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Jan P. Skóra[1, A–F], Tomasz Płonek[2, B–E], Piotr Barć[1, B–D], Dagmara Baczyńska[3, 4, B, C], Agata Radwańska[4, B, C], Artur Pupka[1, A, C], Krzysztof Korta[1, B, D], Marek Ussowicz[5, C, E], Łukasz Strożecki[6, C], Diana Kupczyńska-Markiewicz[1, A], Agnieszka Hałoń[7, A–C, E, F]

The Formation of Blood Vessel After the Administration of the Plasmid Encoding Ang-1 Gene in Fischer Rats

[1] Department of Vascular, General and Transplantation Surgery, Wroclaw Medical University, Poland
[2] Department of Cardiac Surgery, Wroclaw Medical University, Poland
[3] Department of Forensic Medicine, Molecular Techniques Unit, Wroclaw Medical University, Poland
[4] Wrovasc – Integrated Cardiovascular Centre, Regional Specialist Hospital in Wrocław, Research and Development Centre, Poland
[5] Department of Pediatric BMT, Oncology and Hematology, Wroclaw Medical University, Poland
[6] Department of Anaesthesiology and Intensive Therapy, Wroclaw Medical University, Poland
[7] Department of Pathology, Wroclaw Medical University, Poland

A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Chronic limb ischemia is a serious clinical problem. Patients who do not qualify for standard treatment may benefit from novel gene therapies.
Objectives. This study evaluated angiogenesis following intramuscular injections of angiogenic plasmid Ang-1 in Fisher rats.
Material and Methods. Twenty rats had plasmids injected intramuscularly in their hind limbs. The study group consisted of 10 animals which received the Ang-1 plasmid, while the control group consisted of 10 rats that received an empty plasmid. All the animals were euthanized after 12 weeks and tissue samples from the hind limb thigh muscles and internal organs were harvested for histological and immunohistochemical examinations. To assess the angiogenesis the number of vessels in the hind limb muscles visualized by the SMA and FVIII markers was counted for each animal in five separate microscopic fields.
Results. There were no pathological lesions or any signs of neoplastic angiogenesis in any of the 20 rats. The number of vessels visualized by the FVIII marker in the study group was two times higher than in the control group (median: 12, range: 7–25 vs. median: 6, range: 2–15; $p < 0.0001$). The median estimated that the number of vessels visualized by the SMA marker is 63% higher in the study group compared to the control group (median: 6.5, range: 1–12 vs. median: 4, range: 0–10; $p = 0.0008$).
Conclusions. Intramuscular injections of Ang-1 plasmids induced angiogenesis in the rat hind limb muscles (**Adv Clin Exp Med 2016, 25, 4, 611–615**).

Key words: angiogenesis, lower limb ischemia, rats, proangiogenic factors.

Cardiovascular disease is the leading cause of death in the Western world and the World Health Organization estimates that over 17 million people worldwide die of cardiovascular disorders each year [1]. There are more than 25 million people in Europe and the United States who have chronic ischemia affecting lower limbs caused by atherosclerotic lesions in the peripheral arteries. Approximately 1% of patients with peripheral artery disease develop critical lower limb ischemia (CLI) with an unfavorable prognosis. Within one year after the diagnosis, more than 30% of patients with lower limb CLI require amputation [1–4].

The development of endovascular treatment using angioplasty and stents have decreased the amputation rate among patients with CLI. How-

ever, in many cases, the atherosclerosis is too severe to treat the lesions surgically or endovascularly. Therefore, there is a need for other therapeutic options, i.e., stem cells and gene therapy [5–10].

The efficacy of gene therapy was tested in several small clinical studies but has not been examined in large randomized studies [8–10]. A new treatment strategy that aims at the regeneration of endothelial cells uses plasmids encoding proangiogenic cytokines, i.e., the vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and angiopoietin – 1(Ang-1) [5, 8, 11]. Experimental studies suggest that gene therapy can induce vascular growth, cardiomyogenesis and reduce apoptosis and inflammation [5, 11–14].

Angiogenesis is regulated by an interaction between cytokines such as VEGF and Ang-1. Ang-1 stimulates a functional connection between the endothelial cells in microcirculatory blood vessels. Experimental studies have shown that Ang-1 promotes endothelial cell migration and vascular regeneration. VEGF and Ang-1 may also activate the expression of proangiogenic genes. Moreover, Ang-1 is also known to stabilize newly formed vessels [14–17].

The aim of the study was to evaluate the efficacy of the induction of new blood vessel formation by intramuscularly injecting plasmid encoding the angiopoietin-1 (Ang-1) in the hind extremities of Fisher rats.

Material and Methods

The research protocol was approved by the local Ethics Committee (Approval No: 70/2011 of 16 November 2011).

Twenty healthy Fischer rats (weight range: 200–250 g) were selected for the study. The animals were divided into two groups: The study group and the control group. The first group consisted of 10 rats that received 4 mg of naked plasmid Ang-1 in 4 consecutive intramuscular injections in the right hind limb. The control group consisting of 10 rats received 4 intramuscular injections containing an empty plasmid. Three months after the injections, all animals were euthanized and muscle tissue samples from the intramuscular injection site were collected for histological, immunohistochemical and immunomorphometric analyses. A *postmortem* examination was carried out in all animals so as to examine the potential influence of intramuscular injections of the Ang-1 plasmid on other organs.

Plasmid Construction

The proangiogenic plasmid expressing the Ang-1 gene was produced by the local Department of Molecular Technology. Total RNA was extracted from ischemic heart tissue using the EZNA Total RNA Kit I according to the protocol provided by the manufacturer (Omega BIO-TEC). 0.5 μg of RNA was used as a template in the reaction of reverse transcription with oligo (dT) primers and the SuperScript III First-Strand Synthesis System (Invitrogen). cDNA for ANGPT1 (NM_001146.3) was amplified with the use of a specific pair of 5'ATAGCTAGCTGCTGGCAGTACAA and 5'ATAACGCGTCATTGCGCTTTC primers under thermal cycling conditions: Initial denaturation at 95ºC for 2 min, 5 cycles with denaturation at 95ºC for 20 sec, annealing at 51ºC for 15 sec, elongation at 72ºC for 1.5 min and then 30 cycles with denaturation at 95ºC for 20 sec, annealing 62ºC for 15 sec and elongation at 72ºC for 1.5 min. The PCR product was ligated into a pSC-B-amp/kan vector (Stratagene) under conditions recommended by the manufacturer. The ANGPT1 cDNA fragment was purified after digestion with *Eco*RI and *Xho*I and was then cloned into a pcDNA3 vector (Invitrogen) using T4 Ligase (Sigma). The correctness of the structure of the plasmid was confirmed by both restriction and sequence analysis. The apyrogenecity of the plasmid was confirmed using the Limulus amebocyte lysate assay, Pyrochrome Chromogenic Test Kit (Charles River).

Neoangiogenesis Markers

Alpha-SMA and FVIII proteins were used as markers for neovascularization imaging. 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. Endogenous peroxidase was blocked by a 5-min exposure to 3% H_2O_2. All studied sections were boiled for 15 min at 250W in an Antigen Retrieval Solution (Dako, Denmark). Immunohistochemical reactions were then performed using two antibodies: (1) monoclonal mouse anti-human smooth muscle actin (SMA), clone 1A4, catalog number M085, 1:100 dilution (Dako, Danmark) and (2) factor VIII light chain (H-100) antibody, catalog number sc-33584, 1:100 dilution (Santa Cruz Biotechnology, USA). The specific antibodies were incubated for 1 h at room temperature. Subsequent incubations involved biotinylated antibodies (15 min, room temperature) and streptavidin–biotinylated peroxidase complex (15 min, room temperature) (LSAB+, HRP; Dako, Denmark). The DAB+ Liquid kit was used (Dako, Danmark) (7 min, room temperature) to visualize the reaction. The sections were counterstained with Mayer's hematoxylin for 30 sec.

Evaluation of Vessel Density

Histologic specimens of the muscle samples were thinly cut into 5-μm slices and stained with hematoxylin and eosin. The evaluation of neoangiogenesis was performed under the Olympus BX41 light microscope using a computer microscopic image analysis program (AnalySIS DOCU, v. 3.2, Soft Imaging GmbH). Factor VIII and SMA protein expression were evaluated using the quantitative modified Weidner's method [25]. A microscopic image magnified 200 times from one slide was transferred to a computer program. Five randomly selected fields of view with the highest density of stained blood vessels were counted automatically. Each vessel or concentration of endothelial cells (regardless of the presence or absence of the vessel's lumen) was treated as a "hot point" and counted as an individual microvessel. Based on the number of these "hot points", a total number of vessels were counted for each field.

Statistical Analysis

The data was analyzed using STATISTICA 10.0 (StatSoft, Inc. Tulsa, USA). The distribution of variables was tested using the Shapiro-Wilk test prior to further statistical analyses. The normality test failed and the data was analyzed using the nonparametric Mann-Whitney U test. Statistical significance was determined at $p < 0.05$.

Results

All rats survived the 12-week test period after intramuscular plasmid administration and their clinical course was uncomplicated. Necropsy did not reveal any pathological changes in the organs and the tissues of the animals. There were no signs of pathological neoplastic angiogenesis in the examined tissues. A histological examination of the hind limb muscles revealed no abnormalities in any of the 20 rats.

The process of new blood vessel formation in hind limbs was observed in all animals from both groups. However, there was a significantly higher number of vessels in animals receiving Ang-1 plasmid compared to the control group (Table 1). The number of vessels visualized by the FVIII marker in the study group was two times higher than in the control group (median: 12, range: 7–25 vs. median: 6, range: 2–15; $p < 0.0001$). The median estimated number of vessels visualized by the SMA marker was 63% higher in the study group compared to the control group (median: 6.5, range: 1–12 vs. median: 4, range: 0–10; $p = 0.0008$). Moreover, an increased accumulation of FVIII and SMA proteins in the visualized vessels was observed in rats receiving the Ang-1 plasmid compared to those receiving an empty plasmid (Figs. 1, 2).

Discussion

Critical limb ischemia is a serious clinical problem. In severe cases, standard treatment does

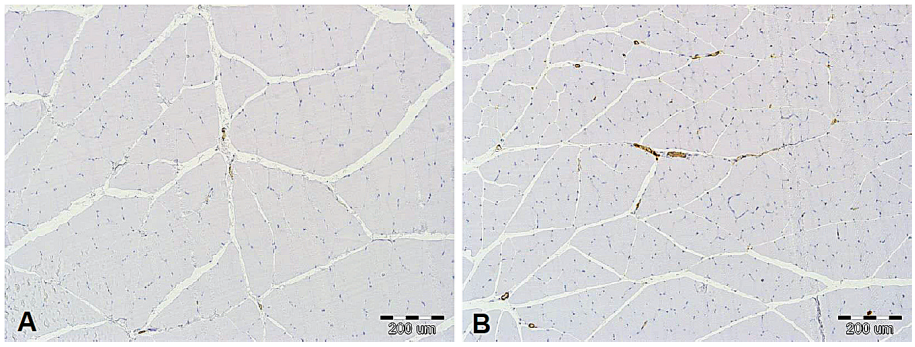

Fig. 1. A comparison of angiogenesis (SMA marker)

A – control group;
B – study group.

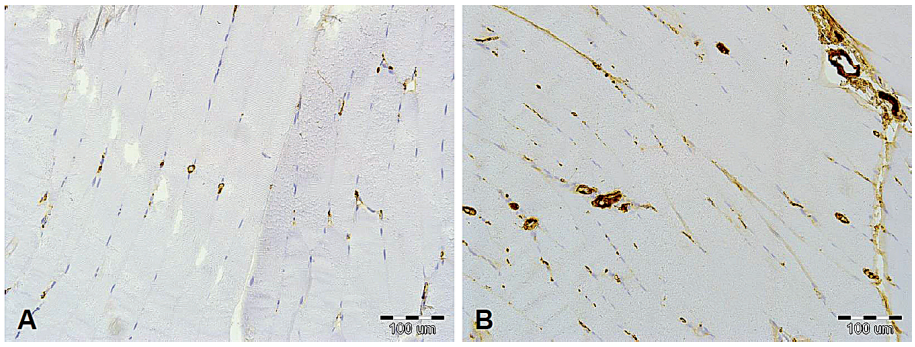

Fig. 2. A comparison of angiogenesis (FVIII marker)

A – control group;
B – study group.

not relieve the patient's symptoms and does not stop the disease. One of the methods to improve the perfusion in the ischemic limb is a gene therapy aimed at inducing angiogenesis [18, 19]. However, angiogenesis is a complex multistage process and it is difficult to predict the results of stimulating it "artificially".

The angiogenesis starts with an increase in the permeability of the blood vessels [8, 13]. This is followed by the degradation of the basement membrane, reorganization of original vessel walls and controlled proteolysis of the matrix molecules [20–23]. Angiopoietin-1 (Ang-1) is of particular interest because receptors for angiopoetin are located in endothelial cells [16, 17]. *In vitro* experiments have shown that Ang-1 has specific effects on endothelial cells: It does not have a mitotic effect but induces a chemotactic response, network formation, sprouting and stops apoptosis [14, 16, 17]. *In vivo* analyses using targeted gene inactivation have suggested that Ang-1 recruits and sustains periendothelial support cells for vessel formation [16, 17, 23, 24]. In acute ischemia, the Ang-1 reduces ischemic injury through antiapoptotic functions leading to a reduction in endothelial cell death and vessel damage, as well as a reduction of vessel leakage and edema caused by ischemia or trauma, which results in better tissue perfusion [16, 19, 20, 23, 24]. At the chronic stage, expression of Ang-1 improves angiogenesis through induction of new vessel formation from stem/progenitor cells.

Several studies have recently examined therapeutic neovascularization using growth factors in critically ischemic limbs and myocardium. In most studies, the application of growth factors significantly induced angiogenesis [16, 17, 23, 24]. In our study, the injection of Ang-1 plasmid significantly increased the number of blood vessels at the injection site compared to the animals which received an empty plasmid.

Intramuscular injections of the Ang-1 plasmid did not stimulate angiogenesis outside the injection site. This was evidenced by an absence of any signs of neoplastic angiogenesis in the necropsy material. Similar results have been observed by other researchers [11, 12, 14, 15]. However, to evaluate the safety of the Ang-1 plasmid administration, longer observation of a larger group of animals is needed.

Molecular technology is rapidly evolving. Gene therapy may become standard treatment not only for genetic disorders but also for common diseases such as limb or myocardial ischemia. The results of both *in vivo* and *in vitro* studies are promising. Nevertheless, large randomized clinical studies are necessary to fully evaluate the safety and efficacy of this treatment method.

In conclusion, an injection of the Ang-1 plasmid in hind limb muscles in rats promoted local angiogenesis and did not lead to general angiogenesis or the stimulation of neoplastic processes in the internal organs of the animals.

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Address for correspondence:

Tomasz Płonek
Department of Cardiac Surgery
ul. Borowska 213
50-556 Wrocław
Poland
E-mail: tomaszplonek@gmail.com

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