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Original Article

A combination of VEGF165/HGF genes is more effective in blood vessels formation than ANGPT1/VEGF165 genes in an *in vivo* rat model

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Abstract: Background: The angiogenic potential of an experimental therapy consisting in an administration of the genes for vascular endothelial growth factor (VEGF165) in combination with hepatocyte growth factor (HGF) or angiopoitein-1 (ANGPT1) has already been studied. However, there is still little known which combination of genes is the most efficient. Methods: Two bicistronic plasmid vectors carrying pIRES/VEGF165/HGF and pIRES/ANGPT1/VEGF165 and an empty plasmid (control group) were constructed. 30 rats were divided into three equal groups: pIRES/VEGF165/HGF, pIRES/ANGPT1/VEGF165 and control group (empty plasmid). The plasmids were injected in the hind limbs of the animals and 12 weeks later they were euthanized and tissue samples from the thigh muscle were harvested. Then, under a microscope, vessels stained for the alpha-smooth muscle actin (α-SMA) and factor VIII (FVIII) were counted. Results: The average number of SMA+ or FVIII+ blood vessels in the pIRES/VEGF/HGF group was 8.96±3.10 and 10.88±2.70, respectively. The angiogenesis was less effective after ANGPT1/VEGF injection and the mean value was 7.12±3.30 for α-SMA+ and 8.63±3.63 for FVIII+ vessels. The average number of SMA+ and FVIII+ vessels in the control group was 4.62±2.46 and 5.82±2.42, respectively. All differences were statistically significant and *P* values were <0.01. Conclusions: In a rat model, the combination VEGF165 and HGF genes is more effective in the neovascularization process than VEGF165 and ANGPT1 and more effective than the injection of an empty plasmid.

Keywords: Blood vessels, gene therapy, ischemia, angiogenesis

Introduction

Critical lower limb ischemia (CLI) is a common disease in the developed countries [1-3]. CLI often results in the need for limb amputation and increases the overall risk of death compared to the general population [3, 4]. An optimal medical therapy, surgical procedures and even modern endovascular procedures used in CLI patients yield very poor results. Several studies indicate a death rate of 25% to 30% per year in CLI patients who do not qualify for surgery [1, 3]. One of the new methods of improving the microcirculation in critically ischemic tissues involves the activation of angiogenesis processes by gene and cell-based therapies [5, 6].

The activation of blood vessel formation in adult tissues requires a cooperation of numerous cells and proteins. Vascular endothelial growth factor (VEGF) is the key, initial player in this multi-step process. It stimulates proliferation and migration of endothelial cells as well as inhibits apoptosis. The activity of VEGF is determined by the presence of VEGF receptors on the cell surface [7, 8]. The VEGF-A overexpression in vivo initiates neovascularization in the damaged or ischemic tissue [9, 10]. However, the formation of mature and stable

blood vessels requires the presence of other growth factors. Angiopoietin-1 (Ang-1) plays the essential role in late phase of angiogenesis and is responsible for the endothelial maturation and stabilization of vascular vessels [11]. In contrast to VEGF, Ang-1 doesn't show mitogenic properties. It inhibits endothelial permeability as well as inflammation [12]. Ang-1 acts on the cells by binding surface, specific receptor, Tie-2 [13]. Hepatocyte Growth Factor (HGF) is heterodimeric protein described as a powerful activator of cell proliferation [14, 15]. The receptor for HGF, c-Met is present on various types of cells including endothelium. It was postulated that HGF may effect on vascular endothelial growth factor receptor-2 (VEGFR2) expression [16, 17]. However, a recent study did not confirm such a relationship [18].

Therapeutic angiogenesis using plasmid vectors as the carriers of growth factor genes are considered to be safe [19]. Our previous experience with VEGF165 gene application using plasmid vectors in animal model as well as CLI patients shows evidence of angiogenesis induction [20, 21]. Nevertheless, the results were more satisfying when a combined gene-cell therapy was utilized [21, 22]. Mesenchymal stem cells (MSC) are a good source of cytokines and growth factors but its isolation from patients with chronic limb ischemia for autologous transplantation is often inefficient. For this reason, we decided to develop a double gene therapy using an optimal set of genes for the induction of angiogenesis process in animal model.

The aim of this study is to compare the efficiency of neoangiogenesis in hind limbs of rats of two bicistronic plasmid vectors carrying pIRES/VEGF165/HGF and pIRES/ANGPT1/VEGF165.

Materials and methods

Plasmid preparation

The total RNA was isolated from ischemic human heart tissue using EZNA RNA isolation Kit (BIoTEC) according to the manufacturer's protocol. The reverse transcription reaction was done with the SuprScript III kit (Invitrogen) for cDNA library synthesis. HGF cDNA was amplified by PCR using specific pair of primers: 5'ATATCTAGACCGTCCAGCAGCACC and 5'-ATAGTCGACCAGACACACTTACTTCAG. The cDNA fragments for ANGPT1 were obtained using a forward primer 5'ATAGCTAGCTGCTGGCAGTACAA and a reverse primer 5'ATAACGCGTCATTGCGCTTTC. Human VEGF165 cDNA was prepared as described previously [21]. All cDNAs were cloned into pIRES bicistronic plasmid (Clontech) with the restriction enzymes. In the case of pIRES/VEGF165/HGF vector VEGF fragment was inserted into multisite cloning place A (MSCA) and HGF in multisite cloning place B (MSCB). In vector pIRES/ANGPT1/VEGF165, VEGF was cloned into MSCB whereas ANGPT1 in MSCA. In vitro studies have shown that VEGF cloning site (A or B) has no effect on the efficiency of protein production.

All plasmids were purified using EndoFree Plasmid Giga Kit (Qiagen) and dissolved in sterile 0.9% NaCl solution. The apyrogeneity of the plasmids was confirmed by a Limulus amebocyte lysate assay, Pyrochrome Chromogenic Test Kit (Charles River). Endotoxin level was below 10 EU per 1 mg DNA in all tested samples.

RT-PCR analysis

COS7 African green monkey fibroblasts (ATCC) was transfected transiently with plasmids pIRES/ANGPT1/VEGF165 and pIRES/VEGF-165/HGF using FuGENE® 6 transfection reagent (Roche) according to the manufacturer's protocol. 48 h after transfection, the cells were harvested and total RNA was extracted using E.Z.N.A™ Total RNA Kit (Omega Biotek) as instructed by the manufacturer. Afterwards, 0.5 μg total miRNA was used as a template into synthesis of cDNA using Thermoscript™ RT-PCR System (Life Technologies). The mRNA expression level was measured in relative real-time PCR method using TaqMan Fast Universal PCR Master Mix (Applied Biosytems) and specific TaqMan Gene Expression Assays (Hs00-900054_m1 for VEGFA, Hs00300159_m1 for HGF, Hs00181613_m1 for ANGPT1 and H99999905_m1 for 18S). All reactions were prepared on 96-well plate in conditions recommended by the manufacturer. The real-time PCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems) under thermal cycling conditions: 20 s at 95°C and 40 cycles of 1 s at 95°C and 20 s at 60°C. For quantification, the samples were normalized against the expression of 18S. Relative quantification (RQ) for the examined mRNAs was cal-

culated using the $2^{-\Delta\Delta CT}$ method. The results were presented as logarithm of RQ.

ELISA of human VEGF, HGF and angiopoietin-1

Human VEGF DuoSet (R&D Systems) was used for ELISA detection of VEGF165 in medium samples. The concentration of human angiopietin-1 and HGF was determined by corresponding Quantikine® kit (R&D Systems) according to the protocols of the manufacturer.

Animals and experimental protocols

The research protocol was approved by the Local Ethics Committee (Ap. No: 70/2011). The study was carried out on30 healthy Fisher female rats. The animals were divided into three, equal groups. The first group received 4 mg of pIRES/VEGF165/HGF plasmid in four intramuscular injections in the right hind limb. The second group was injected with 4 mg of pIRES/ANGPT1/VEGF165 plasmid. The third, control group received 4 mg of empty plasmids.

After 12 weeks all animals were euthanized and muscle tissue samples from the intramuscular injection site were collected for further analyses.

Histology and histopathology

Histologic specimens of the muscle samples were thinly cut into 5-μm sections and stained with hematoxylin and eosin for tissue necrosis analysis. The samples were examined using an Olympus BX51 light microscope.

Immunohistochemistry

Formalin fixed paraffin-embedded tissue was freshly cut into 5-μm sections. The sections were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed with xylene and gradually hydrated. Activity of endogenous peroxidase was blocked by 5-min exposure to 3% H_2O_2. All the studied sections were boiled for 15 min at 250 W in Antigen Retrieval Solution (Dako, Denmark). Then, immunohistochemical reactions were performed using the following antibodies: monoclonal mouse anti-human α-Smooth Muscle Actin-αSMA (clone 1A4, Dako) in dilution 1:100, monoclonal mouse Factor VIII light chain (H-100, Santa Cruz Biotechnology) in dilution 1:100 and monoclonal mouse anti-human VEGF (VG-1, Santa Cruz Biotechnology) in dilution 1:100. The specific antibodies were incubated with studied sections for 1 h at room temperature. Subsequent incubations involved biotinylated antibodies and streptavidin-biotinylated peroxidase complex (LSAB+, HRP; Dako). For visualization of the reaction, the DAB+ Liquid kit was used (Dako). The sections were counterstained with Mayer's hematoxylin for 30 sec.

Assessment of the number of vessels

The evaluation of the neovascularization using the angiogenesis markers was performed under the light microscope Olympus BX41 with AnalySIS DOCU software (Soft Imaging GmbH). Factor VIII and alpha smooth muscle actin (alpha-SMA) protein expression was evaluated using a quantitative modified Weidner's method [18]. A microscopic image from one slide magnified 200 times was transferred to a computer program, and five randomly selected fields of view with the highest density were selected. It means that 50 fields of view were selected for each study group (10 animals × 5 microscopic fields of view). Then, all blood vessels, brown stained for FVIII or SMA were counted automatically.

Statistical analysis

The data were analyzed with Statistica 10.0 software (StatSoft). The distribution of the variables was tested with Shapiro-Wilk's W test prior to any further statistical analyses. Summary statistics are expressed as medians with 25th and 75th percentiles. The Kruskal-Wallis ANOVA on ranks with post-hoc test was used for multi-group comparisons (independent samples). Comparisons within a single group among different time points were performed by using a Wilcoxon rank sum test. Statistical significance was determined as $P<0.05$.

Results

VEGF165, HGF and Ang-1 expression in cell culture

An in vitro evaluation of the expression activity of plasmid constructs was performed through a transfection of the plasmids to the COS7 cells.

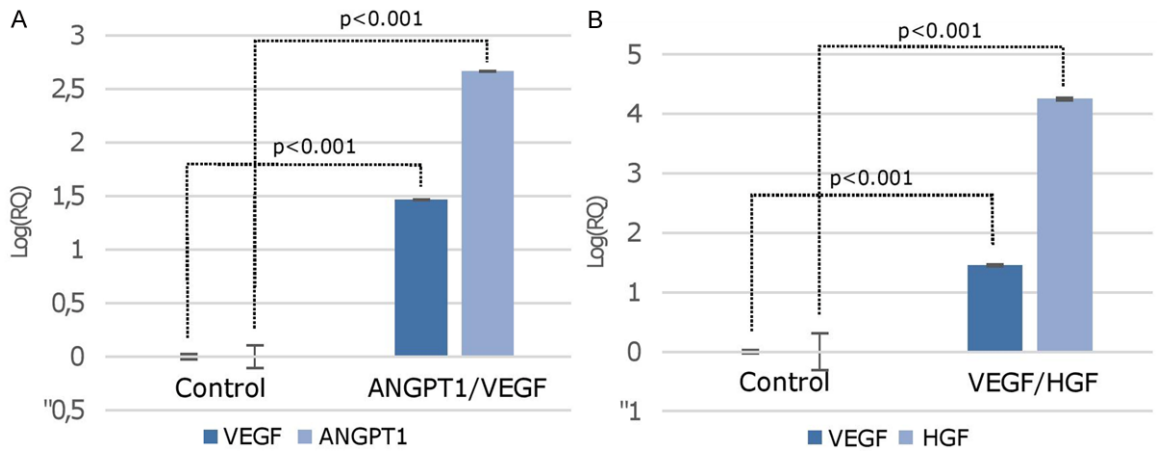

Figure 1. Expression of pIRES/ANGPT1/VEGF and pIRES/VEGF/HGF plasmid constructs in the COS7 cells. Analysis of VEGF, HGF ANGPT1 mRNAs in the COS7 cells transiently transfected with plasmid constructs was performed using real-time RT-PCR method. For relative quantification (RQ) the samples were normalized against 18S rRNA using $2^{-\Delta\Delta Ct}$ method. The COS7 cells transfected with empty plasmid were used as a control. Bars represent the mean ± SD of logarithm of RQ.

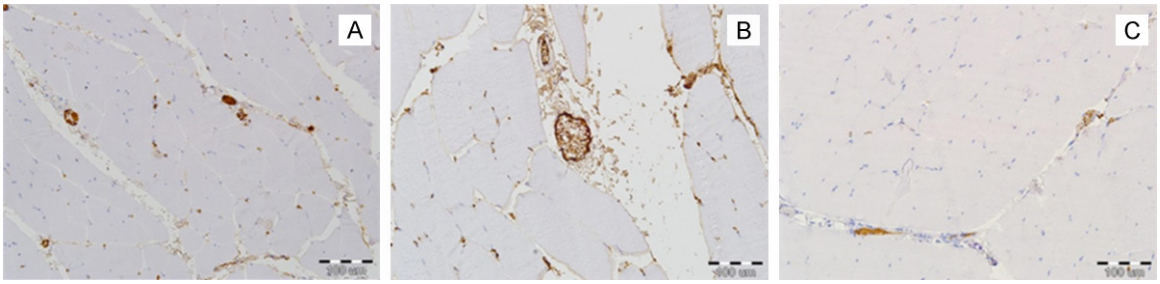

Figure 2. Expression of pIRES/ANGPT1/VEGF and pIRES/VEGF/HGF plasmid constructs in muscle samples from study group of animals. Representative images of VEGF-stained rat muscle cross sections after (A) intramuscular injection of pIRES/VEGF/HGF, (B) pIRES/ANGPT1/VEGF and (C) empty plasmid.

The mRNA level of individual genes in transfected cells was identified using relative real-time PCR method. As shown in **Figure 1**, the transfection with pIRES/ANGPT1/VEGF165 (**Figure 1A**) as well pIRES/VEGF165/HGF (**Figure 1B**) resulted in an increased mRNA level of HGF, VEGF and ANGPT1 compared to control cells transfected with empty plasmid. These results were confirmed on protein level by quantitative ELISA. Both, angiopoietin-1 and HGF were undetectable in the samples of cell culture media, whereas after transfection their concentration increased to 1873±1042 pg/ml and 42.39±28.04 pg/ml respectively. In contrast to HGF and angiopoietin-1 the production of VEGF protein increased slightly or remained at the same level. In case of VEGF we observed positive reaction with media collected from untreated cell culture, which was most likely caused by too low species specificity of the antibodies used in the ELISA test.

Necropsy and histological examination

All rats survived the 12-week test period. The animals developed properly in all groups. Necropsy did not reveal any pathological changes in organs and tissues of the animals. In all animals from all groups a normal histological picture of the striated muscles in the hind limbs was found.

Immunohistochemical evaluation of angiogenesis

There was a significant increase in concentration of VEGF protein and capillary density in animals receiving the pIRES/VEGF165/HGF (**Figure 2A**) and pIRES/ANGPT1/VEGF165 (**Fig-**

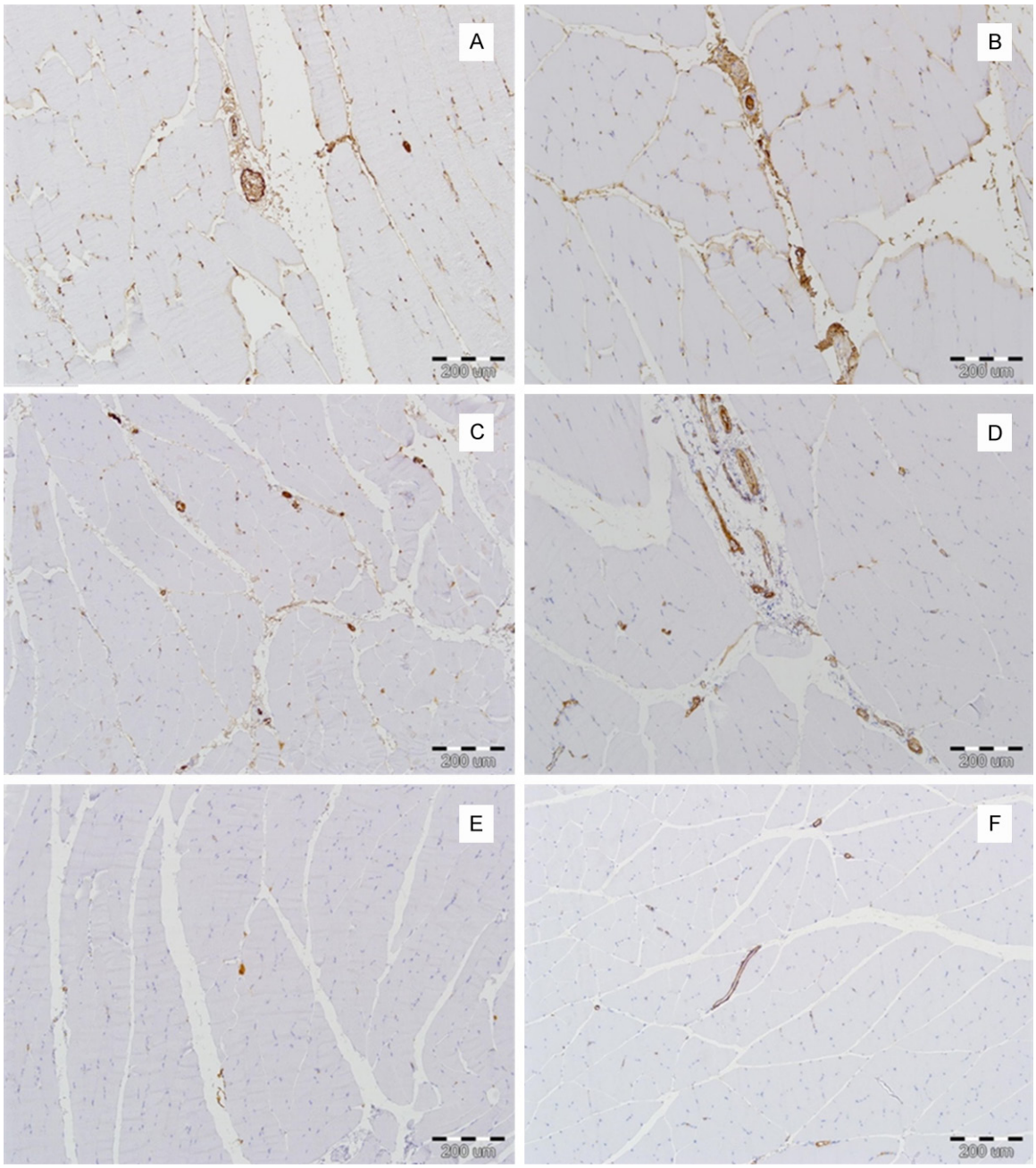

Figure 3. Histological analysis of skeletal muscle neovascularization. Representative photos of sections stained for α-SMA (right) and Factor VIII (left). A and B: Images represented tigh muscles treated with pIRES/VEGF/HGF. C and D: With pIRES/ANGPT1/VEGF. E and F: With empty plasmid, magnification 100 ×.

ure 2B) plasmids compared to the control group (**Figure 2C**).

The administration of pIRES/VEGF165/HGF as well as pIRES/ANGPT1/VEGF165 resulted in an increased new blood vessels formation compared with the empty plasmid administration (**Figure 3**). However, the highest number of α-SMA- and FVIII-positive capillaries was observed after pIRES/VEGF165/HGF genes transfer (**Figure 3A**, **3B**).

The average number of blood SMA+ or FVIII+ vessels in the pIRES/VEGF/HGF group was 8.96±3.10 and 10.88±2.70, respectively (**Figures 4**, **5**). The capillary formation was less ef-

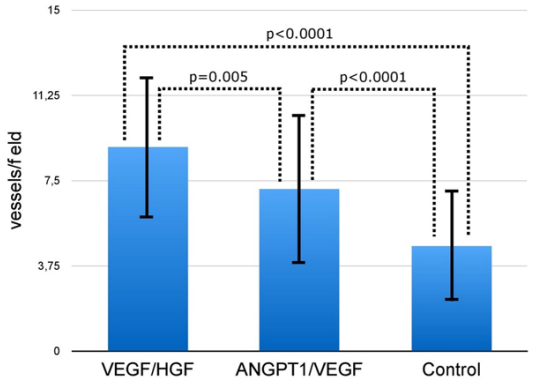

Figure 4. The mean number of capillary vessels under one microscopic field of view (α-SMA-marker).

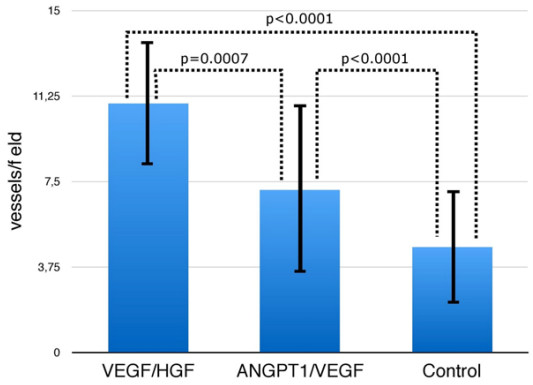

Figure 5. The mean number of capillary vessels under one microscopic field of view (FVIII- marker).

fective after pIRES/ANGPT1/VEGF165 injection and the mean value was 7.12±3.30 for α-SMA+ and 8.63±3.63 for FVIII+ vessels. The average number of SMA+ and FVIII+ capillaries in the control group was 4.62±2.46 and 5.82±2.42, respectively. All differences were statistical significant and *P* values were <0.01.

Discussion

Gene therapy is a promising, alternative treatment option for patients with chronic limb ischemia. Since the first application of naked DNA in CLI therapy by J. Isner, various approaches have developed [24]. The most popular is the use of plasmids and viral vectors. Most of the recent studies involved a single gene, like VEGF, HGF, fibroblast growth factors (FGF1, FGF2, FGF4) [19]. However, a therapy using a single angiogenic factor has been proven to be ineffective, mainly because the angiogenesis requires a cascade of various growth factors [25, 26].

The application of bicistronic vectors with the internal ribosome entry site (IRES) allows simultaneous expression of two genes in the same cell [27]. VEGF, Ang-1 and HGF are regarded as key angiogenic factors [26, 28, 29]. Therefore, the combinations of VEGF165 with ANGPT1 gene and VEGF165 with HGF gene were chosen for the analysis in our study.

VEGF alone is a critical factor for initiation of angiogenesis. However, it has also been shown as key mediator of the inflammation and vascular permeability [30]. An addition of Ang-1 leads to vessel maturation and suppress VEGF-induced permeability and inflammation in endothelial cells [31, 32]. Moreover, Ang-1 has been reported as a chemotactic agent on bone marrow-derived hematopoietic stem cells and inducer of endothelial differentiation [33-35]. Similar to VEGF, HGF is a powerful endothelial mitogen. The benefit of the HGF use is lack of inducing vascular permeability and no proinflammatory effects [36, 37].

In our study, all animals were healthy and developed properly during the 12-week observation period. Necropsy did not reveal any pathological changes in organs and tissues of the rats, including neoplastic lesions. After the injection of both, pIRES/ANGPT1/VEGF165 and pIRES/VEGF165/HGF plasmids, the angiogenesis was more intense than in the rats receiving empty plasmids. The pIRES/VEGF165/HGF plasmid was significantly more effective in inducing the neoangiogenesis than the pIRES/ANGPT1/VEGF165 plasmid. The reason why the improvement in the neovascularization process observed after an injection of the pIRES/VEGF165/HGF was significantly higher better needs to be examined in future studies.

Conclusions

In conclusion, intramuscular administration of bicistronic vectors encoding pIRES/VEGF165/HGF and pIRES/ANGPT1/VEGF165 induced local angiogenesis and did not cause neoplastic processes in the internal organs. The combination of VEGF165 and HGF genesis was more effective in the neovascularization process than VEGF165 and ANGPT1 and more effective than the injection of an empty plasmid.

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Disclosure of conflict of interest

None.

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References

[1] Minar E. Critical limb ischaemia. Hamostaseologie 2009; 29: 102-1099.

[2] Jensen SA, Vatten LJ, Myhre HO. The prevalence of chronic critical lower limb ischaemia in a population of 20,000 subjects 40-69 years of age. Eur J Vasc Endovasc Surg 2006; 32: 60-65.

[3] Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG; TASC II Working Group, Bell K, Caporusso J, Durand-Zaleski I, Komori K, Lammer J, Liapis C, Novo S, Razavi M, Robbs J, Schaper N, Shigematsu H, Sapoval M, White C, White J, Clement D, Creager M, Jaff M, Mohler E 3rd, Rutherford RB, Sheehan P, Sillesen H, Rosenfield K. Inter-society consensus for the management of peripheral arterial disease (TASC II). Eur J Vasc Endovasc Surg 2007; 33: S1-S75.

[4] Mehler PS, Coll JR, Estacio R, Esler A, Schrier RW, Hiatt WR. Intensive blood pressure control reduces the risk of cardiovascular events in patients with peripheral arterial disease and type 2 diabetes. Circulation 2003; 107: 753-756.

[5] Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease, part I: angiogenic cytokines. Circulation 2004; 109: 2487-2491.

[6] Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease, part II: cell-based therapies. Circulation 2004; 109: 2692-2697.

[7] D’Angelo G, Martini JF, Iiri T, Fantl WJ, Martial J, Weiner RI. 16K human prolactin inhibits vascular endothelial growth factor-induced activation of Ras in capillary endothelial cells. Mol Enocrinol 1999; 13: 692-704.

[8] Wu HM, Yuan Y, Zawieja DC, Tinsley J, Granger HJ. Role of phospholipase C, protein kinase C, and calcium in VEGF-induced venular hyperpermeability. Am J Physiol 1999; 276: H535-42.

[9] Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348: 593-600.

[10] Shyu KG, Chang H, Wang BW, Kuan P. Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. Am J Med 2003; 114: 85-92.

[11] Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, Sato TN, Yancopoulos GD. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998; 282: 468-471.

[12] Gamble JR, Drew J, Trezise L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, Vadas MA. Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res 2000; 87: 603-607.

[13] Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996; 87: 1161-1169.

[14] Rubin JS, Chan AM, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW, et al. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci U S A 1991; 88: 415-419.

[15] Kan M, Zhang GH, Zarnegar R, Michalopoulos G, Myoken Y, McKeehan WL, Stevens JI. Hepatocyte growth factor/hepatopoietin A stimulates the growth of rat kidney proximal tubule epithelial cells (RPTE), rat nonparenchymal liver cells, human melanoma cells, mouse keratinocytes and stimulates anchorage-independent growth of SV-40 transformed RPTE. Biochem Biophys Res Commun 1991; 174: 331-337.

[16] Van Belle E, Witzenbichler B, Chen D, Silver M, Chang L, Schwall R, Isner JM. Potentaial angiogenic effect of scatter factor/hepatocyte grpwth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. Circulation 1998; 97: 381-390.

[17] Wojta J, Kaun C, Breuss JM, Koshelnick Y, Beckmann R, Hattey E, Mildner M, Weninger W, Nakamura T, Tschachler E, Binder BR. Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells. Lab Invest 1999; 79: 427-438.

[18] Sengupta S, Gherardi E, Sellers LA, Wood JM, Sasisekharan R, Fan TP. Hepatocyte growth factor/scatter factor can induce angiogenesis independently of vascular endothelial growth factor. Arterioscler Thromb Vasc Biol 2003; 23: 69-75.

[19] Gupta R, Tongers J, Losordo DW. Human studies of angiogenic gene therapy. Circ Res 2009; 105: 724-736.

[20] Skóra J, Barć P, Dawiskiba T, et al. Angiogenesis after plasmid VEGF165 gene transfer in animals model. Centr Eur J Immunol 2013; 38: 305-309.

[21] Sadakierska-Chudy A, Skóra J, Barć P, et al. Angiogenic therapy for clinical lower limb ischemia. Adv Clin Exp Med 2010; 19: 347-359.

[22] Skóra J, Barć P, Pupka A, Dawiskiba T, Korta K, Albert M, Szyber P. Transplantation of autologous bone marrow mononuclear cells with VEGF gene improves diabetic critical limb ischaemia. Endokrynol Pol 2013; 64: 129-138.

[23] Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N Engl J Med 1991; 324: 1-8.

[24] Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes JF. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patients with ischemic limb. Lancet 1996; 348: 370-374.

[25] Smith AH, Kuliszewski MA, Liao C, Rudenko D, Stewart DJ, Leong-Poi H. Sustained improvement in perfusion and flow reserve after temporally separated delivery of vascular endothelial growth factor and angiopoietin-1 plasmid deoxyribonucleic acid. J Am Coll Cardiol 2012; 59: 1320-8.

[26] Makarevich P, Tsokoaeva Z, Shevelev A, Rybalkin I, Shevchenko E, Beloglazova I, Vlasik T, Tkachuk V, Parfyonova Y. Combined transfer of human VEGF165 and HGF genes renders potent angiogenic effect in ischemic skeletal muscle. PLoS One 2012; 7: e38776.

[27] Małecki M, Przybyszewska M, Janik P. Construction of abicistronicproangiogenic expressionvector and its application in experimental angiogenesis in vivo. Acta Biochim Pol 2003; 50: 875-82.

[28] Chen F, Tan Z, Dong ChY, Shieh JH, Dias S, Crystal RG, Rafii S. Combination of VEGF165/Angiopoietin gene and endothelial progenitor cells for therapeutic neovascularisation. Europ J Pharmacol 2007; 568: 22-230.

[29] Samuel SM, Akita Y, Paul D, Thirunavukkarasu M, Zhan L, Sudhakaran PR, Li C, Maulik N. Coadministration of adenoviral vascular endothelial growth factor and angiopoietin-1 enhances vascularization and reduces ventricular remodeling in the infarcted myocardium of type 1 diabetic rats. Diabetes 2010; 59: 51-60.

[30] Ylä-Herttuala S, Rissanen TT, Vajanto I, Hartikainen J. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 2007; 49: 1015-26.

[31] Kim I, Moon SO, Park SK, Chae SW, Koh GY. Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1, and E-selectin expression. Circ Res 2001; 89: 477-479.

[32] Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, Ko JK, Koh GY. Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arterioscler Thromb Vasc Biol 2000; 20: 2573-2578.

[33] Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S. Vascular endothelialgrowth factor and angiopoietin-1 stimulate postnatal hematopoiesis byrecruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001; 193: 1005-1014.

[34] Kim MS, Lee CS, Hur J, Cho HJ, Jun SI, Kim TY, Lee SW, Suh JW, Park KW, Lee HY, Kang HJ, Lee DS, Koh GY, Nakagami H, Morishita R, Park YB, Kim HS. Priming with angiopoietin-1 augments the vasculogenic potential of the peripheral blood stem cells mobilized with granulocyte colony-stimulating factor through a novel Tie2/Ets-1 pathway. Circulation 2009; 120: 2240-2250.

[35] Gomei Y, Nakamura Y, Yoshihara H, Hosokawa K, Iwasaki H, Suda T, Arai F. Functional differences between two Tie2 ligands, angiopoietin-1and -2, in regulation of adult bone marrow hematopoietic stem cells. Exp Hematol 2010; 38: 82-89.

[36] Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S, Uchiyama Y, Ogihara T, Kaneda Y, Morishita R. Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. Circulation 2004; 109: 424-431.

[37] Min JK, Lee YM, Kim JH, Kim YM, Kim SW, Lee SY, Gho YS, Oh GT, Kwon YG. Hepatocyte growth factor suppresses vascular endothelial growth factor-induced expression of endothelial ICAM-1 and VCAM-1 by inhibiting the nuclear factor-kappaB pathway. Circ Res 2005; 96: 300-307.