ORIGINAL PAPER

Nagoya J. Med. Sci. **87**. 285–294, 2025
doi:10.18999/nagjms.87.2.285

Myonectin stimulates endothelial angiogenic activity in vitro and in vivo

Tomohiro Masutomi[1], Noriyuki Ouchi[2], Shukuro Yamaguchi[3], Koji Ohashi[2], Naoya Otaka[1], Zhongyue Pu[1], Yuuki Shimizu[1], Toyoaki Murohara[1] and Rei Shibata[3]

[1]*Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Molecular Medicine and Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[3]*Department of Advanced Cardiovascular Therapeutics, Nagoya University Graduate School of Medicine, Nagoya, Japan*

ABSTRACT

Endurance exercise is known to reduce the risk of cardiovascular disease. Myonectin, a myokine, is increased by endurance exercise and affects remote organs such as the heart. However, the role of myonectin in the blood vessels is unknown. In this study, we investigated the role of myonectin in angiogenesis. Human umbilical vein endothelial cells (HUVECs) were treated with recombinant myonectin to assess tube formation, proliferation, and migration. An in vivo Matrigel plug assay was performed by transplanting Matrigel containing myonectin into myonectin-knockout (Myo-KO) mice, and angiogenic response was evaluated. Mouse models of hindlimb ischemia were developed by ligating and removing the femoral arteries of wild-type (WT), Myo-KO, and myonectin-overexpressing transgenic (Myo-TG) mice, and blood flow was evaluated over time by laser Doppler imaging. In vitro, treatment with myonectin increased the differentiation of HUVECs into vascular-like structures. Myonectin significantly stimulated HUVEC migration, as assessed using a modified Boyden chamber assay. Treatment with myonectin also increased HUVEC proliferation, as assessed by the MTS assay. In the Matrigel plug assay, plugs containing myonectin displayed a significantly higher-degree of endothelial cell infiltration than plugs containing vehicle. Angiogenic repair of ischemic hindlimbs was impaired in Myo-KO mice compared to that in WT mice. However, Myo-TG mice had significantly increased limb perfusion after ischemic surgery compared to that in WT mice. This study showed that myonectin acts directly on vascular endothelial cells and promotes angiogenesis. Treatment aimed at increasing myonectin production may be useful in the treatment of cardiovascular diseases with vascular dysfunction.

Keywords: myonectin, angiogenesis, endothelial cells

Abbreviations:
EBM-2: endothelial cell basal medium-2
HUVECs: human umbilical vein endothelial cells
Myo-KO: myonectin-knockout
Myo-TG: myonectin-overexpressing transgenic
PAD: peripheral arterial disease

Received: August 19, 2024; Accepted: October 2, 2024
Corresponding Authors: Noriyuki Ouchi, MD, PhD; Rei Shibata, MD, PhD
Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
Tel: +81-52-744-2427, Fax: +81-52-744-2427,
E-mail: nouchi@med.nagoya-u.ac.jp (Ouchi); rshibata@med.nagoya-u.ac.jp (Shibata)

WT: wild-type

INTRODUCTION

Endurance exercise is highly effective in reducing the risk of cardiovascular disease.[1] It has also been reported to improve cardiac function and increase the lifespan of patients with ischemic heart disease.[2,3] Exercise therapy has also been shown to be useful for the treatment of intermittent claudication induced by peripheral arterial disease (PAD).[4] Exercise increases collateral circulation and improves blood flow, which allows for increased walking distance and improves prognosis.[5,6] However, the molecular mechanisms underlying the effects of endurance exercise on cardiovascular disease have not been fully elucidated.

The skeletal muscle can secrete bioactive substances called myokines that affect nearby and remote organs.[7,8] These myokines, such as myonectin, interleukin-6, follistatin-like 1, and fibroblast growth factor 21, have drawn attention in recent years as they have been reported to affect the cardiovascular system.[8,9] Myonectin, or C1q/TNF-related protein 15, is an adiponectin paralog with a C-terminal C1q globular domain and a collagen domain.[10] It is expressed at low levels in adipose tissues and distributed abundantly in the skeletal muscle, especially type I muscle fibers.[10,11] Additionally, endurance exercise increases the expression of myonectin in the skeletal muscle and blood.[11] Myonectin has been reported to increase triglyceride uptake in adipocytes, inhibit apoptosis in the liver, and promote the use of stored iron by suppressing hepcidin production in the liver, thereby promoting erythrocyte production.[12-14] In addition, we have reported that myonectin protects against skeletal muscle dysfunction caused by various pathophysiology conditions, such as age-associated, disuse-induced or steroid-induced muscle atrophy.[15]

Recently, we studied the effects of myonectin on ischemic heart disease to determine the role of myonectin in the cardiovascular system.[16] In an ischemia-reperfusion injury model, myonectin-knockout (Myo-KO) mice had larger myocardial infarcts and significantly increased blood troponin levels compared to wild-type (WT) mice. In contrast, the myocardial infarct size after myocardial ischemia-reperfusion injury was significantly reduced in transgenic mice with skeletal muscle-specific overexpression of myonectin (Myo-TG) compared to that in WT mice.[16] It has also been reported that intravenous administration of adeno-associated virus serotype 9-mediated myonectin suppresses pressure overload-induced cardiac hypertrophy and fibrosis.[17] Based on these findings, myonectin is considered to have a protective effect against cardiac diseases; however, its role in blood vessels remains to be elucidated. This study investigated whether myonectin regulates endothelial cell function in vitro and the effect of myonectin on angiogenesis in vivo using both loss- and gain-of-function genetic manipulations.

MATERIALS AND METHODS

Materials

Recombinant mouse myonectin protein produced was purchased from Aviscera Bioscience (CA, USA). The CD31 antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth medium-2 (EGM-2; Basel, Switzerland). Before differentiation and migration assay, the cells

were placed in endothelial cell basal medium-2 (EBM-2; Basel, Switzerland) with 0.5% fetal bovine serum (FBS) for 16 h for serum starvation. Experiments were performed by adding the indicated amounts of recombinant myonectin protein or vehicle for the indicated lengths of time.

Differentiation assay

The formation of vascular-like structures by HUVECs on growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA) was assessed according to the manufacturer's instructions, as previously described.[18] HUVECs were seeded at 1.0×10^4 cells per well on coated 96-well plate in EBM-2 and incubated at 37 °C for 18 h. Network formation was assessed using an inverted phase contrast microscope (Keyence, Osaka, Japan), and photomicrographs were taken at 20× magnification. The degree of tube formation was quantified by measuring the length of the tubes at center fields of each well using the Angiogenesis Analyzer for Image J. The following were also quantitatively evaluated using the Angiogenesis Analyzer: 1) nodes, defined as pixels having at least three neighbors; 2) junctions, defined as groups of nodes forming a bifurcation; 3) segments, defined as binary lines linked with two junctions; 4) total length, sum of branches, and segment length per image (unit: pixel); and 5) total mesh area, defined as the sum of the area enclosed by segments in each image (unit: pixel).[19]

Cell proliferation assay

The proliferation of HUVECs was assessed using the MTS assay. HUVECs were seeded at 3.0×10^3 cells per well in a 96-well plate and treated with myonectin or vehicle for 24 h in EBM-2 with 0.5% FBS. The number of viable cells was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).[20] Twenty microliters of CellTiter 96 Aqueous One Solution reagent was added directly to the wells. The cells were incubated for 2 h, and the absorbance at 490 nm was measured using a 96-well plate reader.

Migration assay

The migration activity of HUVECs was measured using a modified Boyden chamber assay.[21] Serum-starved cells were trypsinized and resuspended in EBM-2 with 0.5% FBS, and 2.0×10^4 cells/well (250 μL) was added to a Transwell gelatin-coated insert (6.5-mm diameter, 3.0-μm pore size, Corning, Kennebunk, ME, USA). Then, 450 μL of EBM-2 with 0.5% FBS supplemented with myonectin (5 μg/mL) or phosphate-buffered saline (PBS) was added to the lower chamber, and the cells were incubated for 4 h. Migrated cells on the lower surface of the membrane were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI). Five random microscopic fields per well were quantified. All assays were performed in duplicate.

Mouse experiments

WT, Myo-KO, and Myo-TG male mice with a C57/BL6 background were used in this study. The study protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University.

Matrigel plug assay

The formation of new vessels in vivo was evaluated using the Matrigel plug assay, as described previously.[22] For these experiments, 400 μL of Matrigel containing myonectin (20 μg/mL) or vehicle were injected into the left inguinal subcutaneous of Myo-KO mice. Mice were euthanized 14 days after injection. Matrigel plugs with adjacent subcutaneous tissues were carefully recovered by en bloc resection, fixed in 4% paraformaldehyde, dehydrated with 30% sucrose, and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA,

USA) in liquid nitrogen. Immunohistochemical staining for CD31 (PECAM-1; Becton Dickinson) was performed on adjacent frozen sections. The primary antibody was used at 1:1000 dilution, followed by incubation with the secondary antibody (Alexa Fluor 594 anti-rat IgG at a 1:100 dilution). CD31-positive capillaries were counted in four randomly chosen high-power (40×) microscopic fields of plug edge. The Matrigel area (μm^2) and endothelial cell area (μm^2) were measured to quantify angiogenesis using ImageJ.

Mouse model of revascularization

Ten-week old WT, Myo-KO, and Myo-TG mice underwent unilateral hindlimb surgery under anesthesia with (medetomidine, midazolam and butorphanol at doses of 0.15, 2.0 and 2.5 mg/kg, respectively).[21,23] The entire left femoral artery and vein were surgically excised.

Hindlimb blood flow was measured using a laser Doppler blood flow (LDBF) analyzer (Moor LDI, Moor Instruments) on postoperative day 14. LDBF analysis was performed on the feet. Blood flow was displayed as changes in the laser frequency using pixels of different colors. After scanning, the stored images were analyzed to quantify blood flow. To avoid data variations due to ambient light and temperature, hindlimb blood flow was expressed as the ratio of LDBF in the left leg (ischemic) to that in the right leg (non-ischemic).

Statistical analysis

Data are shown as mean ± SD. Differences between groups were evaluated by Student's *t*-test or analysis of variance with the Tukey Kramer test. A p value < 0.05 denoted the presence of a statistically significant difference. All statistical calculations were performed using JMP Pro 15 (JMP Statistical Discovery LLC).

RESULTS

Myonectin promotes endothelial cell function in vitro

We first examined whether myonectin affected HUVEC differentiation into vascular-like structures when plated on a Matrigel matrix. HUVECs were treated with recombinant myonectin (5 μg/mL), vehicle (PBS), or EGM-2 as a positive control. Treatment with myonectin promoted the formation of vascular-like tubes (Fig. 1A). Quantitative analyses of the endothelial cell network revealed that treatment with myonectin and EGM-2 significantly increased the numbers of nodes, junctions, and segments, as well as the total length and total mesh area, relative to vehicle (Fig. 1B).

To test the effects of myonectin on endothelial cell migration, a modified Boyden chamber assay was performed. Treatment with myonectin significantly stimulated HUVEC migration (Fig. 2A).

We also examined the effect of myonectin on endothelial cell proliferation. Myonectin significantly increased the proliferation of HUVECs, as assessed by the MTS assay (Fig. 2B). These results suggest that myonectin promotes pro-angiogenic cellular responses in endothelial cells.

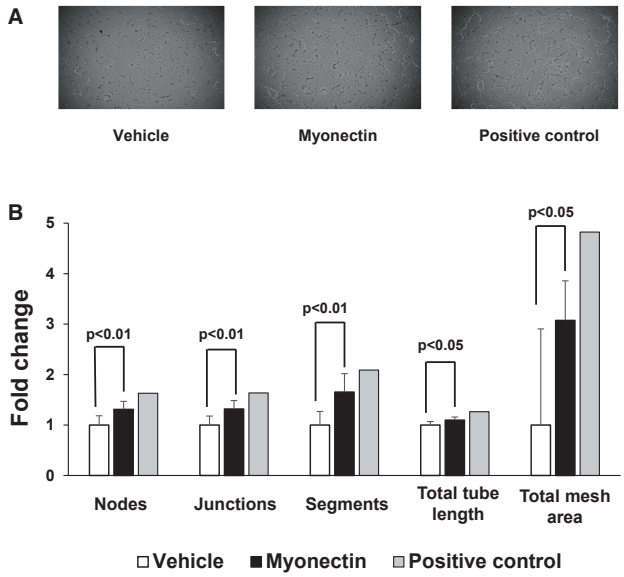

Fig. 1 Myonectin promotes tube-structure formation in endothelial cells

Fig. 1A: Representative photomicrographs of HUVEC network formation. After 16 h of serum deprivation, HUVECs were treated with recombinant myonectin (5 μg/mL), EGM-2 or PBS (vehicle), followed by culture in Matrigel-coated culture dishes.

Fig. 1B: Quantitative analyses of network formation (node, junction, segment, total length, and total mesh area). Results are presented as mean ± standard deviation (SD). Results are expressed relative to the vehicle-treated values (n = 8 in vehicle and myonectin group, n = 2 in positive control group).

HUVEC: human umbilical vein endothelial cell
EGM-2: endothelial cell growth medium-2
PBS: phosphate-buffered saline

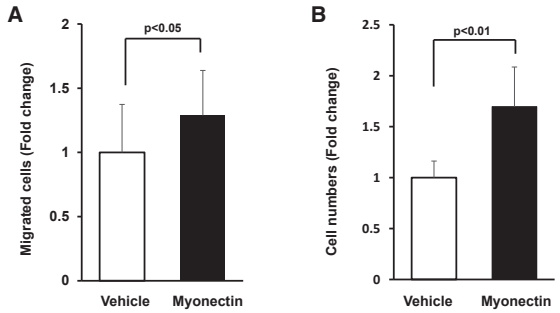

Fig. 2 Myonectin promotes migration and proliferation of cultured endothelial cells

Fig. 2A: Endothelial cell migration after stimulation with myonectin or vehicle. A modified Boyden chamber assay was performed using HUVECs. HUVECs were treated with myonectin (5 μg/mL) or PBS (vehicle).

Fig. 2B: The effect of myonectin on proliferation of HUVECs. The number of viable cells was quantified using an MTS assay. HUVECs were treated with or without recombinant myonectin (5 μg/mL) for 24 h. The CellTiter 96 Aqueous One Solution reagent (Promega) was added to cultured cells. Cells were incubated for 2 h and the absorbance at 490 nm was measured using a 96-well plate reader. Results are presented as mean ± SD and expressed relative to the control values (n = 10 in each group).

HUVEC: human umbilical vein endothelial cell
PBS: phosphate-buffered saline
FBS: fetal bovine serum
SD: standard deviation

Myonectin promotes blood vessel growth in vivo

To examine the in vivo effect of myonectin on angiogenesis, a Matrigel plug assay and a hindlimb ischemia model were performed. For the Matrigel plug assay, Matrigel containing myonectin (20 μg/mL) or vehicle (PBS) was injected into the left inguinal subcutaneous of Myo-KO mice. Myo-KO mice had undetectable plasma myonectin.[16] Endothelial cell infiltration of plugs was assessed by immunohistochemical analysis of CD31-positive cells 14 days later. Figure 3A shows representative photomicrographs of plugs immunostained for CD31. Quantitative analyses of the histological sections revealed that plugs containing myonectin had a significantly higher number of CD31-positive endothelial cells than plugs containing vehicle (Fig. 3B). The areas of CD31-positive endothelial cells in the Matrigel sections containing myonectin were also larger than those in the Matrigel sections containing vehicle (Fig. 3B).

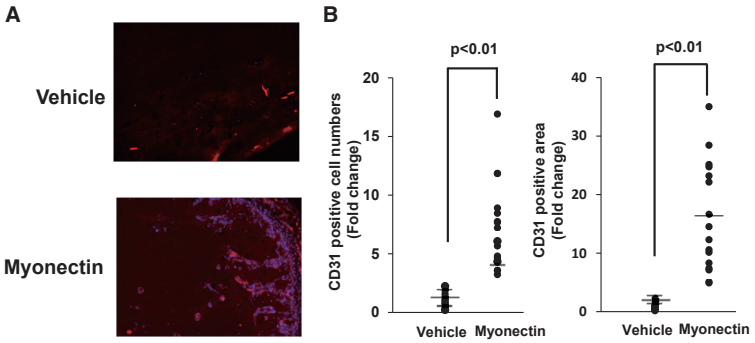

Fig. 3 Matrigel plugs containing myonectin displayed a significantly higher degree of endothelial cell infiltration in vivo

Matrigel containing myonectin (30 μg/mL, n =5) or PBS (control, n =5) were injected subcutaneously into Myo-KO mice.

Fig. 3A: Fluorescence staining of Matrigel plugs with anti-CD31 monoclonal antibody (red).

Fig. 3B: Quantitative analysis of the in vivo Matrigel plug assay. The frequency of CD31-positive endothelial cells (left) and the areas of CD31-positive endothelial cells (right) in four microscopic fields of plug edge was determined for each Matrigel plug. Data are presented as the fold increase of CD31-positive cells relative to vehicle.

PBS: phosphate-buffered saline

Myo-KO: myonectin-knockout

We next investigated whether myonectin modulates the angiogenic process in vivo by employing a hindlimb model of ischemia-induced angiogenesis in WT, Myo-KO, and Myo-TG mice. We previously reported that Myo-KO mice had undetectable plasma myonectin, whereas the levels of myonectin were 4.0-fold higher in Myo-TG mice than in WT mice.[16] Representative images of the blood flow measured by LDBF 14 days after surgery are shown in Fig. 4A. Quantitative analysis revealed that the perfusion 14 days after hindlimb surgery was significantly lower in Myo-KO mice than in WT mice. In contrast, Myo-TG mice showed a significant increase in limb perfusion 14 days after hindlimb surgery compared with that in WT mice (Fig. 4B). These data indicate that myonectin promotes ischemia-induced angiogenesis in vivo.

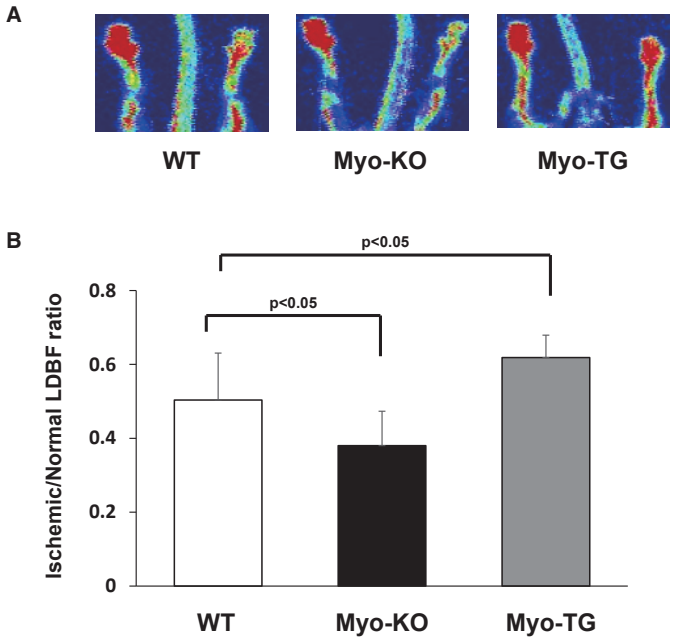

Fig. 4 Myonectin promotes perfusion recovery of ischemic limbs in mice in vivo

Fig. 4A: Representative LDBF images of the ischemic limbs of WT, Myo-KO, or Myo-TG mice. A low perfusion signal (dark blue) was observed in the ischemic hindlimbs of Myo-KO mice, whereas a high perfusion signal (red) was detected in the hindlimbs of Myo-TG mice at postoperative day 14.

Fig. 4B: The ischemic to nonischemic LDBF ratio in the WT, Myo-KO, and Myo-TG mice at day 14 after surgery. Results are shown as the mean ± SD (n = 10 in each group).

WT: wild-type
Myo-KO: myonectin-knockout
Myo-TG: myonectin-overexpressing transgenic
SD: standard deviation
LDBF: laser Doppler blood flow

DISCUSSION

This study revealed a new role of myonectin in the promotion of blood vessel growth. Myonectin stimulated the differentiation of vascular endothelial cells and caused the formation of capillary-like structures, as well as increased their proliferation and migration. Myonectin also promoted angiogenesis in two established animal models of angiogenesis: a mouse Matrigel plug assay model and a hindlimb ischemia model.

Myonectin is a myokine whose production is increased by endurance exercise.[11,16] In our previous study, endurance exercise increased blood myonectin levels by approximately 2.5-fold in WT mice and significantly decreased myocardial infarct size after myocardial ischemia-reperfusion injury.[16] In contrast, endurance exercise was unable to reduce the myocardial infarct size in Myo-KO mice. This suggests that myonectin may be an ischemic heart-protective factor in endurance exercise.

The promotion of angiogenesis has been shown to be beneficial for cardio-vascular protection following ischemia.[24-26] This study demonstrated that myonectin promotes angiogenesis. In vivo, myonectin stimulated blood vessel growth in a mouse Matrigel plug transplantation model, and overexpression of myonectin increased ischemia-induced angiogenesis. Combined with our

previous results,[16] these results suggest that myonectin production is increased by endurance exercise, promotes angiogenesis under pathological conditions, and has beneficial effects on the cardiovascular system.

PAD is a disease in which ischemia occurs in peripheral tissues, causing symptoms of intermittent claudication. Patients frequently require leg amputation, but many still die despite amputation.[27] Exercise therapy has been shown to improve claudication symptoms and extend walking distance and is therefore recommended for patients with PAD.[4,27] Increased angiogenesis in the skeletal muscle after exercise is considered to be a mechanism for improving PAD.[5,6] In a mouse model of hindlimb ischemia that is considered to closely replicate the physiological condition of PAD, endurance exercise promoted angiogenesis.[28] In this study, we demonstrated that myonectin deficiency decreases blood flow in a mouse model of hindlimb ischemia and that blood flow increases in mice overexpressing myonectin after hindlimb ischemia. Therefore, increased myonectin levels due to endurance exercise may contribute to the effect of exercise therapy in patients with PAD.

It has been reported that myonectin promotes free fatty acid uptake and iron metabolism in adipocytes and hepatocytes, has anti-inflammatory effects in macrophages and myocardial cells, and has anti-apoptotic effects in myocardial cells.[12-14,16] This study demonstrates a novel activity of myonectin in vascular endothelial cells. Myonectin stimulated differentiation of vascular endothelial cells into capillary-like structures and increased their proliferation and migration. Therefore, myonectin promotes angiogenesis via direct action on vascular endothelial cells.

However, the present study has several limitations. Our study used an acute limb ischemia model, which may differ from the chronic limb-threatening ischemia observed in patients under clinical setting. Future studies using a chronic limb ischemia model, such as a method in which ischemia is induced using an ameloid contractile device and the artery is gradually occluded, will be required.[29] In addition, the detailed mechanism underlying the effect of myonectin on vascular endothelial cells remains unclear, and further studies are needed.

In conclusion, myonectin promotes angiogenesis by endocrinologically acting on vascular endothelial cells. Therefore, exercise may be a useful treatment for ischemic cardiovascular disease because it increases myonectin. Treatment aimed at increasing myonectin production may be useful in the treatment of cardiovascular diseases with vascular dysfunction, such as PAD.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research A, a Grant-in-Aid for Challenging Exploratory Research, and grants from the Takeda Science Foundation to N. Ouchi. R. Shibata was supported by a Grant-in-Aid for Scientific Research C. The authors gratefully acknowledge the technical assistance provided by Yoko Inoue and Minako Tatsumi.

CONFLICTS OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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