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Review Article

Therapeutic Angiogenesis Using HGF Plasmid

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Hepatocyte growth factor (HGF) is secreted from stromal and mesenchymal cells, and its receptor cMet is expressed on various types of cells such as smooth muscle cells, fibroblast, and endothelial cells. HGF stimulates epithelial and endothelial cell proliferation, motility, and morphogenesis in a paracrine and autocrine manner, organizing multistep of angiogenesis in many organs. In addition, HGF is recognized as a potent anti-inflammatory and anti-fibrotic growth factor, which has been proved in several animal studies, including neointimal hyperplasia and acute myocardial infarction model in rodent. Thus, as compared to other angiogenic growth factors, HGF exerts multiple effects on ischemic tissues, accompanied by the regression of tissue inflammation and fibrosis. These data suggest the therapeutic potential of the HGF for peripheral artery disease as it being accompanied with chronic tissue inflammation and fibrosis. In the present narrative review, the pleiotropic action of the HGF that differentiates it from other angiogenic growth factors is discussed first, and later, outcomes of the human clinical study with gene therapy are overviewed.

Keywords: HGF, gene therapy, peripheral artery disease

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Introduction

Innate regenerative capacity of human lower extremity against progressing artery disease has been reported.[1)] Narrowing of the major artery by atherosclerotic plaque leading to tissue hypoxia stimulates small collateral blood vessels sprouting from preexisting arteries to overcome restricted blood flow. This process is defined as angiogenesis.[2)] Unfortunately, collateral circulation by innate angiogenesis is generally inadequate to fulfill oxygen demand during exercise,[3)] thus limiting physical activity. To supply enough blood to peripheral tissue, interventional or surgical revascularization procedures and medications have been advanced. However, multiple stenotic lesions in the major artery or disease in small peripheral vessels limit repeated revascularization procedures, which remain patients symptomatic. Therefore, researchers and clinicians have long challenged to amplify the innate angiogenic.[4,5)] Cell therapy and gene therapy have been studied for more than 20 years. Cell therapy remains at the primitive stage and is provided marginally positive outcomes in clinical trial of peripheral artery disease (PAD).[6)] Gene therapy targeting angiogenesis for PAD is also at early phase. However, several clinical trials with angiogenic growth factor genes, including vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), and hepatocyte growth factor (HGF), are now showing some progress with positive and negative trials (**Table 1**). In the present review, the multifunctional aspects of HGF on inflammation, fibrosis, and insulin resistance was intensively discussed first. Later, the therapeutic potential of the HGF for PAD with complicated risk factors for cardiovascular disease is deliberated.

Anti-inflammatory and anti-fibrotic function of HGF

Several studies suggest that HGF inhibits both acute and chronic inflammation and reactive oxygen species (ROS) production in a variety of disease models; however, the underlying mechanism has been unclear. For instance, we previously demonstrated that the HGF-cMet system attenuates angiotensin II-induced ROS production and following inflammation signaling by inhibiting the transactivation of epithelial growth factor receptor (EGFR).[7,8)] The

Table 1 Summary of clinical trials using angiogenic growth factor genes for patients with PAD

Trials or author name [reference]	Vector and promoter	Delivery route	Phase	Enrollment	Outcomes
Baumgartner et al. [34]	phVEGF165/ MIEhCMV	Intra-muscular	I	9	Tolerated
Mäkinen et al. [35]	phVEGF165/ MIEhCMV AdVEGF165/ MIEhCMV	Intra-arterial	II	54	Tolerated, increase vascularity
RAVE [36]	AdVEGF121/ MIEhCMV	Intra-muscular	II	95	No improvement of exercise performance or QOL
Groningen [37]	phVEGF165/ not reported	Intra-muscular	II	54	No reduction in amputation rate
Comerota et al. [45]	phFGF-1/MIEhCMV	Intra-muscular	I	107	Tolerated
TALISMAN [46]	phFGF-1/MIEhCMV	Intra-muscular	II	125	Reduction in amputation rate
TAMARIS [47]	phFGF-1/ MIEhCMV	Intra-muscular	III	525	No improvement of QOL or ABI, no reduction in amputation rate or death
Morishita et al. [50]	phHGF/ MIEhCMV	Intra-muscular	I/IIa	22	Tolerated
Makino et al. [53]	phHGF/ MIEhCMV	Intra-muscular	I/IIa	22	Improvement of ABI, reduction in rest pain and ulcer size up to 2 years
HGF-STAT [51]	phHGF/ MIEhCMV	Intra-muscular	II	104	Improvement in TcPO2
TREAT-HGF [52]	phHGF/ MIEhCMV	Intra-muscular	III	40	Improvement in rest pain and ABI, reduction in ulcer size

ABI: ankle-brachial index; TcPO2: transcutaneous oxygen tension; MIEhCMV: major immediate-early enhancer/promoter from human cytomegalovirus

protective effects of HGF against angiotensin II signaling through the activation of cMet receptor. As the HGF-cMet system inhibits the translocation of SH2 domain-containing inositol phosphate 5-phosphatase 2 (SHIP2) to EGFR, EGFR degradation is promoted through EGFR ubiquitination by C-Cbl, E3 ubiquitin ligase, which is normally inhibited to bind EGFR by SHIP2. Thus, HGF reduces Ang II-induced inflammation and ROS production. We further confirmed that ligand-dependent EGFR degradation by HGF is also functioned following the stimulation of transforming growth factor beta (TGF-β), endothelin-1, and epithelial growth factor, which all trans-activate EGFR.[7] In addition, by using a HGF transgenic mouse model (HGF-Tg mice) in which serum human HGF is overexpressed from the heart, we have documented that HGF-Tg mice restricted lipopolysaccharide (LPS)-induced vascular oxidative stress and inflammation in the aortic wall.[9] The protective action of HGF against the LPS was also through the ligand-dependent EGFR degradation mechanism. Hence, HGF can exert its anti-inflammatory and anti-oxidant effects in various pathological conditions, such as diabetes, atherosclerosis, chronic heart failure, and chronic kidney disease (CKD).[10–13] In contrast, VEGF and basic fibroblast growth factor (bFGF) have been shown to initiate tissue inflammation and edema via an activation of nuclear factor-kappa B (NFκB) and its downstream inflammation-related cytokines, such as monocyte chemotactic protein 1 (MCP-1), interleukin-1 (IL-1)β, IL-6, and IL-8 in vascular endothelial and smooth muscle cells.[14,15] After vascular injury, the elevated expression of VEGF recruits monocyte macrophage-lineage cells and exacerbates neointimal formation,[16] while HGF expression is decreased in injured vessels and administration of HGF inhibits the inflammation and the formation of neointima.[17] Intriguingly, Min et al. have documented that HGF considerably increases VEGF expression in endothelial cells (ECs) with decreasing VEGF-induced NFκB activation and leaky vessels or edema in a skin inflammation mouse model. HGF has a synergistic effect with VEGF on neovascularization.[18] Therefore, co-administration of HGF and VEGF could be a better treatment than either factor alone for augmenting therapeutic angiogenesis while avoiding tissue inflammation, which constitute the main pathology of PAD. Of note, HGF can also resolve tissue fibrosis, another complication of PAD. HGF has been repeatedly reported to encounter TGF-β signaling reducing tissue fibrosis in several acute and chronic ischemic models of the heart and kidney. Furthermore, HGF attenuates the process of epithelial to mesenchymal cell transition, EMT, which is considered to be an underlying mechanism of perivascular fibrosis. Considering its strong anti-fibrotic action, HGF gene transfer might lead to better tissue oxygenation, although there is no direct evidence.

Insulin resistance and HGF

A growing body of evidence has demonstrated a correlation between insulin resistance and chronic inflammation.[19] In animal study, chronic inflammation has negative effects on the insulin signaling pathway in adipocytes, hepatocytes, and myocytes.[20] The accumulation of macrophages in the liver and white adipose tissue is known to promote insulin resistance.[21] Thus, the hypothesis that HGF is involved in the mechanism of insulin resistance was tested.[22] In the study, we first demonstrated that HGF inhibits angiotensin II-induced NFκB signaling in mouse macrophages (RAW264 cell), as well as in co-culture

with 3T3-L1 adipocytes, resulting in the reduction of inflammatory cytokine expression including MCP-1, IL-6, IL-1β, and TNF-α in vitro. To prove this in vitro finding in vivo, ApoE KO mice were crossed with HGF-Tg mice. The chronic inflammation in adipose tissue and the liver with macrophage infiltration, adipocyte hypertrophy, and fatty liver observed in ApoE KO mice was significantly ameliorated in the ApoE KO/HGF-Tg mice. Notably, the ApoE KO/HGF-Tg mice increased serum adiponectin levels compared to the ApoE KO mice. These observations indicated that HGF suppresses the pro-inflammatory cytokine from adipocytes, liver, and macrophages and, contrary, increases serum adiponectin, thus inhibiting the vicious cycle of macrophage-adipocyte inflammation. Previously, it was demonstrated that circulating serum HGF levels were associated with existence of type 2 diabetes and obesity.[23] However, its role in obesity-related pathology was unclear. Thus, insulin sensitivity was evaluated in HGF-Tg mice fed with a high-fat diet (HFD).[9] While HFD induced body weight gain in wild-type mice accompanied with insulin resistance, both were prevented in HGF-Tg mice. As compared to wild-type mice, macrophage accumulation in adipose tissue and inflammatory cytokine levels at 14 weeks after HFD was significantly attenuated in HGF-Tg mice. Administration of neutralizing antibody against HGF in wild-type mice with HFD significantly aggravated response to the glucose tolerance test. All together, these studies reinforce the protective role of HGF on glucose metabolism in obesity that has already been presented. Apart from our study, Perdomo et al. have showed that HGF enhances glucose transport and metabolism in myotubes through the PI3K/Akt-mediated increased GLUT-1 and GLUT-4 transporter expression.[24,25] In addition, Flaquer et al. demonstrated that administration of the HGF gene to kidney of a type II diabetic mice (db/db mice) significantly decreased circulating levels of IL-6 and MCP-1 and increased the number of M2 macrophages leading to an improvement in glomeruli inflammation and diabetic nephropathy.[26] Thus, HGF ameliorates obesity- or diabetes-related pathology by preventing the inflammation and insulin resistance in the adipose tissue, liver, skeletal muscle, and even β-cells, further supporting the compensatory mechanism of HGF in insulin resistance. In clinical, peroxisome proliferator activated receptor-γ (PPAR-γ) agonists, such as pioglitazone, irbesartan, and telmisartan, bind to the HGF promoter and increased its expression in several organs.[10,12] Furthermore, our previous experiment demonstrated that a PDE-3 inhibitor, cilostazol, ameliorates insulin resistance and induces HGF expression through the PPAR-γ and the cyclic adenosine monophosphate (cAMP) pathway. It is noteworthy that these drugs have anti-inflammatory and anti-oxidative action in addition to their own pharmacological targets in metabolic syndrome.[27] Basic and clinical evidence show that PPAR-γ agonists could ameliorate renal fibrosis in both diabetic and nondiabetic CKD.[28] Both HGF and PPAR-γ agonists attenuate Smad nuclear translocation by TGF-β1 in renal fibroblasts. Again, these data indicate that HGF might act as a downstream effector of PPAR-γ agonists, improving fibrosis in the heart and kidney.

Angiogenic potential of HGF for PAD

For the treatment of PAD patients, first potential of VEGF and FGF gene therapy has been studied. Later, the angiogenic potential of HGF has been studied by our group and others. **Table 1** summarizes the clinical trials for PAD using angiogenic growth factor gene transfer.

VEGF

The VEGF ligand is a family of six secreted glycoproteins, VEGF-A to VEGF -E, that activate three receptor tyrosine kinases, VEGFR-1 to VEGFR-3. VEGF-A controls angiogenesis and vascular permeability. On the other hand, VEGF-C and VEGF-D largely regulate lymphangiogenesis.[29] VEGF-A is characterized by alternatively spliced variants that generate three principal isoforms, $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. Among them, $VEGF_{165}$ is the best studied and most abundant. In preclinical study, delivery of $VEGF_{165}$ by plasmid or virus vector substantially increased vascular density and tissue oxygenation in hind limb ischemia model.[30,31] VEGF-A is known to stimulate EC mitogenesis and migration, bone marrow-derived endothelial progenitor cell recruitment to the site of vasculogenesis.[32,33] However, VEGF gene therapy for PAD patients so far demonstrated inconsistent data. In initial clinical trial, intra-muscular administration of naked plasmid $VEGF_{165}$ gene significantly promoted collateral artery growth in patients with critical limb ischemia (CLI).[34] Mäkinen et al. also demonstrated that intra-arterial injection of adenovirus vector encoding $VEGF_{165}$ (Ad-$VEGF_{165}$) remarkably enhanced vascular density compared with placebo-controlled group.[35] Unlike $VEGF_{165}$, $VEGF_{121}$ isoform, which is known to be stronger mitogenic alternative splicing isoform than $VEGF_{165}$ or $VEGF_{189}$, has shown no improvement in ankle-brachial index (ABI), intermittent claudication, and quality of life (QOL) in a phase II clinical trial.[36] In addition, $VEGF_{165}$ plasmid shows no improvement in amputation rate in phase II trial.[37] To date, VEGF gene therapy in PAD patients has failed to show evidence of benefit in phase III clinical trial. Notably, VEGF induces vascular permeability through Rac 1-mediated ROS generation.[38] In clinical trials, VEGF gene therapy affects approximately 60% of patients developing dose-dependent leg edema. Recently, it is reported that a new delivery system (α2PI1-8-$VEGF_{121}$) that can release

low-dose VEGF for long term induces non-leaky vessels and ameliorates vascular formation more effectively than native $VEGF_{121}$ gene therapy.[39] Another recent advance is the use of FGF4, which is able to activate VEGFs and orchestrate the downstream cascades involved in angiogenesis more potently than VEGF alone.[40,41] Using a regulatory gene, such as FGF4, seems to be more fruitful than comparing individual angiogenic factor or seeking growth factor combination more suitable for the gene therapy.

FGF

In humans, there are 22 mammalian FGF members, all of which can bind to 4 receptor tyrosine kinase receptors, FGFR1 to FGFR4. FGF signals through these receptors act in a number of embryonic development, such as branching morphogenesis, limb development, and brain patterning. Angiogenic therapeutic potential of FGFs is intensively investigated. Among FGF receptors, FGFR1 is the most abundant in the endothelium.[42] It has demonstrated that endothelial-specific FGFR1 deletion has little influence on vascular development and however impairs vasculogenesis responsive to tissue injury.[43] Among FGFs, FGF-1 (aFGF), FGF-2 (bFGF), and FGF-4 have higher angiogenic potential. Based on the basic research, non-viral naked plasmid (NV1FGF) containing human FGF-1 has been established for angiogenic gene therapy. Preclinical hind limb ischemia models in rodents and following early clinical studies showed its positive effect to induce a functional artery in the ischemic region.[44] A phase I clinical trial conducted by Comerota et al. included 51 no-optional CLI patients with tissue necrosis and rest pain underwent treatment with intra-muscular NV1FGF injection. NV1FGF is well tolerated and significantly improved in ABI, claudication, transcutaneous tissue oxygen, and ulcer size. Thus, NV1FGF is potentially effective for the treatment of patients with end-stage limb ischemia.[45] The following phase II clinical trial (TALISMAN) was carried out enrolling 125 PAD patients in whom revascularization was not considered to be a suitable option. Patients were randomized to receive eight intra-muscular injections of NV1FGF or placebo on days 1, 15, 30, and 45 (total 16 mg: 4×4 mg). Improvements in ulcer healing were comparable for NV1FGF group (19.6%) and placebo-controlled group (14.3%). However, the use of NV1FGF significantly decreased the risk of all amputations ($P = 0.015$) and major amputations ($P = 0.015$). Moreover, tendency for reduced risk of death with the use of NV1FGF was observed.[46] The safety and effectiveness of NV1FGF observed in this study prompted further investigation with a placebo-controlled, double-blind clinical trial. A phase III clinical trial (TAMARIS) was conducted including 525 CLI patients who were not candidate for surgical and catheter-based revascularization.[47] The primary endpoint was time to major amputation or death at 1 year. Unfortunately, neither the primary endpoint nor components of the primary differed between treatment groups. TAMARIS provided no evidence that NV1FGF is effective in reduction of amputation or death in patients with CLI. So far, further human trials using NV1FGF gene transfer have not been conducted. As mentioned above, FGF and VEGF initiate a fundamental transcription factor for inflammation, NFκB, and its downstream inflammation-related cytokine expression in vascular cells with vascular permeability.[13,14] Inflammation-induced angiogenesis might not last long due to the premature cell senescence.[8]

HGF

While HGF is generated and secreted mostly from mesenchymal cells, its targets are cells of both epithelial and mesenchymal origin. Its receptor, cmet, is identified on ECs,[8] endothelial progenitor cells (EPCs),[48] and smooth muscle cells (SMCs)[7] and fibroblasts.[49] The HGF gene therapy using naked human HGF plasmid DNA for PAD patients is particularly interesting, because till now, three randomized placebo-controlled trials confirmed its benefit in rest pain, ulcer healing, and increase in transcutaneous oxygen tension.[50–52] In addition, its long-term efficacy with a reduction of rest pain, an increase in ABI, and ulcer size at 2 years after gene therapy has been reported.[53] Notably, unlike VEGF and FGF, no edema and any adverse side effect by HGF gene therapy was documented. Recently, a biopharmaceutical company obtained conditional approval for HGF gene therapy to treat CLI patients in Japan. HGF is now the first gene therapy product to be approved in Japan for improving ulcers in patients with arteriosclerosis obliterans or Buerger's disease who have had no option for undergoing revascularization. As mentioned, among HGF's multifunction, anti-fibrotic and anti-inflammatory actions differentiate HGF from other angiogenic growth factors. HGF stimulates angiogenesis with reducing inflammation, tissue fibrosis, edema, and cellular senescence and following insulin resistance which are the main pathology of PAD, especially CLI.[54–61] These beneficial functions of HGF might resolve complication of CLI, leading to better tissue oxygenation.

Conclusion

It has been nearly three decades since human HGF complementary DNA (cDNA) was successfully cloned. Since that time, the biological functions of the HGF/cMet axis have been extensively investigated by several groups, including us. The results have provided convincing evidence for the essential physiological functions of HGF, as well as for its therapeutic potential. HGF was originally identified as a

hepatokine; later, its angiogenic, anti-inflammatory, anti-senescence, and anti-fibrotic potential was discovered. Recently, the capacity of the HGF/cMet axis to interfere with energy metabolism has been reported. Similar to what was observed with adipokines and myokines, HGF can ameliorate insulin resistance in basic experiments. Several drugs enhancing HGF production are now available in clinics for the treatment of diabetes. These multifunctional aspects of HGF might result in a different outcome from VEGF and FGF in clinical trial for the treatment of PAD.

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Author Contributions

Study conception: FS, YT, HR, RM
Data collection: FS, TF, KS, YT
Analysis: FS, TF, KS, YT
Investigation: FS, TF, KS, YT
Writing: FS, RM
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