Original paper

Role of vascular endothelial growth factor in inducing production of angiopoetin-1 – *in vivo* study in Fisher rats

Piotr Barć[1], Tomasz Płonek[2], Dagmara Baczyńska[3,4], Artur Pupka[1], Wojciech Witkiewicz[4], Agnieszka Mastalerz-Migas[5], Artur Milnerowicz[1], Maciej Antkiewicz[1], Agnieszka Hałoń[6], Jan P. Skóra[1]

[1]Department of Vascular, General and Transplantation Surgery, Wroclaw Medical University, Poland
[2]Department of Cardiac Surgery, Wroclaw Medical University, Poland
[3]Molecular Techniques Unit, Department of Forensic Medicine, Wroclaw Medical University, Poland
[4]Wrovasc, Integrated Cardiovascular Centre, Provincial Specialist Hospital in Wrocław, Research and Development Centre, Wrocław, Poland
[5]Family Medicine Department, Wroclaw Medical University, Poland
[6]Department of Pathomorphology and Oncological Cytology, Wroclaw Medical University, Poland

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.

40 Healthy Fisher rats received i.m. injections containing plasmids encoding pro-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 assessed under an optical microscope. The anti-ANGPT1 antibodies stained the vessels in all the groups. There were on average 14.1 ±2.3 vessels in the the plANGPT1 group, 32.5 ±10.5 in the pl/RESANGPT1/VEGF group and 30.8 ±13.3 in the plRES/HGV/VEGF group. There were on average 7.3 ±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 plasmids only encoding ANGPT1 does not induce its production.

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 a 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 that assessed the efficiency of factors and genes in promoting angiogenesis, i.e. vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and angiopoetin 1 (ANGPT1) are available. An increased vascularization, extracellular matrix remodelling and an inhibition of apoptosis and inflammatory processes were reported [9, 10, 11, 12, 13, 14, 15].

Angiopoetin 1 mainly acts in the late phase of angiogenesis and is responsible for the stabilization and maturing of the vessels [11]. In contrast to VEGF, ANGPT1 does not show mitogenic features [12]. Angiopoetin1 stimulates the capillary endothelial cells to bind through the Tie2 receptor and stabilizes them [13]. Furthermore, it promotes the migration of the endothelial cells towards damaged vessels and promotes vessel regeneration.

The aim of this study is to investigate how an intramuscular injection of plasmids with genes coding various pro-angiogenic factors (plAPT1, pIRES/ANGPT1/VEGF165 and pIRES/VEGF165/HGF) influences the production of angiopoetin 1 (ANGPT1).

Material and methods

The RNA was extracted from human heart tissue using an 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 monogene plANGPT1, a cDNA fragment was digested using EcoRI and XhoI and subsequently cloned into a pcDNA3 vector (Invitrogen, USA) using T4 Ligase (Sigma, USA). The apyrogenecity of the plasmids was verified using a Limulus amebocyte lysate assay, Pyrochrome Chromogenic Test Kit (Charles River). Each sample contained less than 10EU of endotoxin per 1mg of DNA.

Forty healthy Fischer rats were used in the study (weight: 200-250 g). All rats grew under controlled conditions (temperature 23°C and 12 hour light/dark cycles). The rats were divided into four equal groups: ANGPT1, APGPT1/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 euthanasied using 200 mg/kg of sodium pentobarbital solution. The tissues were extracted from the injection areas and sent 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-angiopoietin 1 antibody (rabbit polyclonal to agiopoietin 1, catalogue number ab8451, ABCAM, dilution 1 : 100). The immunochemically marked samples were assessed under 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 for each sample.

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 Committee.

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 histological 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 ±2.3 vessels in the ANGPT1 group , 32.5 ±10.5 in the ANGPT1/VEGF group and 30.8 ±13.3 vessels in the HGV/VEGF group. There were on average 7.3 ±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.

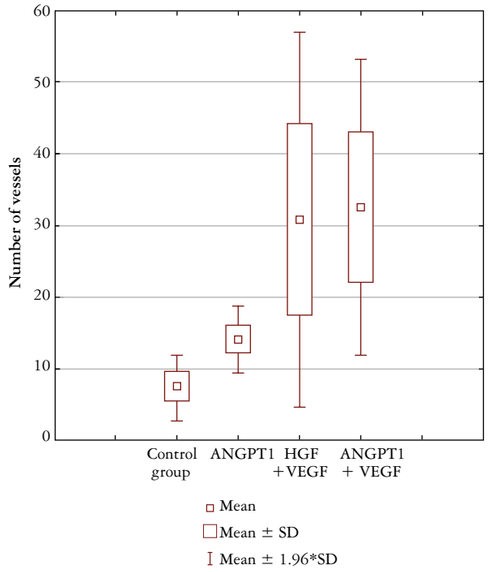

Fig. 1. The number of vessels in each group, stained using anti-APT1 marker

Discussion

Angiogenesis is a complex process that involves the extracellular matrix, the endothelial progenitor cells and cytokines. The extracellular matrix acts as a storage of cytokines, whichare released through the activation of matrix metaloproteinases (MMP). Angiogenesis is regulated mainly by interactions between cytokines, such asthe vascular endothelial growth factor (VEGF), angiopoietin-1 (ANGPT1) and the hepatocyte growth factor (HGF) [4, 5, 6].

VEGF is the critical initiating factor and a mediator of the inflammatory process and vascular permeability [18]. HGF inhibits vascular permeability and pro-inflammatory effects [19, 20]. Bicistronic plasmids coding VEGF with HGF seems to be effective during the first stage of the neoangiogenesis gene therapy [21, 22].

ANGPT1 is an important factor in the late stage of angiogenesis. The function of this cytokine acts in the acute and chronic phase. During the acute phase, the expression of APT1 reduces the ischemic damage due to its anti-apoptotic functions [11]. It acts on the endothelial cells through Tie-2 receptors, improving their survival and maturation [13]. ANGPT 1 also suppresses VEGF-induced permeability and inflammation in endothelial cells [23, 24]. In the chronic phase, it promotes the creation of new vessels from stem/progenitor-like cells and induces the reconstruction of the basal membrane [25]. *In vivo* studies confirmed that ANGPT1 is largely responsible for capillary maturation and the chemotactic response, the formation of blood vessels and their stability but and does not promote mitotic activity [11, 12, 13].

The number of anti-ANGPT1-stained vessels was higher in the ANGPT1/VEGF group than in the

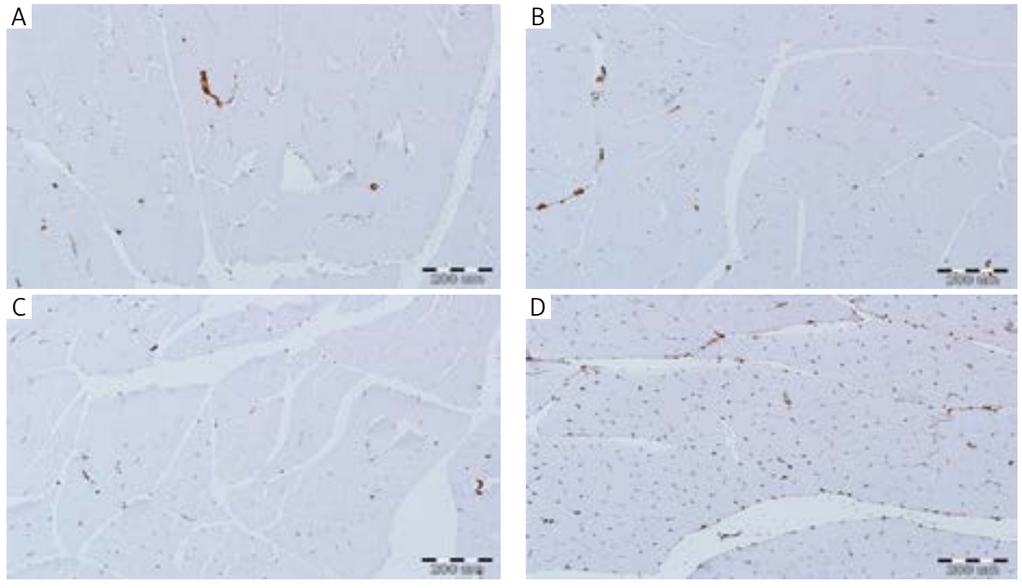

Fig. 2. The immunohistochemical expression of angiopoietin 1 in the vessels: A) control group; B) APT1 group; C) HGF/VEGF group; D) APT1/VEGF group. Hematoxylin and eosin, original magnification: 200×

ANGPT1 group. Surprisingly, the expression of angiopoetin 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 angiopoetin 1 expression compared to the control group that received a naked plasmid. This indicates that the expression of angiopoetin 1 is increased only in the late phase of angiogenesis and the administration of the plasmids encoding angiopoetin 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 angiopoetin 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 angiopoetin 1.

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Address for correspondence

Agnieszka Mastalerz-Migas
Family Medicine Department
Wroclaw Medical University
Syrokomli 1
51-141 Wroclaw, Poland
e-mail: agnieszka.migas@gmail.com