Silencing of int6 gene restores function of the ischaemic hindlimb in a rat model of peripheral arterial disease

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Aims

Intermittent claudication (IC) is one of the serious symptoms of peripheral arterial disease (PAD) and is characterized by pain in the legs or buttocks that worsens with exercise and subsides with rest. The concept of ‘therapeutic angiogenesis’ for PAD has been widely proposed; however, the methodology, including cell transplantation, is still unclear. In this study, we examined the clinical efficacy of silencing the int6 gene, which encodes a protein that stabilizes hypoxia-inducible factor (HIF)-2α, on angiogenesis in PAD.

Methods and results

An animal model for IC was established in Sprague–Dawley rats by external iliac artery ligation and evaluated by quantitative analysis of gait disturbance. Next, we explored the therapeutic effects of int6 siRNA injected into the adductor magnus muscle on IC. Recovery of hindlimb function occurred in the early stages after int6 siRNA injection. The number of blood vessels showed an obvious increase in the int6 siRNA-treated muscles. Angiography revealed the recovery of peripheral circulation at the affected sites. Early up-regulation of HIF-2α and other angiogenic factors, including basic fibroblast growth factor and hepatocyte growth factor, was also apparent in the int6 siRNA-treated sites. We also confirmed the up-regulation of HIF-2α and its translocation to the nucleus in the int6 siRNA-injected muscle.

Conclusion

A single injection of int6 siRNA promoted angiogenesis via up-regulation of HIF-2α-related angiogenic factors in the muscles of the affected hindlimb and reduced gait disturbance. The int6 gene may be a novel therapeutic target for the treatment of IC in patients with PAD.

Keywords

Angiogenesis • Hypoxia • Ischaemia • Peripheral vascular disease • Gene silencing

1. Introduction

Systemic atherosclerosis sometimes results in peripheral arterial disease (PAD), and patients with PAD suffer from both limited function and serious pain of the ischaemic hindlimb.1–4 Intermittent claudication (IC) is one of the serious symptoms caused by PAD and is characterized by pain in the legs or buttocks that occurs with exercise and subsides with rest.1–4 Although PAD is commonly treated pharmacologically, interventional or surgical protocols are sometimes applied to maintain quality of life for patients with serious conditions. Moreover, there are some patients who may ultimately require amputation to obtain relief from the unbearable and life-threatening situation. Promotion of angiogenesis is a recently proposed concept, which has brought a new therapeutic strategy for PAD in the past few years.5–8 However, the exact methodology for cellular and/or cytokine therapy has not been sufficiently developed and defined. Furthermore, the mechanisms by which these cells and/or cytokines exert their angiogenic effects are not completely understood.
Peripheral hypoxia resulting from a type of obstructive ischaemia induces the expression of a series of genes that help recover the oxygen supply to the affected tissues.9−12 One of the most important responses to ischaemia is the induction of angiogenesis triggered by the activation of hypoxia-inducible factor (HIF)-1α and HIF-2α, which bind HIF-1β to form a complex.9−12 Unlike HIF-1α, which is ubiquitously expressed in various cell types, HIF-2α is mainly expressed in vascular endothelial cells, indicating that it may regulate endothelium-specific genes.11−15 The HIF proteins are ubiquitinated and undergo degradation by the 26S proteasome during normoxia; however, in hypoxic conditions, they are stabilized and form heterodimers that transactivate other angiogenic factors.13−15 Recently, Chen et al.16 reported that the silencing of endogenous int6 was sufficient to induce HIF-2α expression even during normoxia, and this enhanced the expression of other angiogenic factors. Ablation of int6 gene expression up-regulated HIF-2α and promoted the function of blood vessels, resulting in acceleration of wound healing in the skin of mice.17 Their investigation suggested that Int6 regulated protein stability by directly binding to HIF-2α.16,17 As the int6 gene may play a critical role in the suppression of HIF-2α, we hypothesized that silencing the int6 gene might promote therapeutic angiogenesis in the hypoxic conditions encountered during peripheral ischaemia.

In this study, we investigated whether therapeutic angiogenesis was induced by silencing of the endogenous int6 gene in a rat model of ischaemia,18 and we evaluated the clinical efficacy of int6 siRNA therapy in PAD. We clearly demonstrated that knock-down of the int6 gene by int6 siRNA increased endogenous HIF-2α expression in a rat model of peripheral ischaemia and induced the expression of several critical angiogenic factors. Moreover, a single treatment with int6 siRNA recovered blood perfusion at the affected sites and decreased gait disturbance. Our results indicate that the int6 gene is a novel and critical determinant of HIF-2α-mediated angiogenesis, and that int6 siRNA transfer may be an effective therapeutic strategy in obstructive vessel diseases.

2. Methods

2.1 Animals and surgical procedure

Male Sprague–Dawley rats, aged 5−7 weeks and weighing 200−250 g, were provided by Japan SLC Inc. (Tokyo, Japan). Under anaesthesia induced and maintained by 2−3% isoflurane inhalation with O2 as the carrier gas, the external iliac artery was approached through the abdomen and carefully ligated at its proximal end using 3−0 silk sutures which are 45 cm long.18 Rats that underwent sham surgery (exposure of the external iliac artery without ligation) were used as control animals. No neuromuscular-blocking or paralytic agents were used in this study. Adequate anaesthesia was maintained using a Small Animal Anesthetizer (MK-A110) and Small Animal Ventilator (MK-100; Murumachikai Co., Ltd, Tokyo, Japan). During and after the surgery, rats were kept on a hot carpet for companion animals (PHK-545; Airis Ohyama, Sendai, Japan) until awakening. The rats’ heart rate was monitored with a stethoscope by research assistants. For isolation of muscle samples, rats were killed by inhalation of 5% isoflurane for 5−10 min. Samples were isolated 3 min following complete cardiopulmonary arrest. All experiments that involved the use of animals were in accordance with the guidelines of the University Animal Care and Use Committee at the Tokyo University of Agriculture and Technology. The investigation also conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.2 Preparation of siRNA and siRNA treatment

Spontaneous expression of int6 gene in L6 rat myoblast cells (purchased from American Type Culture Collection) was confirmed by a standard RT-PCR. The siRNA specific for the rat int6 gene was designed, generated, and provided by alphaGen (Tokyo, Japan). The siDirect™ system was used to select five different alignments of int6 siRNA (ALG-00412, ALG-00417, ALG-00418, ALG-00422, and ALG-00425) that demonstrated high gait hesitation with minimal off-target effects. After introduction of int6 siRNA into L6 cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), the silencing effect of each construct was evaluated by real-time PCR using the Power SYBR Green PCR Master Mix and an ABI Prism 7900 Sequence Detector (Applied Biosystems Inc., Tokyo, Japan). The following primers were used: 5′-TGAAACAACCTCCAGCCAA-3′ and 5′-GGCTAAACCCATGCCTTGCT-3′ for int6, and 5′-AGCCATGTACGTAGCCATCC-3′ and 5′-ACCCCTCATAGATGGGCACAG-3′ for β-actin. The relative expression level of int6 gene was determined using the 2−ΔΔCt calculation method and normalized to the expression level of an endogenous reference gene. In order to maintain stability of siRNA knock-down for in vivo application, we used the siChimera, in which a part of the region of alignment was replaced with DNA. Functional efficacy of int6 siRNA was evaluated by measuring the increased level of HIF-2α in L6 cells transfected with ALG-00425. Briefly, ALG-00425 or scrambled siRNA (10, 25, or 50 nM) was reconstituted in saline and introduced into L6 cells using TransIT-TKO Transfection Reagent (Mirus Bio Corp., Madison, WI, USA) according to the manufacturer’s instructions. To detect HIF-2α expression, cell lysates were collected 6, 24, and 48 h after transfection, and western blot analysis was performed using anti-endothelial PER-ARNT-SIM (PAS) domain protein (EPAS)-1 (a homologue for HIF-2α) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Cell lysate collected from CoCl2-treated L6 cells was used as a positive control.19 According to the results, we selected ALG-00425 (sense, 5′-CCUUUAUUCCGATGATTATTT-3′; anti-sense, 5′-AAATATCAATGGGAAUAAGGGGU-3′; alignment replaced with DNA is underlined) as the most effective siRNA. For in vivo experiments, ALG-00425 was dissolved in sterile saline, and the solution (0.5, 1, or 3 mg/100 g body weight) was injected into the left adductor magnus muscle, immediately after surgery. Saline and scrambled siRNA dissolved in saline were used as controls. Cilostazol is the only therapeutic reagent in vivo that demonstrated improved blood perfusion and promoted the function of blood vessels, resulting in acceleration of wound healing in the skin of mice.20 Therefore, cilostazol (100 mg/kg body weight) was given orally once a day to post-operative rats as a positive control.

2.3 Blood perfusion rate

Blood perfusion rate was measured using a laser-Doppler imager (MoorLDI, Monte System Corp., Tokyo, Japan) and calculated according to the following formula: femoral blood perfusion rate of the affected left limb/femoral blood perfusion rate of the normal right limb × 100 (%).

2.4 Quantitative analysis of gait disturbance

Rats were habituated to the experimental apparatus and trained daily for 2 weeks prior to testing. Briefly, each rat was placed in the acrylic wheel of a GAIT® analyzer (Nolvetec Inc., Kobe, Japan) that revolved at 4.0−10.0 rpm (the speed was raised gradually), and the rats were trained to walk in the wheel. On the day of the experiment, each rat was placed in the wheel and allowed to walk for 1 min prior to examination. Gait disturbance was measured by allowing each rat to walk in the wheel for a 2 min period and recording all of its steps using a DigiOn TVR® (DigiOn Inc., Fukuoka, Japan). Gait analysis was then conducted using GAIT™ software (Noveltec Inc.) according to the manufacturer’s instructions. Swing is defined as the period when the foot is off the ground and is moving forward. As a single hindlimb bears the weight during a swing, the swing time (ST) of one hindlimb shortens if the opposite hindlimb is
experiencing pain. The GAIT analyzer automatically calculated the swing of both hindlimbs in each step cycle, and the ST index (STI) was determined using the following formula: ST ratio (STR) \(=\) (ST of normal hindlimb)/(ST of affected hindlimb). When STR is greater than 1, STI = \(-[(1/STR) - 1]\), and when STR is less than 1, STI = STR – 1. The STI of a normal rat is approximately 0; however, when there is pain in the affected foot, the STI decreases. STI (2 to 1) is a useful parameter for judging the balance of the rat while walking. We have used it as a parameter to calculate the STR of affected and unaffected hindlimbs. The STI represents the oscillation width towards the right and the left, with 0 as a standard, which in turn indicates the balance of the rat. In a normal walk, the STI is distributed around 0. The STI is more efficient than the STR in detecting imbalance, because it analyses the extent of oscillation to the right or the left. The STI of each rat was calculated and averaged every 40 s for the duration of the experimental period (0–40, 40–80, and 80–120 s).

2.5 Angiography

Rats were anaesthetized by inhalation of isoflurane, and heparinized saline was perfused through a cannula placed in the abdominal aorta. Angiography was performed using a 50% barium solution as the contrast medium, and the blood vessels were visualized by computed radiography (FCR XL-2; Fujifilm, Tokyo, Japan).

2.6 Real-time RT-PCR analysis

At the end of the experiment, the adductor magnus muscles were collected from all rats. The frozen tissues were homogenized using a Multi-beads shocker (Yasui-kikai, Tokyo, Japan), and total RNA was extracted. Transcriptional regulation of HIF-2α, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and int6 mRNA was analysed by real-time PCR using target-specific primers purchased from Takara Bio Inc. (Shiga, Japan) as previously described. Histological analysis

The adductor magnus muscles were collected and fixed in paraformaldehyde. Paraffin-embedded sections (thickness 5–6 μm) were treated with either the anti-platelet endothelial cell adhesion molecule (PECAM)-1 antibody (Santa Cruz) or anti-α smooth muscle actin (SMA) antibody (DakoCytomation, Glostrup, Denmark) followed by biotin-conjugated secondary antibodies. To visualize positive results, horseradish peroxidase–streptavidin (Dako) and diaminobenzidine were used as the substrates. Researchers who were blinded to the treatment provided to each rat counted the number of positively stained blood vessels under \(\times 100\) magnification. The number of blood vessels in each preparation was expressed as an average of the numbers in five fields. For the detection of angiogenesis-related proteins, paraffin-embedded sections were incubated with anti-endothelial PAS domain protein (EPAS)-1 (also designated HIF-2α) antibody (#LS-B501; LifeSpan Biosciences, Inc., Seattle, WA, USA), anti-bFGF.

Figure 1 Effects of int6 siRNA on the silencing of the int6 gene. (A) RT-PCR analysis of int6 mRNA expression in L6 cells. β-Actin was used as an endogenous reference gene. (B) Effects of int6 siRNA on the silencing of endogenous int6 gene expression. The ALG-00425 construct demonstrated the strongest silencing effect. (C) Effects of ALG-00425 on HIF-2α expression. As a positive control, CoCl2 (50 μM) was used to induce HIF-2α expression. β-Actin was used as a loading control. The photo represents a typical result. Dose- (10, 25, and 50 nM) and time-dependent effects (6, 24, and 48 h) of ALG-00425 were determined. (D) Relative intensities calculated according to results of three different experiments. Each column represents the mean ± SEM of three different experiments. *P < 0.05, in comparison with the level of HIF-2α expression in untransfected L6 cells.
antibody (Millipore Japan, Tokyo, Japan), anti-HGF antibody (Santa Cruz), and anti-VEGF antibody (Millipore Japan), followed by secondary antibodies conjugated with fluorescent markers. ImageJ software (National Institutes of Health) was used to quantify the increase of each protein.

2.8 Detection of HIF-2α in tissues
Each frozen tissue was disrupted by using a Multi-beads shocker (Yasui-kikai). Nuclear extractions and cytoplasmic extractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). Protein concentrations were measured by a bicinchoninate (BCA) assay (Thermo Fisher Scientific, Yokohama, Japan). A western blot analysis was performed with anti-EPAS-1 (also designated HIF-2α) antibody (LifeSpan Biosciences) according to the manufacturer’s instructions.

2.9 Statistical analysis
Comparisons of two parameters were made using Student’s two-tailed t-test. Comparisons of parameters among groups were made by one- or two-way ANOVA, followed by Tukey’s test. P < 0.05 was considered statistically significant.

3. Results
3.1 Generation and selection of suppressive int6 siRNA
First, we confirmed spontaneous int6 gene expression in L6 rat myoblasts by PCR analysis (Figure 1A). Next, we examined the silencing effect of five different chimeras of rat int6 siRNAs on int6 gene expression by using an in vitro transfection system in L6 cells. Among these five chimeras, ALG-00425 was the most effective at decreasing int6 gene expression in L6 cells (Figure 1B). Therefore, we selected ALG-00425 for use in our subsequent studies, and we analysed the effects of this int6 siRNA construct on the protein abundance of HIF-2α in L6 cells. When various doses of ALG-00425 were transfected into L6 cells, the protein level of HIF-2α was increased in a dose-dependent manner (Figure 1C and D). We found that ALG-00425 could up-regulate HIF-2α in L6 cells to a similar extent to CoCl2 treatment, which is known to induce pseudo-hypoxic conditions14 (Figure 1C and D). The effects of ALG-00425 on the protein intensity of HIF-2α were maintained for 24 h and then decreased by 48 h (Figure 1C and D).

3.2 Generation and evaluation of a rat model for IC
In order to generate an in vivo model for IC, we performed unilateral hindlimb arterial occlusion in rats and evaluated whether this surgery was effective in producing the IC phenotype. Blood flow in the affected left hindlimb was markedly lower than that in the unaffected right hindlimb (Figure 2A). To confirm whether this rat model reproduced the characteristics of IC found in human patients, we performed behavioural analysis to quantify gait disturbance in these rats by using a GAIT® analyzer.21 Gait was monitored as rats walked on a wheel for 2 min, and the procedure was repeated three times at 10 min intervals. We observed that gait disturbance of the affected
hindlimb developed around 30 s to 1 min after the beginning of the walking session and gradually exacerbated (Figure 2B). Normal gait recovered after the 10 min rest period; however, gait disturbances worsened again upon resuming the walking test (Figure 2B). In contrast, the STI of the gait disturbance in sham-operated rats did not change (Figure 2B). These data confirmed that this rat model was extremely useful and suitable for the evaluation of novel IC therapies.

3.3 Dose responses of int6 siRNA on gait disturbance in the rat model for IC
To evaluate the effect of specific int6 knock-down, we injected different doses of int6 siRNA (0.5, 1.0, and 3.0 mg per site) into the left hindlimb of rats immediately following surgical occlusion of the left external iliac artery. Blood flow in the affected hindlimbs of ALG-00425-injected rats was restored to normal levels by day 14, and this recovery was both significant and dose dependent (Figure 3A). We observed an obvious recovery of hindlimb function 14 days after int6 siRNA injection, and a single injection of 3.0 mg int6 siRNA created the strongest and most significant effects (Figure 3B).

3.4 Accelerating effect of int6 siRNA injection on angiogenesis in the rat model for IC
The clinical effects of int6 siRNA treatment on angiogenesis were also evaluated using our novel rat IC model. Immediately after surgery, we injected 3.0 mg of ALG-00425 into the adductor muscle of the affected limb. Angiography of int6 siRNA-injected rats on day 14 revealed that the peripheral lesions of the occluded artery were clearly visible, resembling the angiographic features of a normal rat; however, very few blood vessels could be identified in scrambled siRNA-injected rats (Figure 4A). We also evaluated PECAM-1 expression as a pan-endothelial cell marker by immunohistochemical analysis of the adductor muscle samples collected from each rat on day 14. The number of PECAM-1-positive blood vessels appeared to increase in samples from int6 siRNA-injected rats; however, no statistically significant differences were apparent (Figure 4B and C). In contrast, the number of αSMA-positive blood vessels in the int6 siRNA-injected rats was significantly higher than that in rats with scrambled siRNA injection (Figure 4D and E).

Blood perfusion rate on days 3, 7, and 14 after the treatment revealed that the blood flow in int6 siRNA-injected rats was higher than that in rats injected with saline or random oligonucleotides (Figure 5A). Improvement in blood flow was obvious even on day 7 (Figure 5A). Due to its anti-coagulating effect, cilostazol has been used to alleviate the clinical symptoms of IC. Unexpectedly, in rats administered cilostazol, no significant increase in blood flow in the affected hindlimbs was observed (Figure 5A). Rats treated with int6 siRNA showed obvious functional recovery of the affected hindlimbs (Figure 5B), and the STI of these rats was comparable to that of normal rats that had not undergone surgery. Similar to the int6 siRNA-treated rats, gait disturbance was also significantly less in cilostazol-treated rats (Figure 5B).
3.5 Production of angiogenic factors after injection of int6 siRNA

We performed RT-PCR analysis to detect time-dependent changes in the mRNA expression of angiogenesis-related genes. The mRNA levels of bFGF and HGF were up-regulated at 3 and 6 h after the injection of int6 siRNA, and the expression was restored to normal levels 24 h later (see Supplementary material online, Figure S1A). The increase was seen in the int6 siRNA-injected muscles, but not in the control samples (see Supplementary material online, Figure S1B). However, the mRNA level of VEGF remained unchanged (see Supplementary material online, Figure S1A). As HIF-2α is a target of the int6 protein, we further investigated the kinetics of HIF-2α mRNA expression at the early stages of tissue hypoxia. Increased HIF-2α mRNA expression was detected in samples collected at 30 and 60 min after int6 siRNA injection. We confirmed up-regulation of int6 gene expression immediately after ischaemia in scrambled siRNA-injected rats (see Supplementary material online, Figure S1C). In contrast, int6 gene expression was suppressed during the 3 h following int6 siRNA injection, and was gradually restored to basal levels by 24 h following int6 siRNA injection (see Supplementary material online, Figure S1C). We also examined the kinetics of changes in protein levels for several angiogenesis-related factors following the surgery (Figure 6). HIF-2α levels were elevated at 1, 3, and 6 h after the injection of int6 siRNA (Figure 6B). During the early stages in particular, the elevation of HIF-2α protein expression was quite obvious (Figure 6B). In contrast, increases in bFGF and HGF protein expression were not apparent until a few hours later (Figure 6C and D). Intensive up-regulation of bFGF and HGF was observed at 3, 6, and 12 h after the injection of siRNA (Figure 6C and D). No marked increase was detected in VEGF protein levels after the surgery (Figure 6E).
In our model, rats develop symptoms that more closely resembled with rest. These symptoms were quite similar to those of human patients with stable PAD, is a specific inhibitor of cAMP in platelets and vascular smooth muscle cells and has potent anti-platelet activity. In this study, we demonstrated that int6 siRNA was more effective than cilostazol in the treatment of IC in rats. As previously reported, we found that cilostazol improved walking function, however, our data showed that the efficacy of cilostazol was in fact quite limited when the blood perfusion rate was analysed. Cilostazol has been reported to increase the intracellular concentration of cAMP, leading to the inhibition of smooth muscle cell proliferation. By interfering with the proliferation of arterial smooth muscle cells, cilostazol may prevent arterial recovery.

Finally, we found that a single injection of int6 siRNA induced an immediate increase in HIF-2α mRNA expression in the affected hindlimbs, followed by an increase in the expression of both bFGF and HGF.

### 3.6 Nuclear translocation of HIF-2α after injection of int6 siRNA

Silencing of the int6 gene is expected to stabilize HIF-2α, allowing it to avoid degradation, and thereby enabling HIF-2α to play its role as one of several key transcription factors required for angiogenesis. Therefore, translocation of HIF-2α to the nucleus was examined. We found that nuclear HIF-2α was increased in the muscles at 1 h after injection of int6 siRNA (see Supplementary material online, Figure S2A and B). In the cytosol, the abundance of HIF-2α was gradually increased, reaching maximal expression at 3–6 h following injection of int6 siRNA (see Supplementary material online, Figure S2A and B). In contrast, detection of nuclear HIF-2α indicated that the levels were normalized after 3 h, but increased at 6 h after the injection (see Supplementary material online, Figure S2A and B). No significant up-regulation was observed for either the cytoplasmic accumulation or the nuclear translocation of HIF-2α in muscles isolated from control rats (see Supplementary material online, Figure S2A and B).

### 4. Discussion

In this study, we developed a novel approach to therapeutic angiogenesis in ischaemia accompanying PAD. First, we generated an appropriate animal model for IC and confirmed its suitability. As indicated in Figure 2, peripheral ischaemia immediately after ligation of the proximal portion of the external iliac artery was identified by a laser-Doppler analysis. Moreover, we found that this ischaemia model exhibited progressive gait disturbance while walking, which was recovered with rest. These symptoms were quite similar to those of human IC. In our model, rats develop symptoms that more closely resemble the clinical manifestations of human IC than those of commonly used animal models with severe irreversible limp or peripheral necrosis. Recent reports have revealed that Int6 suppresses HIF-2α, even during normoxia, and that knock-down of the int6 gene leads to up-regulated expression of HIF-2α, promoting blood vessel function. Int6 has been reported to prevent protein stability by binding directly to HIF-2α. Therefore, we attempted to evaluate whether silencing of the int6 gene might promote therapeutic angiogenesis in the hypoxic conditions of peripheral ischaemia.

The biochemical characteristics of siRNA molecules, such as their relatively high molecular weight, negative charge, and rapid degradation by nucleases, make systemic application difficult. Therefore, to deliver int6 siRNA directly to ischaemic sites, we injected it locally into the adductor muscles. Surprisingly, a single injection of 3.0 mg int6 siRNA provided clinical efficacy. Angiography revealed the recovery of blood vessels at the ischaemic site in rats injected with int6 siRNA. Histological analysis of the adductor muscles demonstrated that the number of αSMA-positive blood vessels was markedly increased in the int6 siRNA-injected rats when compared with that in the control rats. As αSMA is expressed in smooth muscle cells, treatment with int6 siRNA may induce the regeneration of arteries. In contrast, we observed no changes in the number of PECAM-1-positive blood vessels in any of the groups. PECAM-1 is a mechanotransduction molecule that is modulated by fluid shear stress and hyper-osmotic shock; therefore, ischaemic stress induced by the operation may have contributed to the up-regulation of PECAM-1. These data suggest that treatment with int6 siRNA might facilitate functional hindlimb recovery by regenerating the appropriate peripheral arteries.

Cilostazol, the only drug approved for the treatment of IC in patients with stable PAD, is a specific inhibitor of cAMP in platelets and vascular smooth muscle cells and has potent anti-platelet activity. In this study, we demonstrated that int6 siRNA was more effective than cilostazol in the treatment of IC in rats. As previously reported, we found that cilostazol improved walking function; however, our data showed that the efficacy of cilostazol was in fact quite limited when the blood perfusion rate was analysed. Cilostazol has been reported to increase the intracellular concentration of cAMP, leading to the inhibition of smooth muscle cell proliferation. By interfering with the proliferation of arterial smooth muscle cells, cilostazol may prevent arterial recovery.

Finally, we found that a single injection of int6 siRNA induced an immediate increase in HIF-2α mRNA expression in the affected hindlimbs, followed by an increase in the expression of both bFGF and HGF.
mRNAs after a short time lag; however, VEGF mRNA expression was unchanged in the affected muscle. Similar results were obtained when protein levels were analysed by immunofluorescence. The HIF-2α, bFGF, and HGF protein levels were increased following the up-regulation of mRNA expression for each target. Both HGF and bFGF are multifunctional cytokines that play an important role in endothelial cell regeneration; thus, they may have a synergistic effect on angiogenesis and tissue repair following int6 siRNA injection. Although HIF-1α shows broad tissue distribution and activity, the response of HIF-2α may be restricted to specific cell types. HIF-2α, which is regulated by int6, is responsible for VEGF induction, particularly in endothelial cells. In contrast, VEGF is induced mainly through HIF-1α activation in skeletal muscle. VEGF up-regulation was not obvious in this study, suggesting that HIF-2α might not be involved in the regulation of VEGF expression in the muscles, as was previously reported. Abundance of HIF-2α protein in the nucleus was markedly increased at 1 h after int6 siRNA injection, suggesting that nuclear translocation of HIF-2α was promoted. The results also allow us to speculate that degradation of HIF-2α by int6 was suppressed, and stabilized HIF-2α might induce the immediate response of nuclear translocation. Moreover, there is a possibility that not only stabilization, but also de novo synthesis of HIF-2α was up-regulated 0.5–1 h after int6 siRNA injection. Newly synthesized HIF-2α mRNA might be translated into protein, which may then accumulate in cytosol at 3–6 h following injection and then translocate to the nucleus 6 h following injection. The early induction and stabilization of HIF-2α was thought to be indispensable for the promotion of functional blood vessels immediately after ischaemia. Cytosolic accumulation and nuclear translocation of HIF-2α were also up-regulated 12 h after injection of int6 siRNA, suggesting that the angiogenic factors induced by early activation of HIF-2α may be responsible for the later HIF-2α response.

Figure 6 Histological analysis of time-dependent changes in the expression of angiogenesis-related proteins. (A) Protein expression of EPAS-1 (HIF-2α), bFGF, HGF, and VEGF in adductor muscle samples collected from the rat model of IC after surgery. Scale bars represent 50 μm. (B–E) Quantification of protein levels for EPAS-1 (B), bFGF (C), HGF (D), and VEGF (E). Data represent the means ± SEM from five experiments. *P < 0.05, in comparison with the values for scrambled siRNA-injected control rats.
Previous studies have evaluated the therapeutic effects of novel medicines on PAD by measuring the outgrowth of blood vessels and the recovery of local circulation. However, functional recovery of limbs has not been well explored, most probably due to the lack of precise methods to determine recovery. In the present study, we use the GAIT® system to clearly demonstrate an improvement in hindlimb function resulting from local injection of int6 siRNA in rats. STI data indicated that rats treated with int6 siRNA were relieved from gait disturbance and regained their ability to walk. Our results indicate that the silencing of endogenous int6 by specific siRNA constructs might cause steady-state stability and initial induction of HIF-2α expression, followed by later expression of other angiogenic factors, resulting in the modulation of angiogenesis. Therefore, we conclude that treatment with int6 siRNA may provide a novel strategy to induce therapeutic angiogenesis and promote arterial recovery, leading to the improvement of clinical symptoms in patients with PAD.

**Supplementary material**

Supplementary material is available at Cardiovascular Research Online.

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