Clinical and Population Studies

Long-Term Follow-Up Evaluation of Results From Clinical Trial Using Hepatocyte Growth Factor Gene to Treat Severe Peripheral Arterial Disease

Hirofumi Makino, Motokuni Aoki, Naotaka Hashiya, Keita Yamasaki, Junya Azuma, Yoshiki Sawa, Yasufumi Kaneda, Toshio Oghara, Ryuichi Morishita

Objective—As angiogenic growth factors can stimulate the development of collateral arteries, a concept called therapeutic angiogenesis, we performed a phase I/IIa open-label clinical trial using intramuscular injection of naked plasmid DNA encoding hepatocyte growth factor (HGF). We reported long-term evaluation of 2 years after HGF gene therapy in 22 patients with severe peripheral arterial disease.

Methods and Results—Twenty-two patients with peripheral arterial disease or Buerger disease staged by Fontaine IIb (n=7), III (n=4), and IV (n=11) were treated with HGF plasmid, either 2 mg or 4 mg x2. Increase in ankle-branchial pressure index >0.1 was observed in 11 of 14 patients (79 %) at 2 years after gene therapy and in 11 of the 17 patients (65%) at 2 months. Reduction in rest pain (>2 cm in visual analog scale) was observed in 9 of 9 patients (100%) at 2 years and in 8 of 13 (62%) patients at 2 months. At 2 years, 9 of 10 (90%) ischemic ulcers reduced by >25%, accompanied by a reduction in the size of ulcer. Severe complications and adverse effects caused by gene transfer were not detected in any patient throughout the period up to 2 years.

Conclusion—Overall, the present study demonstrated long-term efficacy of HGF gene therapy up to 2 years. These findings may be cautiously interpreted to indicate that intramuscular injection of naked HGF plasmid is safe, feasible, and can achieve successful improvement of ischemic limbs as sole therapy. (Arterioscler Thromb Vasc Biol. 2012;32:2503-2509.)

Key Words: angiogenesis ■ peripheral arterial disease ■ plasmid DNA ■ hepatocyte growth factor ■ gene therapy

The clinical consequences of peripheral arterial disease (PAD) include pain on walking (claudication), pain at rest, and loss of tissue integrity in the distal ischemic limbs. Recent progress in molecular biology has led to the development of gene therapy as a new strategy to treat a variety of cardiovascular diseases using angiogenic growth factors, such as vascular endothelial growth factor (VEGF).1-3 However, recent reports have documented the disadvantage of VEGF, such as edema formation,1,2 and recent studies have demonstrated that VEGF121 did not show improvement in clinical end points. In contrast, although phase II nonviral fibroblast growth factor-1 clinical trial revealed a significant decrease in the amputation rate, large-scale phase III trial failed to show its efficacy.

Our previous reports demonstrated that intramuscular gene transfer of naked plasmid DNA containing the sequence encoding human hepatocyte growth factor (HGF), a potent angiogenic growth factor,4-5 in patients with critical limb ischemia (CLI) resulted in a significant increase in ankle-branchial pressure index (ABI) and improved clinical symptoms, such as ischemic ulcer at 2 months after transfection. In addition, 3 randomized placebo control studies (1 phase III trial in Japan and 2 phase II trials in the United States) demonstrated the healing of ulcer, decrease in rest pain, increase in transcutaneous oxygen tension, or toe pressure at 2 or 6 months after the treatment. These clinical trials including ours revealed clinical therapeutic values of HGF gene therapy to treat CLI. Of particular interests, different from VEGF, HGF gene therapy did not show edema as side effects. However, previous studies only showed short-term efficacy of HGF gene therapy but not long-term efficacy, amputation rate, and mortality. Thus, we sought to evaluate the long-term safety and clinical effects of direct intramuscular gene transfer of naked human HGF plasmid DNA on ABI, ulcer, and rest pain as functional end points, and amputation and death rate as hard end points up to 2 years after gene therapy in 22 no-option patients with severe PAD refractory to maximal medical therapy and not amenable to conventional revascularization. The present study documented the continuous improvement in clinical symptoms, such as the healing of ulcers. Despite
this limitation of the open-label trial, we demonstrated that intramuscular injection of naked HGF plasmid might decrease amputation rate and mortality up to 2 years after gene therapy compared with historical data.

Materials and Methods

Clinical Trial Design

This study was conducted as a phase I/IIa investigator-initiated clinical trial, as previously described.\textsuperscript{16,17} Part of the information included in this article has been published in the previous reports.\textsuperscript{16,17} Briefly, the pVAX1 plasmid vector (Invitrogen Corporation, Carlsbad, CA) was selected for HGF construct. To minimize the possibility of chromosomal integration, insertional mutagenesis through the activation of oncogenes, or inactivation of tumor suppressor genes, all sequences with possible homology to the human genome have been removed from the pVAX1 DNA, along with any sequence not necessary for replication in \textit{Escherichia coli} or for expression of recombinant protein in mammalian cells. The pVAX1 HGF plasmid consists of a cDNA fragment of human HGF inserted into the pVAX1 vector.

In this single-center prospective, open-label study, patients were enrolled if they (1) had chronic CLI, including rest pain and a non-healing ischemic ulcer, or had severe intermittent claudication with maximum walking distance $<200$ m for a minimum of 4 weeks; (2) had been resistant to conventional drug therapy for $>4$ weeks; (3) were not candidates for surgical or percutaneous revascularization based on usual standards of practice; (4) did not have previous or current neoplasms; and (5) did not have severe retinopathy, as documented previously.\textsuperscript{16,17} Objective documentation of ischemia, including resting ABI of $>0.6$ in the affected limb on 2 consecutive examinations performed 1 week apart, was necessary. Patients were observed for 4 weeks under conventional drug therapy to confirm that their clinical symptoms and objective parameters were not improved. The study was approved by the Ministry of Health, Labor, and Welfare, and Ministry of Education, Culture, Sports, Science, and Technology. Twenty-two limbs of 17 patients (13 men and 4 women; 59.6±12.0 years) with arteriosclerosis obliterans (ASO; n=14) or Buerger disease (thromboangiitis obliterans [TAO]; n=8) staged as Fontaine IIb, III, or IV underwent direct intramuscular gene transfer of naked plasmid DNA encoding HGF. The characteristics of the patients are shown in Table 1 and in a previous article.\textsuperscript{17} In 4 patients, plasmid was administrated to bilateral legs one-by-one at an interval $>3$ months. One patient was treated bilaterally at the same time.

### Intramuscular Injection of Naked Plasmid DNA Encoding Human HGF

Each patient received an intramuscular injection of naked plasmid HGF DNA, as described previously.\textsuperscript{16,17} This study was divided into 2 stages. As stage 1, for the initial 6 patients with Fontaine stage III or IV, a test intramuscular injection of a small dose (test injection) (0.4 mg plasmid DNA) was performed to examine acute or subacute allergy to plasmid DNA. After confirmation of no allergic reaction or anaphylaxis, 2 mg of naked HGF plasmid DNA was intramuscularly injected 2 weeks after test injection into the calf or distal thigh muscles of the ischemic limb by direct intramuscular injection under ultrasonic guidance. The injection sites of plasmid DNA are described in our previous preliminary report.\textsuperscript{17} Four weeks after the initial injection, a second injection (2 mg) was similarly administered, giving a total dose of 4 mg plasmid DNA per patient. Results of this study up to 6 months were previously published.\textsuperscript{17} As stage 2, 16 limbs of 12 patients with Fontaine stage IIb, III, or IV were intramuscularly injected with a therapeutic dose (2 mg/4 sites or 4 mg/8 sites) of naked HGF plasmid DNA. The doses of HGF plasmid were randomly allocated using an envelope method. HGF plasmid DNA (0.5 mg) was diluted in sterile saline solution up to 3 mL, and 4 or 8 aliquots (total 2 or 4 mg/12 or 24 mL) were administered as stage 1. Four or 8 injection sites were selected arbitrarily, according to the angiographic findings and the available muscle mass. Four weeks after the initial injection, the second injection was similarly administered, giving a total dose of 4 or 8 mg plasmid DNA per patient.

### Patient Follow-Up and Assessment

Briefly, patients were followed by physical examination (including change in ischemic ulcers), blood analysis, visual analog scale pain scale (VAS), and measurements of ABI weekly during the first 12 weeks, every other week for the next 8 weeks, monthly for the next 12 weeks, and then every 3 months up to 2 years after the first injection of plasmid DNA. ABI was measured using plethysmographic wave or Doppler wave system. IMEX (Getz Bros. Co). Maximum walking distance was measured at baseline, 1, 2, 3, and 24 months using treadmill at 2.4 km/h with 5% incline. During the study period, the development of malignant tumors and progression of retinopathy were carefully investigated by various examinations.

### Statistical Analysis

All values are expressed as mean±SD. To compare the clinical parameters (ABI, ulcer size, VAS, and maximum walking distance) between baseline and each time point, the Dunnet test was used. To test the long-term increasing or decreasing tendency of these clinical parameters, the regression coefficient of the time course was calculated. To assess the time-dependent change in these parameters.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Disease</th>
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<th>Sex</th>
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<th>Injection Dose, mg</th>
<th>Background Factors</th>
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<td>III</td>
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<td>HT and current smoking</td>
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<td>4</td>
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<td>F</td>
<td>III</td>
<td>2</td>
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<td>HT, HL, and past smoking</td>
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<td>DM and HL</td>
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<td>Iib</td>
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<td>HL and past smoking</td>
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<td>III</td>
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<td>DM, HT, and CRF on HD</td>
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<td>27</td>
<td>F</td>
<td>IV</td>
<td>2</td>
<td>past smoking</td>
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<td>DM, HT, and HL</td>
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<td>M</td>
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<td>63</td>
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<td>IV</td>
<td>4</td>
<td>DM, HT, and CRF on HD</td>
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<td>63</td>
<td>M</td>
<td>IV</td>
<td>2</td>
<td>DM, HT, and CRF on HD</td>
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<tr>
<td>22</td>
<td>ASO</td>
<td>66</td>
<td>M</td>
<td>Iib</td>
<td>4</td>
<td>HT, HL, DM, and past smoking</td>
</tr>
</tbody>
</table>

ASO indicates arteriosclerosis obliterans; TAO, thromboangiitis obliterans (Buerger disease); M, male; F, female; HT, hypertension; DM, diabetes mellitus; CRF, chronic renal failure; HD, hemodialysis; HL, hyperlipidemia.

Case Nos. 5 and 7, 9 and 16, 11 and 18, and 19 and 22 represent same patients, respectively. In those 4 patients, plasmid was administrated to bilateral legs one-by-one at an interval $>3$ months. Case No. 20 and 21 also represent the same patient, who was treated bilaterally at the same time.
between 2 groups, such as ASO and TAO, or CLI and intermittent claudication, the regression coefficient of time course was compared using ANCOVA. \( P < 0.05 \) was considered statistically significant. All tests were 2-tailed. To avoid any bias, an evaluation committee independent from the trial investigators checked all the data. The JMP statistical software was used for calculations.

**Results**

**Long-Term Efficacy of HGF Gene Therapy**

In this study, we evaluated ABI as a functional end point and resting pain, as assessed by VAS or the size of ischemic ulcer, as a clinical end point. As previously reported, \(^{17} \) ABI significantly increased from 0.46±0.08 \((n=17)\) at baseline (before administration) to 0.59±0.13 \((P=0.0135; n=17)\) at 8 weeks after injection (Figure 1A). Even at 2 years after gene therapy, ABI still increased significantly \((0.61±0.13; P=0.0053; n=14)\), whereas the peak increase in ABI was observed at 6 months after treatment \((0.65±0.15)\). There was no significant time dependency in ABI increase (regression coefficient, 0.00359; \( P = 0.051 \)). We also analyzed the results of ABI according to Fontaine stage III–IV (CLI) and stage II (intermittent claudication). There was no significant difference in the improvement of ABI between the 2 groups \((P=0.1620)\) (Figure I in the online-only Data Supplement). When an increase in ABI of >0.1 was assumed to be an improvement, according to the standard of Rutherford, 11 of 17 patients \((65\%)\) showed a positive response at 2 months after transfection (Table 2). The efficacy rate of ABI after 2 years reached 79\% \((11 of 14 patients)\). These results demonstrated that functional improvement in blood flow induced by HGF still continued up to 2 years after transfection, despite the short duration of gene expression.

To evaluate the effects of HGF gene therapy on clinical symptoms, we evaluated rest pain using visual analog scale, as a standard method for evaluation of pain. As shown in Figure 1B, rest pain had significantly improved at 2 months after transfection \((P=0.0042)\). Importantly, rest pain gradually improved in a time-dependent manner (regression coefficient, \(-0.184; P=0.0001\)). Rest pain reduced from 5.92±1.67 \((n=13)\) at baseline to 3.99±0.99 \((n=9)\) at 2 years after transfection. In this trial, 9 of 9 patients \((100\%)\) demonstrated improvement in rest pain over 2 cm at 2 years after transfection and in 8 of 13 patients \((62\%)\) at 2 months after transfection (Table 2). In this trial, a total of 25 ischemic ulcers were found in 11 patients at baseline. The size of the largest ulcer had significantly reduced from 3.08±1.53 \((n=11)\) at baseline to 0.61±1.27 \((n=10)\) at 2 years (Figure 1C). Consistent with the gradual improvement in rest pain, ischemic ulcer also significantly reduced in a time-dependent manner (regression coefficient, \(-0.0926; P=0.0004; \)Figure 1C). At 2 years after transfection, the reduction in ischemic ulcer reached 0.61±1.27 \((n=10)\). Considering an improvement in ischemic ulcers by >25\% to be evaluated as positive, 18 of 25 ulcers \((72\%)\) had improved at 2 months after transfection. It is noteworthy that at 2 years after transfection, 17 of 18 ulcers \((94\%)\) reduced by >25\%.

As shown in Figure 1C, the largest ischemic ulcer diameter also reduced after transfection. Seven of 11 patients \((efficacy rate 64\%)\) demonstrated an improvement in the largest ischemic ulcer diameter by >25\% at 2 months and 9 of 10 patients \((efficacy rate 90\%)\) at 2 years (Table 2). At 2 months, one of 11 ulcers completely healed. Typical examples of the changes in ischemic ulcers in patients with ASO and Buerger disease are shown in Figure 2. Two years after transfection, 7 of the 11 patients with ulcers achieved complete healing (Table 3). In this trial, 7 patients with Fontaine IIb stage were also tested. Peak walking time had significantly increased at 2 and 3 months after second transfection (Figure 1D); however, this effect was diminished at 2 years. In addition, we compared the time-dependent changes in patients with ASO and TAO, because these 2 groups have different characteristics in pathophysiology and clinical course. Both groups showed an improvement in ABI, ulcer size, and VAS (Figure II in the online-only Data Supplement). However, there was no significant difference in the tendency between the 2 groups. We also evaluated the transition of the Fontaine stage. As shown in Table 4, 5 of 11 patients with

![Table 2. Serial Changes in Efficacy Rate](image-url)

<table>
<thead>
<tr>
<th>Condition</th>
<th>2M</th>
<th>6M</th>
<th>12M</th>
<th>24M</th>
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</thead>
<tbody>
<tr>
<td>ABI (increase &gt;0.1)</td>
<td>11/17</td>
<td>13/16</td>
<td>10/14</td>
<td>7/11</td>
</tr>
<tr>
<td>Rest pain (reduction &gt;2 cm)</td>
<td>8/13</td>
<td>9/12</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td>Largest ulcer size (reduction &gt;25%)</td>
<td>7/11</td>
<td>8/10</td>
<td>9/10</td>
<td>9/10</td>
</tr>
</tbody>
</table>

ABI indicates ankle-branchial pressure index.

Efficacy rates are indicated as effective cases/total cases (%). An ABI increase by >0.1, a visual analog scale pain scale reduction by >2 cm, and an ulcer diameter reduction by >25\% were defined as effective cases. 2M, 6M, 12M, 24M indicate 2, 6, 12, and 24 months, respectively, after the second injection.

**Figure 1.** Serial changes in parameters. **A**, Changes in ankle-branchial pressure index (ABI). **B**, Sizes of largest ulcer. **C**, Rest pain assessed with visual analog scale pain scale (VAS). **D**, Maximum walking distance. Data are expressed as means±SD. For comparison between baseline and each time point, data were analyzed using Dunnett test. Asterisks indicate statistically significant. Pre indicates baseline; 2M, 6M, 12M, and 24M indicate 2, 6, 12, and 24 months, respectively, after the second injection; N.S., not significant.
2 months after transfection exhibited a significant improvement in functional end point as assessed by ABI measurement and clinical end points as assessed by VAS and ischemic ulcer. After the initial analysis, we performed the long-term analysis up to 2 years after transfection in this study. The most striking point from the present study is that an improvement in ABI, ulcer size, and rest pain continued up to 2 years after transfection. It is well known that the duration of gene expression driven by naked plasmid DNA is usually not longer than 1 month after injection. Indeed, our preclinical study also confirmed the transient expression of transgene using naked plasmid HGF DNA. Nevertheless, the present study demonstrated that the efficacy of HGF gene therapy was sustained up to 2 years after transfection. Especially, ABI as a functional end point to reflect an improvement in blood flow was significantly increased up to 2 years after transfection. To our knowledge, such an improvement has not previously been achieved spontaneously or with medical therapy in patients with CLI. One of the possible explanations is that the increase in blood vessels induced by HGF at early time point might enlarge the collateral vessels at later time point over the transient expression of exogenous HGF. Alternatively, as exogenous HGF upregulated endogenous HGF and VEGF through an essential transcription factor for angiogenesis, ets-1,11,21 increase in endogenous HGF and VEGF might work for the continuous growth of collateral formation. Moreover, the lymphangio genetic effects of HGF might contribute the reduction in edematous lesion in Buerger disease.22 For example, the fingers of case 3 patients were edematous at baseline and 2 months, whereas his edema almost disappeared at 24 months after the therapy (Figure 2, lower). However, 2 years after transfection, ABI seems to be slightly decreased compared with 6 months. Another treatment using HGF gene might be necessary to develop and maintain the collateral formation, although further studies must be done.

It is more important to consider the clinical application that clinical symptoms, such as rest pain and ischemic ulcer, were gradually decreased in a time-dependent manner. The improvement in these clinical symptoms was different from the time course of ABI. ABI and maximum walking distance peaked at around 3 to 6 months and then decreased a little, whereas rest pain (VAS scale) and ulcer size improved continuously until 2 years after gene therapy. Possible explanation for this discrepancy includes that improvement in blood flow by HGF gene therapy might increase the local flow, resulting in the continuous improvement in resting pain and ulcer size. In patients with CLI, it is well known that critical point to improve resting pain and ulcer would have existed. Probably, the increase in ABI over the critical value 0.5 might lead to the

Discussion
The present study reported 2-year follow-up study of HGF gene therapy in 22 cases. The initial end points evaluated at

Table 3. Healing of Ulcers

<table>
<thead>
<tr>
<th>Time</th>
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<th>All Ulcers</th>
<th>Complete Healing</th>
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<tr>
<td></td>
<td>1M</td>
<td>2M</td>
<td>3M</td>
</tr>
<tr>
<td>1M</td>
<td>0/11 (9)</td>
<td>7/25 (28)</td>
<td>0/11 (9)</td>
</tr>
<tr>
<td>2M</td>
<td>1/11 (9)</td>
<td>7/25 (28)</td>
<td>1/11 (9)</td>
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<td>6M</td>
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<tr>
<td>12M</td>
<td>6/10 (60)</td>
<td>16/21 (76)</td>
<td>6/10 (60)</td>
</tr>
<tr>
<td>24M</td>
<td>7/10 (70)</td>
<td>18/21 (86)</td>
<td>7/10 (70)</td>
</tr>
</tbody>
</table>

Largest ulcers and all ulcers are indicated as number of healed ulcers/number of ulcers at baseline (%). Complete healing shows the number of patients whose ulcers were completely healed. One patient (case no. 2) with 4 ulcers was excluded at 4 months because of participation for other angiogenic trial and died at 7 months due to hyperkalemia on the day that the patient underwent peripheral artery bypass surgery. 2M, 6M, 12M, and 24M indicate 2, 6, 12, and 24 months, respectively, after the second injection.
continuous improvement in clinical symptoms up to 2 years after gene therapy. In this study, 8 of 9 patients with CLI whose ABI was measurable achieved continuous improvements in rest pain and ulcers at 2 years. The ABI was maintained over 0.5 in 8 of 9 CLIs, although, in 7 of 9 ABI peaked out from 6-12 months to 24 months. It was suggested that in the patients whose ABI was over 0.5, local blood was sufficient to exceed the critical point to improve rest pain and ulcer. However, from this study, it is not clear how long the improvement in clinical symptoms would be continued over 2 years.

It should be noted that 3 randomized placebo-controlled double-blinded studies (1 phase III trial in Japan and 2 phase II trials in the United States) successfully demonstrated the effectiveness of HGF gene therapy. HGF-STAT trial, a phase II randomized placebo-controlled double-blinded study, demonstrated that transcutaneous oxygen tension significantly increased at 6 months in the high-dose HGF group compared with the placebo, low-dose, and middle-dose groups. Additional phase II study in the United States also demonstrated that change in toe brachial pressure index, as well as VAS, had significantly improved from baseline at 6 months in HGF-treated group compared with placebo. More dramatic evidence of the clinical efficacy of HGF gene therapy was obtained from phase III study in Japan. In this study enrolling 44 patients, the overall improvement rate of the primary end point was 70.4% in the HGF group and 30.8% in the placebo group, showing a significant difference. Especially, the HGF group achieved a significantly higher improvement rate (100%) than the placebo group (40%) to reduce ischemic ulcer.

Up to 6 months after HGF gene therapy, all clinical studies demonstrated the safety of this treatment. In this study, even up to 2 years after HGF gene therapy, few safety concerns were documented. In this study, 1228 adverse effects based on good clinical practice guideline were reported during the 2 years. However, some of the moderate adverse effects, such as pain or small hemorrhages at the injected sites, were only considered serious adverse events of case Nos. 20 and 21 were doubly counted because this patient was treated bilaterally at the same time.
to be related to gene therapy. But most of them were not considered to be related to gene therapy. Potential side effects, such as hemangioma, cancer, or worsening diabetic retinopathy, were not observed during the 2 years after transfaction. Indeed, the previous study demonstrated no increase in the serum HGF concentration during gene therapy.\textsuperscript{15} More exciting data of the present long-term follow-up study are the incidence of major amputation and mortality. Previous HGF-0205 trial (phase II) documented that complete ulcer healing at 12 months occurred in 31\% of patients in the HGF group and 0\% in the placebo group.\textsuperscript{20}

In this study, major amputation of the treated limb with HGF plasmid was 0\% through 2-year follow-up, or mortality was 9.5\% at 12 months and up to 2 years (Table 6). In contrast, historical control studies demonstrated higher amputation and mortality rate. TransAtlantic Inter-Society Consensus-II stated that 1-year natural history of CLI included 30\% major amputation and 25\% death.\textsuperscript{21} Data from control group of other angiogenic gene therapy could represent a natural history of nonrevascularized CLI. For example, phase II fibroblast growth factor gene therapy study showed 33.9\% major amputation rate and 23.2\% death rate at 1 year in the placebo group,\textsuperscript{4} whereas in the phase III fibroblast growth factor gene therapy major amputation was 21\% and mortality was 15\% at 1 year.\textsuperscript{4} However, it was reported that the age-adjusted major amputation rate was lower in Japanese population than in north of England and North America.\textsuperscript{24} In Japan, although few reports mentioned the fate of CLI, Shigematsu et al\textsuperscript{25} reported 15\% major amputation rate and 18.5\% mortality at 1 year, and Kumakura et al\textsuperscript{26} reported 27.3\% death rate at 1 year. Compared with these natural history data of CLI in Japan, in the current study, the rate of amputation-free survival and mortality in patients treated with HGF gene seemed to be favorable.

However, the long-term clinical outcome of angiogenic therapy using bone marrow mononuclear cells implantation reported that the mortality rate at 2 years was 20\% and 0\% in ASO and TAO group, respectively, whereas the amputation rate at 2 years was 33\% and 9\% in ASO and TAO, respectively.\textsuperscript{27} Although the direct comparison is difficult because of the differences in the patient population, HGF gene therapy seems to be superior to cell therapy in event-free survival. In the cell therapy, ABI was not significantly improved, whereas ulcer size and rest pain (VAS) were reduced, and its effect was sustained up to 2 years. In the present study, HGF gene therapy significantly improved ABI up to 2 years. In the HGF study, baseline ABI (0.46) was lower than that in cell therapy (0.58 in ASO group). These results suggest that HGF gene therapy might be more suitable to treat patients with lower ABI.

This study has several limitations. First, this is not a randomized or placebo-controlled study. Second, this trial was the preliminary study before phase III randomized trial, and the number of involved patients was small. Thus, statistical values might be weak, even if P values were calculable and significant. Third, the long-term results from the present study might be biased because of some unanalyzable dropout patients. During the 2-year follow-up, there were 4 unanalyzable dropout cases: Nos 2, 4, 8, and 14, all of whom were patients with CLI, whereas the remaining group had 7 cases of intermittent claudication and 11 of CLI. The dropout group could have included more severe patients than the remaining group. Thus, it remains possible that more severe patients tend to drop out, and the results from the current study may have potential for bias to be overestimated.

Overall, the present study demonstrated that the 2-year long-term efficacy of phase I/IIa open-label study using intramuscular injection of naked HGF plasmid is safe, feasible, and might achieve successful improvement in ischemic limbs as sole therapy. Larger studies to determine whether HGF plasmid can avoid major amputation and decrease the mortality in patients with CLI are warranted.

Sources of Funding

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Disclosures

Ryuichi Morishita is a member of the Board of AnGes MG that has developed the HGF gene therapy drug and has stocks of AnGes MG. Part of the information included in this manuscript has been previously published in Hypertension, 2004, and Arteriosclerosis Thrombosis Vascular Biology, 2011.

References


Long-Term Follow-Up Evaluation of Results From Clinical Trial Using Hepatocyte Growth Factor Gene to Treat Severe Peripheral Arterial Disease
Hirofumi Makino, Motokuni Aoki, Naotaka Hashiya, Keita Yamasaki, Junya Azuma, Yoshiki Sawa, Yasufumi Kaneda, Toshio Ogihara and Ryuichi Morishita

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Moderate to High Concentrations of High Density Lipoprotein from Healthy Subjects Paradoxically Impair Human Endothelial Progenitor Cells and Related Angiogenesis by Activating Rho-associated Kinase Pathways

Materials and Methods

In vitro study

Reagents and antibodies

The rabbit anti-hCD31, goat anti-hCD34, rabbit anti-hCD133, and goat anti-hVE-cadherin antibodies were purchased from Santa Cruz Co. (Santa Cruz, CA, USA). The rabbit anti-phospho-eNOS and total-eNOS antibodies were purchased from Millipore Co. (North Billerica, MA, USA). The anti-Akt and anti-MAPKs antibodies were all purchased from Cell Signaling Co. (Danvers, MA, USA). All chemical reagents, including LY294002, PD98059, SB203580, SP600125 were purchased from Calbiochem-Merck (KGaA, Darmstadt, Germany). 3-Hydroxyl-3-methyl coenzyme A (HMG-CoA) reductase inhibitor such as Rosuvastatin (Crestor®) was purchased from AstraZeneca Co. (Wilmington, DE, USA), and atorvastatin (Lipitor®) was purchased from Pfizer Co. (Cambridge, MA, USA).

Preparation of oxidized low density lipoprotein and high density lipoprotein

The LDL and HDL fraction of human serum was isolated and characterized as previously described.1, 2 In brief, plasma was taken from blood withdrawn into 0.38% sodium citrate from healthy young adult males. The major lipoprotein classes of HDL (d=1.063-1.210 g/mL) and LDL (d= 1.019-1.063 g/mL) prepared by sequential ultracentrifugation. Density was adjusted with solid NaBr and extensively dialyzed at
4°C for 24 h against phosphate-buffered saline (PBS, 5 mM phosphate buffer and 125 mM NaCl, pH 7.4). LDL was oxidized by dialysis for 24 h at 37°C against 10 μM CuSO₄ in PBS, then the oxidized LDL (oxLDL) was dialyzed for 24 h at 4°C against PBS containing 0.3 μM EDTA. The extracted oxLDL and HDL was stored in the dark at -80°C until use. In our experiments, the extent of oxidation was monitored by measuring thiobarbituric acidreactive substance (TBARS) and horizontal electrophoresis; additionally, in order to avoid that some minor contaminations (e.g. forming oxidized-HDL during the storage of the HDL) when present at higher HDL concentrations may also result in EPC function impairment, the horizontal electrophoresis and TBARS assay were also used to analysis the ingredient and oxidative situation of HDL before each study. To avoid the possible effects of heavy metal contamination in HDL samples, the isolated HDL was dialyzed for 24 h at 4°C against PBS containing 0.3 μM EDTA. Besides, in order to avoid the contamination of endotoxin, the isolated lipoproteins were acquired from healthy volunteers (not at ill) and under sterile environments. We also culture the isolated lipoprotein samples on bacterial culture disc to exclude the possibility of bacterial contamination. In this study, only oxLDL and HDL prepared within 3 days were used.

**EPC isolation and cultivation**

Total mononuclear cells (MNCs) were isolated from 40 ml of peripheral blood from healthy young male volunteers by density gradient centrifugation with Histopaq-1077 (density 1.077 g/mL; Sigma). MNCs (1x10⁷ cells) were plated in 2 ml of endothelial growth medium (EGM-2 MV; Cambrex, Charles, IA, USA) with supplements (hydrocortisone, R3-insulin-like growth factor 1, human vascular endothelial growth factor, human fibroblast growth factor, gentamicin, amphotericin
B, vitamin C, and 20% fetal bovine serum) on fibronectin-coated six-well plates at 37°C in a 5% CO2 incubator. The cultures were observed daily, and after 4 days of culture, the media were changed and nonadherent cells were removed; attached early EPCs were elongated with a spindle shape. Thereafter, media were replaced every 3 days, and each colony/cluster was observed. A certain number of early EPCs can continue to grow into colonies of late EPCs, which emerge 2-4 weeks after the start of MNC culture. The late EPCs exhibited the “cobblestone” morphology and a monolayer growth pattern typical of mature endothelial cells at confluence.

**EPC characterization**

The late EPC-derived outgrowth EC population was also characterized by immunofluorescent staining for the expression of lectin, VE-cadherin, von Willebrand factor, PE-CAM (platelet/endothelial cell adhesion molecule), CD31, CD34, kinase insert domain receptor /VEGF receptor 2, CD133 and the endocytic portion of Dil-acLDL. Alpha-smooth muscle actin was undetectable. The fluorescent images were recorded using a laser scanning confocal microscope.

**Measurement of cytotoxicity by MTT assay**

The cytotoxicity of oxLDL and HDL was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. EPCs (2x10^4 cells) were grown in 96-well plates and incubated with oxLDL (25-100 µg/mL) or HDL (5-800 µg/mL) in various concentrations for 12 hours. Additionally, EPCs were preincubated with HDL for 1 h followed by 100µM/mL of oxLDL treatment for 12 hours. Subsequently, MTT (0.5 µg/mL) was applied to cells for 4 h to allow the conversion of MTT into formazan crystals. After washing with PBS, the cells were
lysed with dimethyl sulfoxide, and the absorbance was read at 530 nm with a DIAS Microplate Reader (Dynex Technologies, Chantilly, VA, USA).

**EPC senescence assay**

Senescence was characterized as limited capacity to replicate of normal cells, and means an arrested state in which the cell remains viable. Senescence invokes a specific cell cycle profile that differs from most damage-induced arrest processes or contact inhibition. The β-galactosidase Staining could detect β-galactosidase activity at pH 6, a known characteristic of senescent cells not found in presenescent, quiescent or immortal cells. Cellular aging was determined with a Senescent Cells Staining Kit (Sigma-Aldrich, CA, USA). Briefly, after washing with PBS, EPCs were fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS and then incubated for 12 h at 37°C without CO₂ in the presence of fresh X-gal staining solution (1 mg/mL X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂). After staining, the blue-stained cells and the total number of cells were counted, and the percentage of β-galactosidase-positive cells was calculated.

**EPCs tube formation assay**

The tube formation assay was performed on EPCs to assess angiogenesis capacity, which is believed to be important for new vessel formation. The *in vitro* tube formation assay was performed using the Angiogenesis Assay Kit (Chemicon, CA, USA) according to the manufacturer’s protocol. In brief, ECMMatrix gel solution was thawed at 4°C overnight, mixed with ECMMatrix diluent buffer, and placed in a 96-well plate at 37°C for 1 hour to allow the matrix solution to solidify. EPCs were treated with 10-800 µg/mL HDL for 8 hours and then harvested. A total of 10⁴ cells were
placed on the matrix solution with HDL, and the samples were incubated at 37°C for 12 hours. Tubule formation was inspected under an inverted light microscope. Four representative fields were taken, and the average of the total area of complete tubes formed by the cells was compared using the Image-Pro Plus computer software.

**Western blot analysis**

Cells were lysed with lysis buffer (0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05% SDS, 0.5% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride). The protein concentration in the supernatants was measured using a Bio-Rad protein determination kit (Bio-Rad, CA, USA). The supernatants were subjected to 8% SDS-PAGE and transferred for 1 hour at room temperature to polyvinylidene difluoride membranes. The membranes were treated for 1 hour at room temperature with PBS containing 0.05% Tween-20 and 2% skimmed milk and incubated separately for 1 hour at room temperature with the primary antibodies. The membranes were incubated with horseradish peroxidase-conjugated IgG. Immunodetection was performed using a chemiluminescence reagent and exposure to Biomax MR Film (Kodak, NY, USA).

**Pull-down assay for detection of Rho activity**

The activation of Rho protein was analyzed using the Rho Activation Assay Kit (Catalog #17-294, Millipore, MA, USA). EPCs were lysed and then centrifuged at 14,000 g for 10 min at 4 °C. The supernatant was collected and incubated with Rhotekin Rho Binding Domain. The protein-bead complexes were then recovered by centrifugation and washed. Following the last wash, the protein-bead complexes were resuspended in SDS reducing sample buffer and resolved by 12% SDS-PAGE. The
proteins were transferred to PVDF membranes, and the membranes were incubated with a mouse anti-Rho A/B/C antibody (Millpore, MA, USA) and an HRP-conjugated secondary antibody. Activated Rho was then detected using an ECL detection kit.

Manufacture of neoangiogenesis gel

The manufacture of the neoangiogenesis gel was modified by from a previous study. The neoangiogenesis gel solution contains 1.5 mg/mL of rat-tail type 1 collagen, 90 µg/mL human plasma fibronectin, 25 mM Hepes, 1.5 mg/mL NaHCO₃, and EGM-2 medium, which was stored at 4°C until use. Isolated EPCs were suspended in neoangiogenesis gel solution and then pipetted into 12-transwell plates and warmed to 37°C for 10 minutes to allow polymerization of the neoangiogenesis gel. In some experiments, neoangiogenesis/EPC gels contained 400 µg/mL of HDL and 100 µM of Y27632. After the polymerization was completed, warmed EGM-2 was added to cover the solidified gels, and the gels were cultured at 37°C and 5% CO₂ for 24 hours. For implantation, the neoangiogenesis/EPC gels were harvested from the transwell plates and approximately 1x1x0.5 cm gel segments were dissected.

In vivo study

Preparation of animals

All animals were treated according to protocols approved by the Institutional Animal Care Committee of the Taipei Medical University (Taiwan). Twenty adult male NOD SCID mice (6-week old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept in microisolation cages on a 12-h day/night cycle and fed a commercial mouse chow diet (Scientific Diet Services, Essex, UK) with water ad libitum. Experimental procedures and animal care conformed to the “Guide
Study protocol

The animals were anesthetized by intraperitoneal injection of Xylocaine (2 mg/kg of BW) plus Zoletil (containing a dissociative anesthetic, Tiletamine/Zolazepam at a ratio of 1:1; 5 mg/kg of BW). The neoangiogenesis/EPCs gels were subcutaneously implanted into bluntly dissected subcutaneous pouches in the backs of the NOD SCID mice. The wounds were closed with skin sutures. At the time indicated (21 days), the mice were anesthetized and the implanted constructs were harvested. The implanted constructs were determined in 5-µm frozen sections, and vascular-like density was identified using a goat monoclonal antibody directed against human CD31 (Santa Cruz, CA, USA). Four representative fields were taken, and the average of the total area of complete tubes formed by the cells was compared using the Image-Pro Plus computer software.

Discussion

Concentration-dependent EPC protection of HDL in the presence of oxLDL

The HDL particle is comprised of apolipoprotein (mostly apoA-I and apoA-II), phospholipid and a cholesterol shell surrounding a lipid core that includes cholesterol, triglycerides, cholesterol esters and lysophospholipids including sphingosine-1-phosphate (S1P). It has been previously demonstrated in experimental models that HDL may exhibit multiple anti-atherosclerosis properties including that HDL could reverse cholesterol transport from the peripheral tissue to the liver and inhibit the plaque progression which is mediated by the interaction between apoA-I on HDL and lipid transporter molecules and scavenger receptor B-I on macrophages or...
endothelial cells;\textsuperscript{8} and that HDL could inhibit LDL-induced cytotoxicity,\textsuperscript{9} prevent
LDL oxidation, and inhibit the accumulation of lipid hydroperoxides in LDL via the
paraoxonase.\textsuperscript{10} Further, HDL was shown to inhibit the expression of endothelial cell
adhesion molecules and monocyte chemotactic protein-1;\textsuperscript{11} and may stimulate the
generation of NO to improve endothelial function.\textsuperscript{12} However, these studies were
usually done with the concentrations (10-100 μg/mL, equal to 1-10 mg/dL in human)
of HDL that are far below the normal range of serum HDL level (>40 mg/dL)
suggested in clinical guideline. In the present study, the cell protective effects of HDL
on late-outgrowth EPCs were concentration-dependently with the presence of oxLDL,
which is concordant to the previous findings that HDL may exert anti-atherosclerosis
properties in endothelial cells with the presence of LDL or other atherogenesis
stimulators.\textsuperscript{37-39} Furthermore, the cell protective effects of HDL could be increased
with the concentrations up to 400-800 μg/mL (equal to 40-80 mg/dL in human).
Taken together, our findings did support the current concept of increasing serum HDL
level for vascular protection in the presence of atherosclerosis risk factors or insults.

\textbf{Study limitations}

There are some issues that may be further addressed. First, given the similarity
between late-outgrowth EPCs and mature endothelial cells, future study may help to
validate the effects of high concentrations of HDL on mature endothelial cells. Second,
given the experimental nature, the current \textit{in vitro} and \textit{in vivo} studies are
hypothesis-driven for concept-proving. Since the \textit{in vivo} interactions among different
lipoproteins (HDL, triglyceride, LDL, oxLDL, etc) could be very complex, one may
not simply conclude that HDL has no beneficial effects on cardiovascular diseases at
this point. Alternatively, our findings might provide some preclinical evidence that
HDL could exert its beneficial effects on EPCs in a dose-dependent fashion in the
presence of ox-LDL but may not be always helpful in a relatively high concentration when the harmful lipids such as ox-LDL are absent. Additional animal study is required to assess the in vivo effects of different serum HDL levels. Third, given the potential adverse effects of higher concentrations of HDL on late-outgrowth EPCs in the absence of oxLDL, one may speculate that the optimal level of HDL might be lower in the absence of atherosclerosis risk factors or stress, which could be similar to the risk-dependent optimal serum LDL levels suggested by current guidelines. Future large-scaled clinical studies are required to validate the concepts of current study.

References


### Supplement Table I

The level of endotoxin in culture medium and high density lipoprotein (HDL)

<table>
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<th>OD</th>
<th>Endotoxin-free water 50 μL (Thermo Scientific Co., PO, USA)</th>
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<th>HDL2 350 μg/50 μL</th>
<th>HDL3 350 μg/50 μL</th>
<th>Culture medium 50 μL (LOZA Co., CA, USA)</th>
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The effects of HDL on LDH expression in EPC-cultured medium. After treatment of EPCs with 25-800 μg/mL of HDL for 12 hours, the concentration of LDH was also measured using a SPOTCHEMTM automatic dry chemistry system (SP-4410; Arkray, Shanghai, Japan).
The effects of oxLDL and HDL on EPCs tube formation. After treatment of EPCs with 5-50 µg/mL of oxLDL for 12 hours and/or pretreatment with 5-100 µg/mL of HDL for 1 hour before treatment of oxLDL. An *in vitro* angiogenesis assay was used with the ECMMatrix gel to investigate the EPC neovascularization.
The effects of oxLDL and HDL on EPCs activation on eNOS. After treatment of EPCs with 5-50 μg/mL of oxLDL for 2 hours and/or pretreatment with 5-100 μg/mL of HDL for 1 hour before treatment of oxLDL. The eNOS activation (phosphorylation) was analyzed by western blotting. The density of bands was quantified by densitometer.
The effects of HDL on EPCs senescence. After treatment of EPCs with 400 µg/mL of HDL for 0.5-24 hours, the acidic β-galactosidase expression in EPCs was detected as a biochemical marker for acidification that is typical of EPC senescence. The diagram shows the quantification of senescent EPCs. Data were expressed as the mean ± SEM of three experiments performed in triplicate. *p < 0.05 was considered significant.
Supplement Figure IVa

The effects of HDL from CAD patient on EPCs tube formation. EPCs were pretreated with the 25-100 μg/mL of HDL for 12 hours, an in vitro angiogenesis assay was used with the ECMatrix gel to investigate the effect of HDL on EPC neovascularization. Tube formation assay was performed for 6 hours. Representative photos of in vitro angiogenesis are shown. Data were expressed as the mean ± SEM of three experiments performed in triplicate. *p < 0.05 was considered significant.
The effects of HDL from CAD patient on EPCs senescence. EPCs were pretreated with 25-800 μg/mL of HDL for 12 hours, the acidic β-galactosidase was detected as a biochemical marker for acidification that is typical of EPC senescence. Data were expressed as the mean ± SEM of three experiments performed in triplicate. *p < 0.05 was considered significant.
The effects of HDL from healthy donor or CAD patient on EPCs eNOS activation. EPCs were pretreated with or without 10 μM LY294002 and Y27632 for 1 hour prior to HDL treatment for 2 hours. The eNOS activation were analyzed by western blotting. The total-eNOS and β-actin protein levels were used as loading controls.
The effects of HDL2 and HDL3 from healthy donor on EPCs tube formation. EPCs were pretreated with the 50-800 μg/mL of HDL for 12 hours, an in vitro angiogenesis assay was used with the ECMatrix gel to investigate the effect of HDL on EPC neovascularization. Tube formation assay was performed for 6 hours. Representative photos of in vitro angiogenesis are shown. Data were expressed as the mean ± SEM of three experiments performed in triplicate. *p < 0.05 was considered significant.
The effects of HDL2 and HDL3 from healthy donor on EPCs senescence. EPCs were pretreated with 50-800 μg/mL of HDL for 12 hours, the acidic β-galactosidase was detected as a biochemical marker for acidification that is typical of EPC senescence. Data were expressed as the mean ± SEM of three experiments performed in triplicate. *p < 0.05 was considered significant.
Suppl. Figure 1. Comparison of changes in ABI between Fontaine stage III-IV and stage II

FIII-IV: Fontaine stage III-IV, FII: Fontaine stage II.
Data were analyzed using ANCOVA. There was no difference between two groups.
Suppl. Figure II. Serial changes in ABI, ulcer size and VAS in ASO and TAO

(A) ABI

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(B) Ulcer size

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(C) VAS

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ASO: arteriosclerosis obliterans, TAO: Buerger’s disease (thromboangiitis obliterans).
Data were analyzed using ANCOVA. There was no difference in the parameters between two groups (ASO vs TAO). (A) ABI; $P = 0.1264$, (B) Ulcer size; $P = 0.7732$, (C) VAS; $P = 0.6023$