The aim of the study was to investigate how an intramuscular injection of plasmids with genes coding various pro-angiogenic factors: angiopoetin-1 (ANGPT1), vascular endothelial growth factor (VEGF165) and hepatic growth factor (HGF), influences the production of ANGPT1. Forty healthy Fisher rats received i.m. injections containing plasmids encoding angiogenic genes in thigh muscles. They were divided into four equal groups. The first group received the plANGPT1 plasmid and the second group—the pIRES/ANGPT1/VEGF165 bicistronic plasmid. The pIRES/VEGF165/HGF bicistronic plasmid was administered to the third group and an empty plasmid (control group) to the fourth group. The animals were euthanized after 12 weeks. In each group, the number of vessels stained with the anti-ANGPT1 antibody was assayed under an optical microscope. The anti-ANGPT1 antibodies stained the vessels in all the groups. There were on average $14.1 \pm 2.3$ vessels in the the plANGPT1 group, $32.5 \pm 10.5$ in the pIRES/ANGPT1/VEGF165 group and $30.8 \pm 13.3$ in the pIRES/VEGF165/HGF group. There were on average $7.3 \pm 2.3$ stained vessels (p < 0.0001) in the control group. The VEGF plays a role in the induction of the production of ANGPT1. The administration of genes does not require surgical intervention and is considered safe for the patients [9].

Materials and methods

The RNA was extracted from human heart tissue using a BioTEC EZNA RNA isolation Kit. The plasmids, pIRES/ANGPT1/VEGF165 and pIRES/VEGF165/HGF were prepared according to a previously described protocol [16]. In order to produce monogenic plANGPT1, a DNA fragment was digested using EcoRI and Xhol and subsequently cloned into a pcDNA3 vector (Invitrogen, USA) using T4 Ligase (Sigma, USA). The aprotinine of the plasmids was verified using a Lumulus amboccy lyase assay, Pyrochrome Chromogenic Test Kit (Charles River). Each sample contained less than 10EU of endotoxin per 1mg of DNA.

Forty healthy Fisher rats were used in the study (weight: 200-250 g). All rats grew under controlled conditions (temperature $23^\circ$C and 12 hour light/dark cycles). The rats were divided into four equal groups: ANGPT1, ANGPT1/VEGF, VEGF/HGF and the control group. Each animal received four consecutive intramuscular injections into the right hind limb containing: 4mg of the plANGPT1 plasmid in the ANGPT1 group, 4 mg of the pIRES/ANGPT1/VEGF165 plasmid in the ANGPT1/VEGF group, 4 mg of the pIRES/VEGF165/HGF plasmid in the VEGF/HGF group and 4mg of a naked plasmid in the control group. After 12 weeks, the rats were euthanized using 200 mg/kg of sodium pentobarbital solution. The tissues were extracted from the anterior region of the limb (for a histological and immunohistochemical analysis).

The muscle samples were divided in 5-μm slices and stained using hematoxylin and eosin. The number of the vessels stained with the anti-ANGPT1 antibodies were counted in each group in order to assess the induction of the production of angiopoetin-1. The immunohistochemical reactions were performed using the anti-angiopoetin 1 antibody (rabbit polyclonal to angiopoetin 1, catalogue number ab451, ABCAM, dilution 1 : 100). The immunohistochemically marked samples were assessed using the Olympus BX41 light microscope with computer microscopic image analysis software (AnalySIS DOCU).

The ANGPT1 protein expression was evaluated by recording multiple random fields of view using the modified Weidner’s method [17]. Microscopic images of the slices were analyzed digitally at a 200× magnification. Five randomly selected fields of view with the highest density of positive (brown) stained foci that marked blood vessels were selected automatically. Each vessel or concentration of endothelial cells (regardless of the presence or absence of a full lumen) was counted as an individual micro-vessel. The average number of vessels was calculated.

The data were analysed using Statistica 10.0 (StatSoft, Inc. Tulsa, USA). Prior to any further statistical analyses, the distribution of the variables was estimated using the Shapiro-Wilk’s W test. The normality test failed and data were analysed using nonparametric tests. The Kruskal-Wallis ANOVA on ranks with post-hoc test was used for multi-group comparisons (independent samples). Statistical significance was determined as p < 0.05.

The study was approved by the local Ethical Committe.

Results

All the rats survived the 12 week experiment period. Their development was normal. All the animals were similar in appearance and behaviour. Post mortem analysis showed no morphological pathologies in the muscles or the internal organs. The pathological assessment of all of the harvested muscle specimens confirmed as the presence of normal skeletal striated muscles.

The anti-ANGPT1 antibodies stained the vessels in all the groups. There were on average $14.1 \pm 2.3$ vessels in the ANGPT1 group, $32.5 \pm 10.5$ in the ANGPT1/VEGF group and $30.8 \pm 13.3$ vessels in the HG/HGF group. There were on average $7.3 \pm 2.3$ vessels in the control group (p < 0.0001). The results are shown in Fig. 1. The microscopic images with the stained vessels are presented in Fig. 2.

Key words: blood vessels, gene therapy, ischaemia, angiogenesis.

Introduction

Peripheral artery disease (PAD) affects over 25 mln people in Europe and USA [1]. Many patients do not qualify for standard surgery or endovascular treatment. In those patients, optimal palliative medical therapy is the only available treatment. Such patients still suffer from chronic pain, ulcerations and often require limb amputations [2].

Hence, alternative methods of treatment, including therapeutic angiogenesis, are sought [3, 4]. One therapeutic concept is based on the use of genes encoding angiogenic factors delivered to the ischaemic muscle tissues [5, 6]. The clinical benefits (reduction of pain, accelerated healing of ischaemic ulcerations, reduction in the incidence of limb amputation) of such therapy supports its use in patients suffering from critical limb ischemia and with no other therapeutic options [7, 8].

The administration of genes does not require surgical intervention and is considered safe for the patients [9]. Several studies assessed the efficiency of factors and genes in promoting angiogenesis, i.e. vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and angiopoetin 1 antibody (rabbit polyclonal to angiopoetin 1, catalogue number ab451, ABCAM, dilution 1:100). The immunohistochemically marked samples were assessed using the modified Weidner’s method [17]. Microscopic images of the slices were analyzed digitally at a 200× magnification. Five randomly selected fields of view with the highest density of positive (brown) stained foci that marked blood vessels were selected automatically. Each vessel or concentration of endothelial cells (regardless of the presence or absence of a full lumen) was counted as an individual micro-vessel. The average number of vessels was calculated.

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ANGPT1 group. Surprisingly, the expression of angiopoietin 1 increased in the VEGF/HGF group, which did not receive the ANGPT1 gene. Moreover, the administration of the ANGPT1 plasmid did not induce a significant angiopoietin 1 expression compared to the control group that received a naked plasmid. This indicates that the expression of angiopoietin 1 is increased only in the late phase of angiogenesis and that the administration of the plasmids encoding angiopoietin 1 alone do not promote sufficient angiogenesis. To increase the production of ANGPT1, additional factors such as VEGF, HGF, which act in the early phases of angiogenesis, are necessary. Hence, an optimal therapeutic set of genes to accelerate angiogenesis should include late mediators of angiogenesis as well as factors that induce this process.

In summary, an administration of the plasmid encoding the ANGPT1 gene did not significantly increase the production of angiopoietin 1 compared to the control group in Fisher rats. Additional factors acting in the early phase of angiogenesis (VEGF, HGF) were necessary to accelerate the production of angiopoietin 1.

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