Effect of Angiogenesis Induced by Consecutive Intramuscular Injections of Vascular Endothelial Growth Factor in a Hindlimb Ischemic Mouse Model

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Abstract

Purpose Angiogenesis plays a major role in various physiological and pathological situations. Thus, an angiogenic therapy with vascular endothelial growth factor (VEGF) has been commonly recommended as a representative therapeutic solution to recover the insufficient blood supply of collateral vessels in an ischemic lesion. In this study, the injection method and injection time point of VEGF proteins were focused to discover how to enhance the angiogenic effect with VEGF.

Methods Mouse models ($n=15$) were divided into control, VEGF treatment by intra-venous injection (VEGF-IV) and VEGF treatment by intra-muscular injection (VEGF-IM). Right proximal femoral arteries of mice were firmly sutured to obstruct arterial blood-flow. In the VEGF-IV treatment group, VEGF proteins were injected into the tail vein and, in the VEGF-IM treatment group, VEGF proteins were directly injected into the ischemic site of the right thigh after postoperative day 5, 10, 15, 20 and 25 follow-ups. Blood-flow images were acquired by ^{99m}Tc Gamma Image Acquisition

System to compare the ischemic-to-non-ischemic blood-stream ratio at postoperative days 5, 15, and 30.

Results VEGF-IM treatment significantly induced higher an angiogenic effect rather than both the control group ($P=0.008$) and VEGF-IV treatment group ($P=0.039$) at the 30th day.

Conclusion During all experiments, angiogenesis of VEGF-IM treatment represented the most evident effect compared with control and VEGF-IV group in a mouse model of hindlimb ischemia.

Keywords VEGF · Angiogenesis · Consecutive injection · Perfusion · Gamma imaging · Hindlimb ischemia

Introduction

Atherosclerosis affects many people globally. Atherosclerotic disease is causally related with coronary artery disease, stroke, and peripheral vascular disease [1]. Peripheral artery disease (PAD), which is one of the typical clinical conditions of atherosclerosis, is treated using anti-coagulant and anti-platelet drugs, percutaneous trans-luminal angioplasty, and bypass surgery. However, such treatments may carry an unfavorable surgical prognosis and drug-related side effects. Consequently, angiogenic treatment is commonly required to improve atherosclerotic symptoms [2].

Angiogenesis, the formation of new blood vessels from damaged vessels, ordinarily occurs during wound healing or organ regeneration and is significant in many physiological and pathological conditions, such as tumor growth, rheumatoid arthritis, and diabetic retinopathy [3, 4].

Vascular endothelial growth factor (VEGF) is a well-established angiogenic factor that stimulates new blood vessel formation during clinical situations including blood vessel occlusion, vascularization after injury of muscle following exercise, and bypassing blocked vessels [5].

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Simple injection of angiogenic factors into an ischemic lesion is insufficient for therapeutic angiogenesis [3]. To enhance the effect of VEGF for angiogenic therapy, VEGF release at the target region must be sustained [6]. This study examined the effectiveness of periodical intramuscular injections of VEGF compared with natural angiogenesis in a hindlimb ischemic mouse model.

Materials and Methods

Materials

Recombinant rat VEGF was obtained from Peprotech (St. Rocky Hill, NJ, USA). Isoflurane was obtained from Hana Pharmaceutical (Hwasung, Korea). Surgical suture was purchased from ETHICON (Somerville, NJ, USA). ^{99m}Tc -pertechnetate was eluted from a technetium generator produced by Covidien (Dublin, Ireland).

Preparation of Hindlimb Ischemic Mouse Models

All animal experiments were performed in compliance with the policies and procedures of the Institutional Animal Care and Use Committee for animal treatment of Chonbuk National University.

Female Balb/c mice ($n=15$, 8 weeks old) from Orient Bio (Seongnam, Korea) were allocated into three groups, which involve non-treatment (control, $n=5$), VEGF ($1\text{ }\mu\text{g}/150\text{ }\mu\text{l}$ in DIW3) treatment by intravenous injection (VEGF-IV, $n=5$) and VEGF treatment by intramuscular injection (VEGF-IM, $n=5$). All right femoral arteries were firmly occluded by surgical suture (5-0, silk) to obstruct inward flow of blood. At post-operative days 5, 10, 15, 20, and 25, VEGF was injected through a tail vein in the VEGF-IV group and directly approximately 5 mm away from the ischemic lesion in the right thigh of mice in the VEGF-IM group. The injections were done while mice were under anesthetic condition with inhalation of isoflurane in the supine position.

Assessment of Blood Perfusion in Hindlimb Ischemic Lesion

The tail vein was catheterized with a needle (27 G) and ^{99m}Tc (37 MBq) was injected. All blood perfusion images were acquired and measured by a Symbia TruePoint SPECT-CT gamma camera image acquisition system (Siemens Healthcare, Erlangen, Germany) at postoperative days 5, 15, and 30. A low-energy high-resolution (LEHR) collimator was used for the acquisition. For this procedure, the mice were anesthetized by inhalation in the supine position. ^{99m}Tc (37 MBq) was injected into a tail vein via the catheter needle. After ^{99m}Tc injection, the acquisition for blood perfusion was performed for 1 min and blood circulation images were

displayed on an LCD monitor as changes in intensity of ^{99m}Tc gamma energy by different pixels on a gray scale or a spectrum with colors. For the analysis of blood perfusion ratio, the region of interest (ROI) of ischemic and non-ischemic lesions was drawn as hindlimb shape (Fig. 1). Mean values and ischemic-to-non-ischemic ratio were calculated from the acquired ROI information.

Assessment of Immunohistochemistry (IHC) Staining

Myectomy of each ischemic right thigh was done at post-operative day 30. Paraffin-embedded sections were used to assess the expression of von Willebrand Factor/Factor VIII according to a standard histological protocol. Briefly, femoral muscle tissues were fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and embedded in paraffin. Femoral muscle tissue sections $4\text{ }\mu\text{m}$ in thickness were cut. Paraffin was removed from the tissue by immersion of sections in xylene prior to rehydration in a graded series of alcohols. The tissue sections were incubated in peroxidase-blocking solution (Dako, Glostrup, Denmark) to eliminate endogenous peroxidase activity, followed by protein block solution (Dako) and incubation with von Willebrand Factor/Factor VIII rabbit polyclonal antibody (Dako) in antibody diluent solution (Dako) overnight at $4\text{ }^{\circ}\text{C}$. Sections were then rinsed with Tris-buffered saline-Tween (TBST), incubated with secondary antibody at room temperature for 30 min, rinsed again with TBST and incubated with streptavidin peroxidase (Thermo Fisher Scientific, Basingstoke, UK) at room temperature for 30 min. Slices were developed using diaminobenzidine as 3-amino-9-ethylcarbazole (AEC) substrate chromogen (Dako) and counterstained with hematoxylin.

Statistical Analysis

Data are expressed as mean $\pm$ SD. The statistical difference between three groups was analyzed by the one-way ANOVA

Fig. 1 ROI drawing location for the estimation of bloodstream recovery ratio. A blue-colored arrow indicates an ischemic hindlimb and yellow one indicates a non-ischemic hindlimb. The area of both circles and number of pixels are equal to each other

test using the least significant difference (LSD) test available in SPSS software (SPSS, Chicago, IL, USA). A P value ≤ 0.05 was considered as statistically significant.

Results

^{99m}Tc Gamma Images of Blood Perfusion

Blood perfusion images revealed high intensity in the non-ischemic regions in the left limbs compared with the ischemic regions in right limbs. The intensity increased or decreased with time, whether treated or not (Fig. 2).

Analysis of Bloodstream Recovery Ratio

Bloodstream recovery ratio with VEGF-IV and VEGF-IM treatments was markedly increased within 15 days and either decreased rapidly thereafter (VEGF-IV) or was maintained throughout the 30-day follow-up (VEGF-IM), compared with the slight increase in the control group over 30 days (Fig. 3).

Findings of Immunohistochemistry Stain

An increment of VEGF expression has been observed by IHC staining with factor VIII at the fascia or vacuolating region following ischemic injury (Fig. 4). Number of blood vessels stained was increased against von Willebrand Factor/Factor VIII in VEGF treatment groups compared with the control group at the postoperative day 30. The VEGF-IM group especially showed more increased blood vessels than the VEGF-IV group and control group.

Discussion

Fundamentally, if perfusion of normal tissues is impaired by inadequate blood supply, several angiogenic factors including

Fig. 3 Ischemic to non-ischemic bloodstream recovery ratio in the three groups. Consecutive VEGF-IM treatments significantly induced higher bloodstream recovery ratio during all postoperative periods rather than both control group ($P_{15D}=0.03$, $P_{30D}=0.008$) and VEGF-IV treatment group ($P_{30D}=0.039$)

VEGF and corresponding receptor (VEGFR) of endothelial cells are increased to activate neovascularization in ischemic tissues with an enhanced permeability and retention (EPR) effect, which is induced by damaged or cancerous tissue [7]. For example, the control group displays an ordinary angiogenesis derived from the naturally synthesized VEGF.

Natural angiogenesis induced from the ischemic situation has the limitation of recovery to normal when the severity is beyond the threshold of spontaneous healing [4]. In this case, medical or surgical treatments should be provided. The ideal method of treatment is required to lower pain and simple application in the clinical situation. In general, the medical approach is the first option, except when surgical or interventional treatment is mandatory. In an effort to improve angiogenic effect, angiogenic factors can be exclusively used to treat ischemic diseases.

We used VEGF proteins, which are well-known and characterized angiogenic factors that produce an angiogenic effect. In this study, we anticipated that injection of VEGF enhances

Fig. 2 Gamma image of ^{99m}Tc at postoperative days 5, 15, and 30. The yellow-colored arrows indicate each of ischemic lesion sites. The scale bar that indicates an intensity of radioactivity is on the right side of the images

Fig. 4 IHC staining images of three groups. Most VEGF expression occurred at the fascia or vacuolating site following damage of the ischemic femoral muscle. The scale bar shows 50 μm (magnification $\times 200$). The arrows indicate microvessels which are stained with vWF

angiogenic effect relative to natural angiogenesis induced by naturally synthesized VEGF. The adjustment of injection period and the expansion of injection method were considered as two ways of treatment.

In this study, mice received VEGF injections at postoperative days 5, 10, 15, and 25. The amount of natural VEGF induced by ischemic condition might not be enough to recover that condition [8, 9]. We expected that if the VEGF is injected consecutively, the time to reach maximum recovery is going to be even shorter and more effective rather than the natural angiogenesis [10].

Moreover, the amount of VEGF from blood vessels may not be enough to enter the ischemic lesion because the expanded gaps caused by EPR effect of ischemic tissues may

return to normal during angiogenesis [7]. In case of peripheral ischemic lesions, intramuscular injection of VEGF adjacent to an ischemic lesion was considered as another method including intravenous injection to improve therapeutic effect. Intravenous injection can cause unexpected side effect because the factor can go and stay for a while by enhanced permeability and retention effect of vascular system anywhere vascularity is abnormal, but intramuscular injection can avoid such a problem. In addition, intramuscular injection is even simpler and faster to process than intravenous injection. We have compared the angiogenic effect between intravenous and intramuscular injection and identified the result that intramuscular injection of VEGF induces higher therapeutic effect than an intravenous injection of VEGF in this study.

As expected, the present results demonstrated that consecutively injected VEGF enhances the angiogenic effect compared with natural angiogenesis. Moreover, in case of intramuscular injection, the angiogenic effect remains stable. In terms of intravenous injection, the amount of VEGF factors being supplied to the ischemic lesion may not be sufficient to reach damaged tissue as followed by the formation rate and the distribution ratio of peripheral blood vessels. On the other hand, in case of intramuscular injection, we estimated that the angiogenic effect could be highly enhanced by direct injection of VEGF into the damaged tissue in relatively lower spatiotemporal limitation.

Follow-up of blood perfusion studies acquired from various radiopharmaceuticals, such as $^{99\text{m}}\text{Tc}$ -pertechnetate, $^{99\text{m}}\text{Tc}$ -labeled albumin, etc., offers accurate information on a diagnosis of whether blood perfusion is increased or not [11]. In addition, quantification of sample materials injected has powerful advantages of radionuclide analytic methods [12]. We used a gamma camera imaging system to evaluate blood perfusion and recovery status from ischemic conditions by natural angiogenesis and treatments of injected VEGF.

Finally, the ischemic lesions are developed from stenosis or occlusion of the proximal part of blood vessels. After ischemic pathologic process is distally progressed to overcome or adapt to that, microenvironments of peripheral tissue would be changed. Therefore, gross approach to a proximal culprit site such as via bypassing, stenting, or ballooning may not provide the complete solution. In terms of evaluation of microenvironments, sensitive diagnostic tools such as a gamma imaging system and additional therapeutic supplies of angiogenic factors such as VEGF after a gross approach, as used in this study, can provide more advanced solution to ischemic conditions.

Conclusion

Consecutive VEGF injections with the interval of 5 days produced an effective therapeutic result in peripheral ischemia,

although natural recovery power did not. Consecutive intramuscular injection was superior to intravenous injection in terms of blood restoration ratio.

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Conflicts of Interest Tai Kyoung Lee, Chang-Moon Lee, Hyosook Hwang, Kyung Sook Na, JeongIl Kwon, Hwan-Seok Jeong, Philsun Oh, Hee Kwon Kim, Seok Tae Lim, Myung-Hee Sohn, and Hwan-Jeong Jeong declare that they have no conflict of interest.

Ethical Statement This study was approved by the animal ethics committee in our hospital and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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