toe-brachial pres-
sure index might be more reliable than the ABI to the patient.23 In contrast, a high ABI in Case 7 might be caused by the non-compressible leg arteries with severe athero-
sclerosis, which is often observed in patients with long-
standing diabetes mellitus, in elderly patients, and patients

Co-Investigators

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References


ulcers were completely healed in 3 patients, reduced in 1, and no change in 1. Fig 4 shows the drastic improvement of a non-healed foot ulcer in Case 1. One patient (Case 2) did not show healing of the ulcer progressed stenosis of the superficial femoral artery during the study period.

Discussion

We have shown for the first time that the sustained release of bFGF from gelatin hydrogel microspheres effect-
ively increased blood flow in the ischemic limbs, as assessed by substantial increases in ABI, TcO2, LDPI, and skin temperature. The treatment also significantly improved the distance walked in 6 min and rest pain (complete regression in half of the patients). Ischemic ulcers were completely or partially improved except in 1 case that showed stenosis of the superficial femoral artery during the study period. In addition, the sustained release of bFGF from gelatin hydrogel did not induce focal inflammation at the injected site. Thus, we believe that the method is promising.

As a carrier biomaterial, gelatin hydrogel might be suitable for clinical use in terms of ease processability and the versatile controlled release of various growth factors.15,16 They enable a sustained release by a single administration across various fields of regenerative medicine. We used a sustained release of bFGF because bFGF shows not only potential for angiogenesis and arteriogene-
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