

***In Vivo* Evidence of Enhancement of HGF-induced Angiogenesis by Fluvastatin**

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Abstract: Currently, therapeutic angiogenesis using gene transfer of angiogenic growth factors has entered the realm of novel therapy for patients with critical limb ischemia. However, its clinical utility might be enhanced by combination with classical pharmacological approaches. Thus, in this study, we examined adjunctive therapy to enhance the angiogenic effects of gene transfer, with HMG-CoA reductase inhibitors, statins, which have pleiotropic vascular-protective effects. Here, we evaluated the effects of a combination of hepatocyte growth factor (HGF) gene therapy with fluvastatin in a mouse hindlimb ischemia model.

Hindlimb ischemia model mice were given a normal diet, high-cholesterol diet (HCD) or HCD with fluvastatin, after intramuscular injection of human HGF plasmid DNA. Intramuscular injection of HGF plasmid into ischemic limbs significantly increased blood flow and capillary density. However, HCD diminished the increase in blood flow and capillary density induced by HGF gene transfer. Administration of fluvastatin significantly attenuated the reduction in blood flow and decrease in capillary density, while it did not change the serum cholesterol level.

Overall, these results demonstrated that fluvastatin significantly enhanced the increase in blood flow and capillary density induced by HGF gene transfer in a hindlimb ischemia mouse model with HCD, through a non-cholesterol-lowering effect. Clinical use of fluvastatin might be possible adjunctive therapy to enhance therapeutic angiogenesis.

Key Words: Angiogenesis, gene therapy, HGF, statin, critical limb ischemia.

INTRODUCTION

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. Despite the development of technology for vascular intervention and drugs such as prostaglandins, more than a few patients are still resistant to conventional therapy, and worsening of disease and limb amputation are unavoidable for them. In these circumstances, therapeutic angiogenesis using genes of angiogenic growth factors is expected to be a new treatment for patients with severe ischemic disease. 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], basic fibroblast growth factor (FGF) [2], and hepatocyte growth factor (HGF) [3, 4]. Several clinical trials of gene therapy using these angiogenic factors have been performed [5-10]. Although some clinical trials using the VEGF121 gene [11] or FGF4 gene [12] did not show a significant improvement in clinical endpoints, we conducted a phase I/IIa clinical trial of gene therapy using HGF [10], which showed that direct intramuscular gene

transfer of naked plasmid DNA encoding human HGF in patients with critical limb ischemia was safe, increased ankle-brachial pressure index, and improved clinical symptoms such as ischemic ulcers. Moreover, a placebo double-blinded multi-center phase III clinical trial demonstrated significant improvement of the primary endpoints (resting pain and ischemic ulcer). An HGF gene therapy drug is now under new-drug application in Japan, and will be launched in 2009-2010. Nevertheless, it can be anticipated that therapeutic angiogenesis might be enhanced by combination with classical pharmaceutical drugs to treat patients with critical limb ischemia. Thus, it is important to evaluate supplementary and practical treatment in gene-transfer-based angiogenesis.

In this study, we focused on 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, statins, which are used as lipid-lowering agents. Statins are reported to have lipid-independent beneficial effects including improvement of endothelial function [13] and inhibition of inflammation [14]. Moreover, it is recently reported that statins enhance collateral vessel development in the ischemic muscle of animal models [15, 16]. However, the effects of statins on therapeutic angiogenesis gene therapy have not been elucidated. Here, we investigated the effects of fluvastatin, which is well known as a vascular statin, on peripheral blood flow in a hypercholesterolemic condition and on angiogenesis induced by HGF in a mouse hindlimb ischemia model.

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MATERIALS AND METHODS

Mouse Ischemic Hindlimb Model

Ten-week-old male C57/BL6 mice were purchased from Charles River Breeding Laboratories (Yokohama, Japan). All animal experiments were conducted under protocols approved by the Institutional Animal Care Committee of Osaka University. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (30mg/ kg body weight). A longitudinal incision was then made, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Through this incision, using surgical loupes, the operator dissected free the left femoral artery along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries, were also dissected free. After dissection of the popliteal and saphenous arteries distally, the external iliac artery and all of the mentioned arteries were ligated with 4-0 silk. Finally, the right femoral artery was completely excised to create the ischemic limb model, from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates to form the saphenous and popliteal arteries. Excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Excision of the femoral artery 1 cm below the peritoneum created an ischemia model. Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery.

Administration of Fluvastatin

Mice were divided into four groups: (1) control group fed a normal diet (ND), (2) high-cholesterol diet (HCD), (3) HCD + 1 mg/kg/day fluvastatin, (4) HCD + 5 mg/kg/day fluvastatin. At 1 week after surgery, high-cholesterol diet (containing 1.25% cholesterol) and fluvastatin (Novartis Pharma Japan, Tokyo, Japan) were started. Fluvastatin was continuously administered by an osmotic mini-pump at a dose of 1 or 5 mg/kg/day. After 4 weeks of high-cholesterol diet and fluvastatin, blood flow and capillary density in the hindlimb were measured. Plasma level of total cholesterol was also measured.

In Vivo Gene Transfer to Ischemic Limb Model Using Direct Intramuscular Injection

To produce an HGF expression vector, human HGF cDNA (2.2 kbp) was inserted into a simple eukaryotic expression plasmid that utilizes the cytomegalovirus promoter/enhancer [4, 17]. This promoter/enhancer has been used to express reporter genes in a variety of cell types and can be considered to be constitutive. Cytomegalovirus expression vector plasmid without HGF cDNA sequence was used as a control vector.

We injected 500 µg naked plasmid DNA directly into the ischemic limb of mice with a 27 G needle (Terumo, Tokyo, Japan) at 1 week after surgery. The injection volume of plasmid was 100 µL. After gene transfection, high-cholesterol feeding and administration of fluvastatin were performed as described above.

Measurement of Blood Flow by Laser Doppler Imager

Measurement of blood flow with a laser Doppler imager (LDI) has been previously described [4, 17]. As it is clearly

demonstrated that laser Doppler flow velocity correlates well with capillary density, we measured blood flow using a laser Doppler imager blood flowmeter (Moor Instruments, England). Excess hairs were removed from the limb with depilatory cream before imaging, and rats were placed on a heating plate at 37°C to minimize temperature variation. Consecutive measurements were obtained over the same regions of interest (leg and foot). The LDI uses a 12-mW helium-neon laser beam that sequentially scans a 5 x 5 cm surface area at extremely high speed to be able to measure blood flow in the ischemic hindlimb. Blood flow at 1 mm under the surface can be measured. While scanning, the motion shift of blood cells is converted to the frequency of projected light according to the Doppler principle. Upon the termination of scanning, a color-coded image representing blood flow distribution is displayed on a monitor. The perfusion signal is subdivided into 14 different intervals, and each interval is displayed as a separate color. Low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as white. The stored perfusion values behind the color-coded pixels remain available for data analysis. These laser images were quantitatively converted into histograms with blood flow on the x-axis and the number of pixels on the y-axis in the traced area. The average blood flow in each histogram was calculated for evaluation.

Measurement of Capillary Density

Alkaline phosphatase staining was used as a specific marker for endothelial cells in paraffin-embedded sections, as previously described [4, 17]. To analyze the number of vessels in the ischemic hindlimb transfected with HGF vector or control vector, mice were sacrificed and the muscles were removed. Three individual sections from the middle of the transfected muscle were analyzed. The number of vessels was counted under light microscopy (magnification, x100) in a blinded manner. The total number of vessels in each section was summed and expressed as the number per section. At least ten individual sections were evaluated in each muscle. The areas in which the number of vessels was quantified were randomly selected in the injected site and around the injected site. The animals were coded so that analysis was performed without any knowledge of which treatment each animal had received. The reproducibility of the results was assessed.

Table 1. Cholesterol Levels in Mice Treated with Vehicle or Fluvastatin

	Total Cholesterol (mg/dL)
1) ND	83 ± 10
2) HCD	177 ± 23 #
3) HCD+Flu 1	180 ± 18 #
4) HCD+Flu 5	164 ± 16 #

p<0.05 vs ND, ND = ischemic hindlimb mice fed normal diet, HCD = ischemic hindlimb mice fed high-cholesterol diet, HCD+Flu 1 = ischemic hindlimb mice fed high-cholesterol diet with fluvastatin (1 mg/kg/day), HCD+Flu 5 = ischemic hindlimb mice fed high-cholesterol diet with fluvastatin (5 mg/kg/day). Each group contains 5-7 animals.

Statistical Analysis

All values are expressed as mean + SEM. Analysis of variance with subsequent Duncan’s test was used to determine the significance of differences in multiple comparisons. Differences with a p value less than 0.05 were considered significant.

RESULTS

Effect of Fluvastatin on Serum Cholesterol Level

Initially, we measured plasma cholesterol level after administration of fluvastatin at 1 and 5 mg/kg/day to mice fed a high-cholesterol diet. As shown in Table 1, these doses of fluvastatin did not affect serum total cholesterol level.

Effects of Fluvastatin on Peripheral Blood Flow

To assess the effects of a high-cholesterol diet and fluvastatin on recovery of peripheral blood flow, we measured peripheral perfusion in the ischemic limb using a laser Doppler imager. As shown in Fig. (1a) & (1b), peripheral perfusion ratio was significantly reduced in mice fed HCD as compared to those with a normal diet. Interestingly, administration of fluvastatin alone attenuated the reduction of blood flow by HCD in a dose-dependent manner (Fig. 1b), although a low dose of fluvastatin did not affect peripheral blood flow. Perfusion ratio was significantly increased in

mice administered a high dose of fluvastatin as compared to those with HCD only.

Enhancement of HGF-Induced Blood Flow Recovery and Angiogenesis by Fluvastatin

Next, we performed transfection of the human HGF gene to the ischemic limb. In the normal diet group, over-expression of the human HGF gene resulted in a significant increase in blood flow in the ischemic hindlimb as compared to control vector (Fig. 2a and 2b), consistent with our previous data [4, 17]. Interestingly, the recovery of blood flow was significantly diminished in hypercholesterolemic mice as compared to those with a normal diet (Fig. 2-b). Importantly, administration of fluvastatin enhanced the increase in blood flow induced by HGF in a dose-dependent manner.

Angiogenesis was also confirmed by measurement of capillary density as assessed by alkaline phosphatase staining. As shown in Fig. (3), capillary density after 4 weeks of HCD was also significantly lower in hypercholesterolemic mice than in mice fed a normal diet, although transfection of human HGF plasmid DNA significantly increased the capillary density. Importantly, capillary density was significantly higher in mice treated with a high dose of fluvastatin as compared to mice without fluvastatin (Fig. 3). These data suggest that fluvastatin enhanced HGF-induced angiogenesis in a hypercholesterolemic condition.

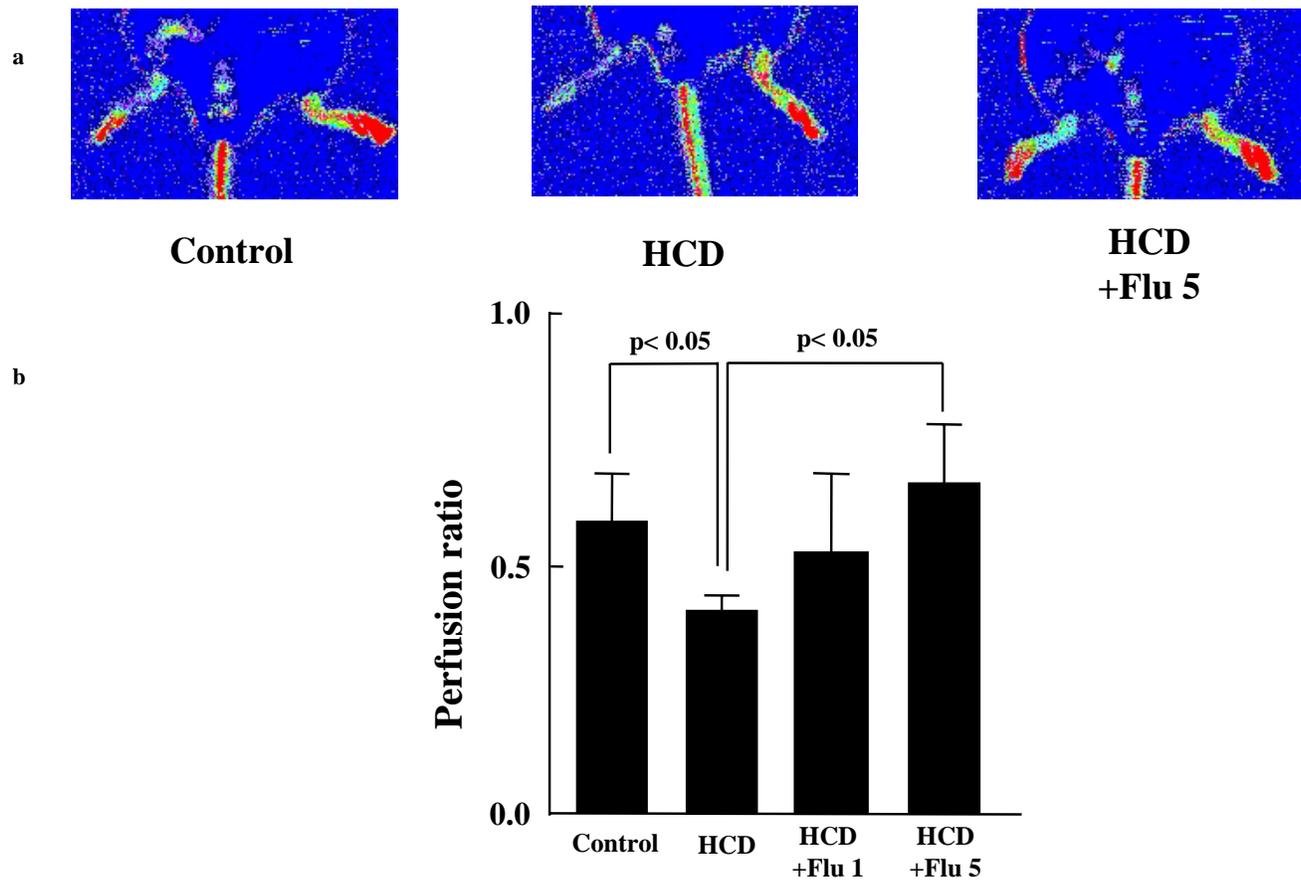


Fig. (1). Effect of 4 weeks of high-cholesterol diet and/or fluvastatin on recovery of blood flow in mouse ischemic hindlimb model. (1-a). Representative photographs of LDI. (1-b). Quantitative analysis of blood flow in hindlimb expressed as perfusion ratio of ischemic hindlimb to untreated opposite limb. ND = normal diet, HCD = high-cholesterol diet, HCD+Flu 1 = high-cholesterol diet with fluvastatin (1 mg/kg/day), HCD+Flu 5 = high-cholesterol diet with fluvastatin (5 mg/kg/day). Each group contains 5-7 animals.

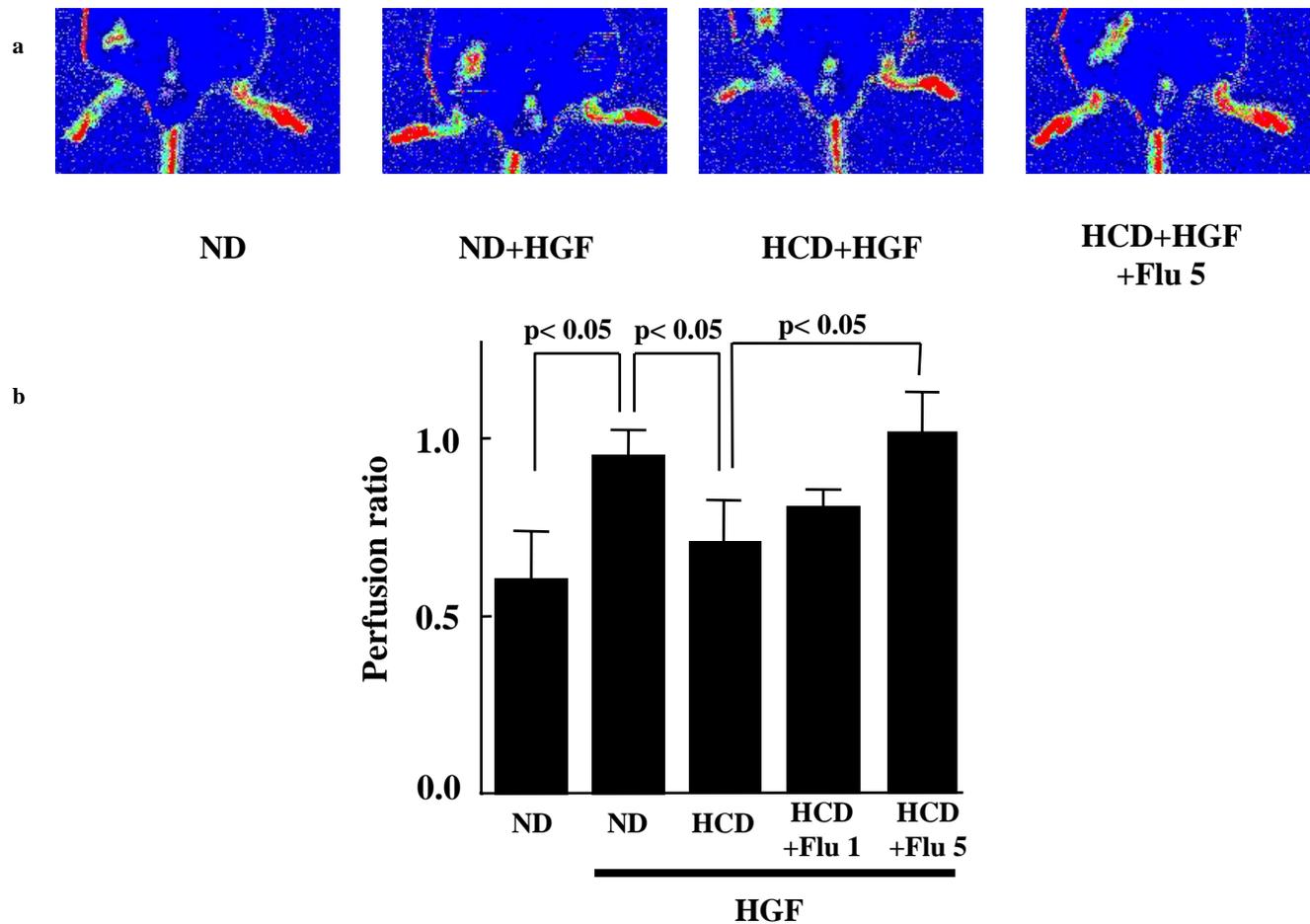


Fig. (2). Effect of fluvastatin on increase in blood flow induced by transfection of human HGF gene in ischemic hindlimb mouse model at 4 weeks after transfection. (2-a) Representative photographs of LDI. (2-b) Quantitative analysis of blood flow in hindlimb expressed as perfusion ratio of ischemic hindlimb to untreated opposite limb. ND = normal diet, HCD = high-cholesterol diet, HCD+Flu 1 = high-cholesterol diet with fluvastatin (1 mg/kg/day), HCD+Flu 5 = high-cholesterol diet with fluvastatin (5 mg/kg/day), HGF = human HGF plasmid (500 μ g). Each group contains 5-7 animals.

DISCUSSION

Critical limb ischemia is estimated to develop in 220 persons per million per year [18], and the natural history of critical limb ischemia has been well documented to be an inexorable downhill course [19, 20]. Therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia. Several growth factors such as HGF, VEGF, FGF and others have been tested in clinical trials. The first gene therapy drug to stimulate angiogenesis will be launched within a few years, since HGF gene therapy trials have been successfully completed and a FGF phase III trial is now ongoing. However, angiogenesis induced by single gene transfer of an angiogenic growth factor might be not ideal for patients who have multiple risk factors for atherosclerosis and severe critical limb ischemia. Thus, to consider the practical and clinical situation, additional treatment to enhance angiogenesis should be investigated.

In the present study, we employed fluvastatin as an adjunct treatment combined with therapeutic angiogenesis induced by HGF, because statins are well known to improve endothelial function independently of their lipid-lowering effect [13]. Hypercholesterolemia induces endothelial dys-

function through vascular inflammation and/or oxidative stress [21, 22], and leads to a delay of angiogenesis in ischemic lesions. Indeed, endothelial dysfunction in hypercholesterolemic animals results in poor collateral formation and a delay of recovery of peripheral blood flow in ischemic lesions [23, 24]. Endothelial function is closely related to collateral formation. Given that more than 50% of patients with peripheral arterial disease have hypercholesterolemia [25], restoring endothelial dysfunction is an important factor in considering treatment for atherosclerotic disease. Thus, statins are expected to be suitable for concomitant use with therapeutic angiogenesis. Especially, fluvastatin is well known as a vascular statin, and might have superior effects by a direct action on the blood vessel wall.

The present study demonstrated that administration of fluvastatin enhanced angiogenesis either alone or in combination with HGF in the ischemic hindlimb under a hypercholesterolemic condition. As fluvastatin did not affect serum cholesterol level, these effects might be independent of its lipid-lowering effect. It is reported that statins promote ischemia-induced angiogenesis through induction of endothelial nitric oxide synthase (eNOS) gene expression [15, 16]. Statins have been shown to promote angiogenesis *via*

up-regulation of eNOS expression [26, 27], activation of Akt [28] or inactivate Rho [29]. In addition, transfection of the eNOS gene induces angiogenesis in ischemic muscle through up-regulation of VEGF [30]. Moreover it is also reported that statins up-regulate VEGF expression and augment proliferation of microvessels in diabetic mice model [31].

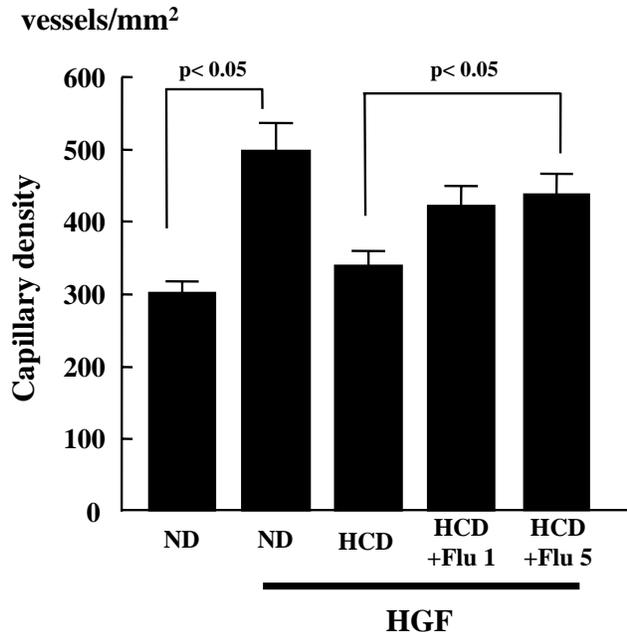


Fig. (3). Effect of transfection of HGF plasmid on capillary density in ischemic hindlimb of mice at 4 weeks after transfection. ND = normal diet, HCD = high-cholesterol diet, HCD+Flu 1 = high-cholesterol diet with fluvastatin (1 mg/kg/day), HCD+Flu 5 = high-cholesterol diet with fluvastatin (5 mg/kg/day), HGF = human HGF plasmid (500 µg). Each group contains 5-7 animals.

Furthermore, statins may improve blood flow through vasodilatation induced by its effect of improvement in endothelial function. Statins improve endothelial dysfunction through various mechanisms without lipid-lowering effects. One is their anti-inflammatory effect. Vascular inflammation is regarded as important in the pathogenesis of atherosclerosis. Statins are reported to inhibit inflammation in the vascular system. This concept is supported by recent findings that statins decreased high-sensitive C reactive protein [32, 33] and inhibited the progression of atherosclerosis [34]. It is also reported that statins attenuated activation of the transcription factor nuclear factor-kappa B (NF-κB), which regulates various cytokines, chemokines and adhesion molecules to cause adhesion of monocytes, neutrophils and macrophages [35]. Moreover, as mentioned above, statins up-regulate eNOS gene expression [26, 27], which induce an increase in NO and may subsequently lead to vasodilatation. Both of the mechanisms, vasodilating effect *via* an improvement in endothelial function and angiogenic effect, could contribute to augmentation of blood flow.

CONCLUSION

Here, the present study demonstrated that the combination of fluvastatin with HGF gene transfer enhanced the increase in peripheral blood flow induced by HGF in a hyper-

cholesterolemic condition. These data suggest that statins such as fluvastatin might have therapeutic potential for supplementary treatment in therapeutic angiogenesis.

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