学位論文

Nerve growth factor stimulates regeneration of the perivascular nerve, and induces the maturation of microvessels around the injured artery.

（神経成長因子は末梢神経の再生を介して障害血管周囲の微小血管の成熟化を促進する）

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浅野目　晃

（　川辺 淳一、松木 孝樹、鹿原 真樹、平 義樹、暮地本 宙己、
山内 敦司、青沼 達也、竹原 有史、渡部 剛、長谷部 直幸　）

Title

Nerve growth factor stimulates regeneration of perivascular nerve, and induces the maturation of microvessels around the injured artery.

Akira Asanome[a],
Jun-ichi Kawabe[b*], Motoki Matsuki[a], Maki Kabara[a], Yoshiki Hira[c], Hiroki Bochimoto[c], Atsushi Yamauchi[a], Tatsuya Aonuma[a], Naofumi Takehara[b], Tsuyoshi Watanabe[c], and Naoyuki Hasebe[a, b]

a Department of Medicine, Division of Cardiovascular, Respiratory and Neurology
b Department of Cardiovascular Regeneration and Innovation
c Department of Anatomy, Asahikawa
Asahikawa Medical University, Asahikawa, Japan

*** Corresponding Author; Jun-ichi Kawabe. MD, PhD,**
2-1-1 Midorigaoka-higashi, Asahikawa, 078-8510, Japan
FAX +81-166-68-2449, TEL +81-166-68-2442,
e-mail kawabeju@asahikawa-med.ac.jp

Abstract

An immature vasa vasorum in the adventitia of arteries has been implicated in induction of the formation of unstable atherosclerotic plaques. Normalization/maturation of the vasa vasorum may be an attractive therapeutic approach for arteriosclerotic diseases. Nerve growth factor (NGF) is a pleotropic molecule with angiogenic activity in addition to neural growth effects. However, whether NGF affects the formation of microvessels in addition to innervation during pathological angiogenesis is unclear. In the present study, we show a new role for NGF in neovessels around injured arterial walls using a novel *in vivo* angiogenesis assay.

The vasa vasorum around arterial walls was induced to grow using wire-mediated mouse femoral arterial injury. When collagen-coated tube (CCT) was placed beside the injured artery for 7-14 days, microvessels grew two-dimensionally in a thin layer on the CCT (CCT-membrane) in accordance with the development of the vasa vasorum. The perivascular nerve was found at not only arterioles but also capillaries in the CCT-membrane. Biodegradable hydrogels containing VEGF and NGF were applied around the injured artery/CCT. VEGF significantly increased the total length and instability of microvessels within the CCT-membrane. In contrast, NGF induced regeneration of the peripheral nerve around the microvessels and induced the maturation and stabilization of microvessels. In an *ex vivo* nerve-free angiogenesis assay, although NGF potentially stimulated vascular sprouting from aorta tissues, no effects of NGF on vascular maturation were observed.

These data demonstrated that NGF had potent angiogenic effects on the microvessels around the injured artery, and especially induced the maturation/stabilization of microvessels in accordance with the regeneration of perivascular nerves.

Keywords

angiogenesis, neural growth factors, innervation, vasa vasorum, atherosclerosis,

Abbreviations

VEGF: vascular endothelial growth factors; NGF: nerve growth factor; CCT: collagen-coated tubes

Introduction

The vasa vasorum forms a microvascular network in the adventitial layer of large arteries and supplies oxygen and nutrients to the outer layers of the vessel walls[1]. As vessel walls thickness increases in the setting of atherosclerotic diseases, the vasa vasorum within the arterial walls grows[2]. The vasa vasorum within atherosclerotic lesion has relatively thin and fragile walls, usually with fewer pericytes around the microvessels compared to normal capillaries[3]. These properties of the immature vasa vasorum contributes to the progression of unstable plaques[4,5]. Therefore, the vasa vasorum in plaques is an attractive therapeutic target for arteriosclerotic diseases, and improving our understanding of the processes involved in the maturation and remodeling of the vasa vasorum is necessary. However, the mechanism of vasa vasorum angiogenesis in the pathophysiological setting is poorly understood because of limited appropriate methods for observing the microvessels in the arterial walls.

Perivascular nerves play an important role in the maintenance and regulation of vascular tone[6]. Regeneration of the perivascular vasomotor nerve occures during angiogenesis to form the functional vasculature. In contrast to postnatal stages, peripheral nerve play an alternative role in angiogenesis during embryonic development. During development of embryonic mouse limb skin, peripheral nerves provide a template that determines the organo-typic pattern of vessel branching and arterial differentiation[7,8]. Neurotrophic and neural guidance factors contribute to embryonic vessel formations, forming the vascular and neural network[9] [10]. However, whether the perivascular nerve is involved in neovessels formation in postnatal physiological and pathological conditions is unclear.

Neurotrophic factors such as nerve growth factor (NGF) are well known for their roles in regulating growth and functional maintenance of peripheral and central nervous system cells[11]. NGF also has potent angiogenic activity[12], although the mechanism of the angiogenic effects of NGF is still controversial. NGF increases the density of not only capillaries but also matured vessels such as arterioles in response to hindlimb ischemia[13,14]. The angiogenic effects of NGF are mediated through direct effects on vascular endothelial cells or indirectly by influencing the action of other endogenous growth factors such as vascular endothelial growth factor (VEGF)[12,15]. However, the effects of NGF on vascular maturation cannot be fully explained by the previously proposed mechanisms.

Material and methods

Animals Male C57BL/6 mice aged 10 to 12 weeks were used for the experiments. Animals were maintained in a temperature- and light-controlled room and fed normal chow. All animal interventions were approved by the Animal Care and Use Committee of Asahikawa Medical College.

Wire Injury-Mediated Vascular Remodeling To induce vascular injury, we employed a wire-mediated endovascular injury model as described previously[16]. Briefly, a spring wire (0.38 mm in diameter, Cook, Bloomington, IN) was inserted into the left femoral artery, and left in place for 1 minutes to induce trans-luminal arterial injury. The CCT, which consisted of a polypuropiren tube (5mm length, 1.3 mm inner diameter) coated with collagen I, was sutured just beside the injured femoral artery. The mice were randomly divided into four groups (VEGF, NGF, VEGF+NGF, no treatment), and biodegradable gelatin hydrogels containing VEGF (0.2 mg/mg dry gel) (Peprotech, London) and/or NGF (0.4 mg/mg dry gel) (Biomedical Technologies, Stoughton, MA) were placed on the femoral artery of each group.

Histological Assessment Two weeks after the operation, the mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.). CCT was isolated and fixed in 4% paraformaldehyde at 4°C overnight. The fixed thin collagen layers on the CCT (CCT-membrane) were stripped off and laid onto glass plates. The CCT-membranes were stained with hematoxylin & eosin and examined in a blinded fashion. The total vasculature, which developed in two dimensions within the CCT-membrane, was traced using an angiogenesis image analyzer (Kurabo, Osaka, Japan), and the lengths of both the full vasculature and large vessels (outer diameter > 20 μm) were measured. The ratio of enlarged vessels was calculated as the percent of the total length of vessels within the CCT layers.

Immunocytochemical Analysis In some experiments, at 7-10 days after the operation, just before euthanasia, the vascular intraluminal endothelial layer was stained by infusing FITC-conjugated *Griffonia simplicifolia* Lectin (Vector Laboratory, Burlingame, CA) (0.5 mg/ml saline; 300 μl/mouse) via the tail vein. After 5 minutes, the mice were perfused through the left cardiac ventricle with phosphate-buffered saline (PBS) and 4% paraformaldehyde/PBS. Then the CCT-membrane was fixed in 4% paraformaldehyde at 4°C overnight. The fixed sample was washed with PBS and immersed in PBS containing 0.5% TritonX-100 overnight and then incubated in PBS

containing 0.5% bovine serum albumin for 60 minutes. The CCT-membrane was then incubated in primary antibodies: anti-PGP9.5 (1: 300; raised in rabbits; Affinity BioReagents, Golden, CO), anti-tyrosine hydroxylase (1:200; raised in rabbits; Chemicon international, Temecula, CA) or anti-NG2 (Abcam, Cambridge, UK) for 72 hours at 4°C. After incubation, the sample was washed with PBS and incubated with Alexa488-, 568- or 647-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). The nuclei were counterstained with Hoechst 33258. The sample was mounted in glycerol/PBS 2:1 (v/v) and observed with a 3D-deconvolution fluorescence microscopy (AF6000, Leica, Mannheim, Germany) and a confocal laser scanning microscope (FV1000D, Olympus, Tokyo, Japan). The lectin-stained immature microvessels and PGP-positive peripheral nerve fibers were traced using the image analyzer as described above. Twelve fields of each CCT-membrane at 400× magnification were randomly examined and averaged. The ratio of the lengths of the nerve fiber-associated microvessels to the total microvessels was calculated.

Aorta Ring Ex vivo Angiogenesis Assay The aorta ring culture was performed as previously reported[17]. Briefly, aorta rings were embedded in matri-gel (BD Biosciences, Bedford, MA) and incubated in endothelial basal medium-2 (PromoCell, Heidelberg, Germany) containing 2.5% autologous mouse serum with or without 10 ng/ml VEGF and/or 50 ng/ml NGF. Quantitative analysis of sprouting microvessels per aortic ring was performed using angiogenesis image analyzer software (Kurabo); the lengths of total sprouting microvessels and enlarged microvessels (outer diameter >10 μm) were measured. In a previous study, we found that these enlarged microvessels were surrounded by α-smooth muscle-actin, or NG2-positive pericytes[17].

Electron microscopy For electron microscopic observation, CCT-membranes were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1M PBS (pH 7.4) at 4°C overnight. After fixation, CCT-membranes were cut into small pieces of tissue blocks and post-fixed with 1% OsO_4 in 0.1 M PBS containing 7.5% sucrose for 1 hour at 4°C. The tissue blocks were then washed thoroughly with 0.1 M PBS containing 7.5% sucrose, dehydrated in graded ethanol and embedded in epoxy resin (Epon 812). Ultrathin sections from the tissue blocks embedded in Epon 812 were contrasted with saturated aqueous solutions of uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-7650, Hitachi High Technologies, Tokyo, Japan).

Statistical Analysis All experimental values were expressed as the mean ± SEM. Multiple comparisons were evaluated through the use of ANOVA, followed by Fisher's test. All statistical analyses were performed using Statview software (Abacus, Grand Rapids, MI). Statistical significance was defined as a *p* value of less than 0.05.

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Results and Discussion

1. Formation of neovessels on the CCT around the injured femoral artery.

We developed an *in vivo* angiogenesis assay to observe the microvessels around the injured arterial walls. The mouse femoral artery was injured by inserting a coiled wire, and a CCT was placed beside the injured artery. Two weeks after the operation, enhanced angiogenesis was observed around the injured femoral artery and the CCT (Figure 1A). The CCT was extracted and fixed, and the coated collagen-layer on the CCT (CCT-membrane) was removed. As shown in Figure 1B, microvessels within the CCT-membrane (200-300 μm thickness) were distributed two-dimensionally and can be examined quantitatively in detail. The vasculature within the CCT-membrane was composed of neovessels at various stages of maturation, *i.e.* capillaries, endothelial tubes partially covered with mural cells, pericytes (< 20 μm in diameter), and arterioles with walls composed of smooth muscle cellular layers(>50 μm) (Figure 1B).

The microvasculature on the CCT-membrane was not identical to the vasa vasorum of vascular walls. However, these microvasculature formed in parallel with the growth of the vasa vasorum in response to vascular injury. When a CCT was placed beside a non-injured artery, angiogenesis on the CCT-membrane was rarely observed (data not shown). Therefore, the characteristics of neovessels on the CCT-membrane are expected to be similar to those of the vasa vasorum in injured vascular walls.

2. Peripheral nerve fibers were distributed around the microvessels

Vascular maturation occurs when nascent microvessels become enlarged and mature when an endothelial tube is surrounded by pericytes or smooth muscle cells. Finally, the mature vessels obtain vasomotor properties following regeneration of perivascular nerves[18]. However, when perivascular nerves innervate the vessels during angiogenesis, especially in pathophysiological condition is unclear.

Observing the innervation of peripheral nerve fibers, especially within peripheral tissues, is difficult because of their small size and complex distribution pattern. Because neovessels in the CCT-membrane were distributed two-dimensionally, a CCT angiogenesis assay would be beneficial for observing peripheral nerve fibers around microvessels. Interestingly, using electron microscopy,

peripheral unmyelinated nerve fibers were frequently observed around capillaries and were composed of endothelial cell tubes covered by pericytes (Figure 1C). To test if peripheral nerves were distributed around premature capillaries, innervating peripheral nerves within the CCT-membrane were immunostained; tyrosine hydroxylase-positive sympathetic nerve fibers were distributed around nascent capillaries with small diameters (about 10-20 μm), and pericytes were partially attached (Figure 1D). Because capillaries have few vasoconstrictive properties, innervated perivascular nerves may contribute to angiogenesis in addition to their vasomotor action within injured vascular walls.

3. Effects of VEGF and NGF on the formation of microvessels in CCT-membrane

NGF facilitates innervation of perivascular nerves around mesenteric arteries[19,20]. Thus, to examine the effects of peripheral nerves innervation on angiogenesis around injured vascular walls, a biodegradable gel containing VEGF and/or NGF was placed around the CCT and injured artery. Neovessels in the CCT-membrane can be traced and were divided into two groups: large, mature (≥20 μm in diameter) and small, immature microvessels (< 20 μm). The total length of neovessels and the ratio of large microvessels among total neovessels in the CCT-membrane were measured. As shown in Figure 2A, VEGF significantly stimulated the growth of microvessels, estimated as the total length of microvessels (Figure 2B). NGF alone or in combination with VEGF tended to enhance angiogenesis. More interestingly, NGF apparently increased the ratio of large matured vessels, whereas VEGF decreased the ratio of large matured vessels (Figure 2A,B). NGF also enhanced the maturation of VEGF-induced neovessels(Figure 2B).

Blood leakage from microvessels was also observed (Figure 2A), indicating their instability. VEGF apparently increased blood leakage in parallel with the formation of microvessels. In contrast, NGF did not enhance blood leakage, and further blocked the VEGF-induced blood leakage (Figure 2A).

4. NGF stimulated innervation by perivascular nerve fibers around the microvessels.

To estimate the innervation by perivascular nerves during angiogenesis around the injured arterial walls, PGP-positive peripheral nerve fibers in

CCT-membrane of each group were immunostained (Figure 3). Most mature arterioles (≥100 μm in diameter) were strongly associated with PGP-positive nerve fibers in all four groups (data not shown). Consistent with the results shown in Figure 1, some premature microvessels (<100 μm in diameter) including capillaries were also associated with peripheral nerves (Figure 3A). NGF significantly increased the ratio of peripheral nerve fibers-associated microvessels, whereas VEGF reduced the ratio of the innervated microvessels within CCT-membrane (Figure 3B).

5. Effects of NGF on the formation of microvessels in *ex vivo* angiogenesis.

The effects of NGF on the maturation of microvessels may be mediated through a direct action on vascular cells or by indirectly affecting other factors within arterial tissues. Accordingly, next we examined the angiogenic effects of NGF in the absence of nerve innervation.

The *ex vivo* angiogenesis assay using isolated aorta ring have been frequently utilized to study angiogenesis. In particular, this assay can be used to estimate vascular maturation[21]. Endothelial cell tubes sprouting from the aorta ring were covered by endogenous pericytes or vascular smooth muscle cells to form enlarged microvessels-like vessel structures (Figure 4A). We estimated the length of sprouting tubes including enlarged vessel tubes (≥10 μm in diameter) and calculated the total length of sprouting vessels and the ratio of enlarged vessels per aorta ring. As shown in Figure 4, VEGF and NGF significantly stimulated the growth of sprouting vessels. Although VEGF had potent angiogenic effects, VEGF did not increase the ratio of enlarged vessels. In contrast to the result of the *in vivo* CCT angiogenesis assay, NGF did not affect the formation of enlarged vessels with or without VEGF (Figure 4). Thus, the effects of NGF on vascular maturation are not be mediated through actions on the vascular cells or other unknown factors within isolated arterial tissues.

Immature vasa vasorum in the adventitial wall of arteries has been implicated in inducing the formation of unstable plaques. Thus, NGF-mediated normalization and maturation of microvessels would affect neointimal remodeling in injured arterial walls. We used matrigel to chronically release growth factors around the injured femoral artery for less than 2 weeks. However, significant vascular remodeling and neointimal thickening occurred after 2 weeks of vascular injury[16]. Accordingly, we were not able to estimate the role of NGF on the vascular remodeling in the present study.

Furthermore, endothelial injury-mediated neointimal thickening is different from arteriosclerotic plaque from the point of view of the pathological process and even the role of the vasa vasorum. Therefore, to examine the role of NGF on growth of the vasa vasorum and vascular remodeling, an appropriate arteriosclerotic model with a system for releasing NGF for a longer period is necessary.

Several neurotransmitters including norepinephrine and acetylcholine have angiogenic effects[22,23]. Neuropeptide Y, which is a nonadrenergic neurotransmitters rereleased from sympathetic nerves innervating the cardiovascular system, has potent angiogenic effects[24,25]. Substance P, which is secreted from peripheral sensory nerve and act as a neurotransmitter or hormones, induces the recruitment of bone marrow-derived cells, which have angiogenic activities, resulting in enhanced angiogenesis[26,27]. What kind of peripheral nerves and how peripheral nerves mediates the maturation of vasa vasorum microvessels within the remodeling arterial walls should be investigated, *e.g.*, the effects of neurotransmitters released from perivascular nerves on the formation of the vasa vasorum.

In summary, using a newly developed CCT angiogenesis assay, we demonstrated for the first time that peripheral nerves were already present at early time points around premature microvessels in injured arterial walls. We propose a new mechanism in which the effects of NGF on vascular maturation in adult pathophysiological angiogenesis are mediated through innervated perivascular nerves. This present finding also provides insight into new treatments for atherosclerotic diseases.

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Figure 1

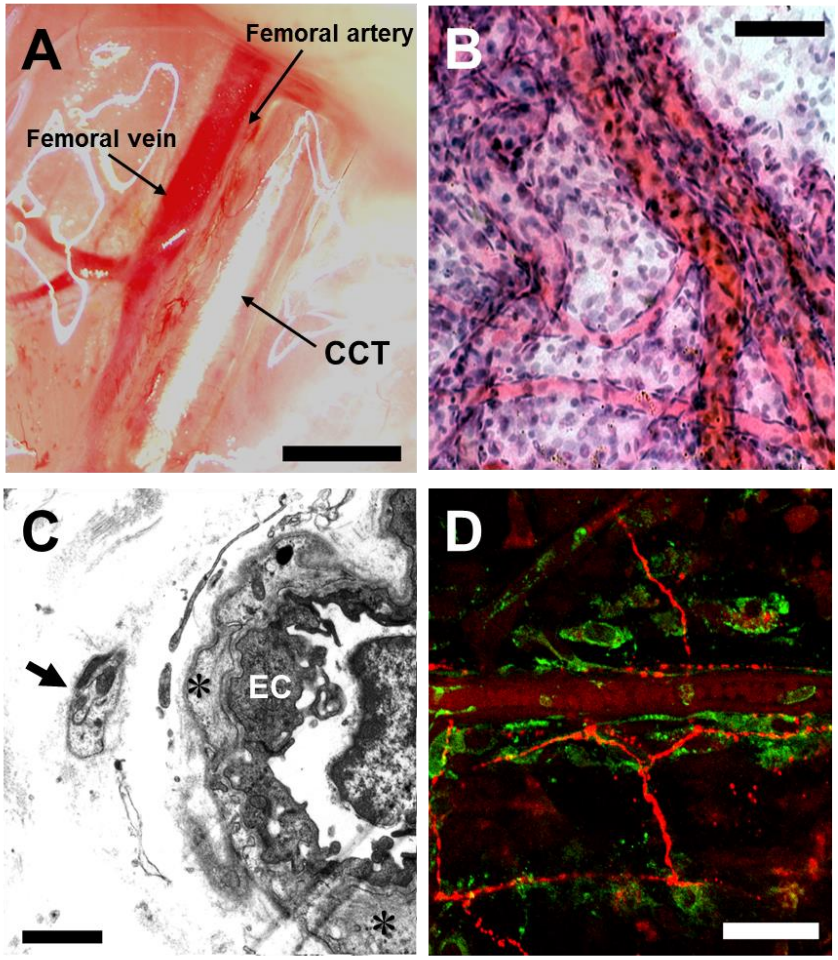

Figure 1 ***In vivo*** **CCT angiogenesis assay.** A collagen-coated tube (CCT) was placed just beside the injured femoral artery. Two weeks after the operation, the growth of microvessels around the injured artery and on the CCT was observed (**A**). The thin collagen layer on the CCT (CCT-membrane) was fixed and unfolded onto a glass plate. Microvessels at different maturation stages including capillaries and arterioles that had grown within the CCT-membrane were observed two-dimensionally following hematoxylin and eosin staining (**B**). Representative electron microscopic view showing an unmyelinated nerve fiber (arrow) located beside a capillary of which the endothelial tube (EC) was partially covered by pericytes (asterisks) (**C**). Whole-mount immunostaining of a CCT-membrane demonstrated that tyrosine hydroxylase-positive sympathetic nerve fibers (red) were distributed around the capillary (**D**). Endothelial cells were stained with CD31 (green). Bars indicate 1 mm (A), 20 μm (B), 2 μm (C), and 50 μm (D).

Figure 2

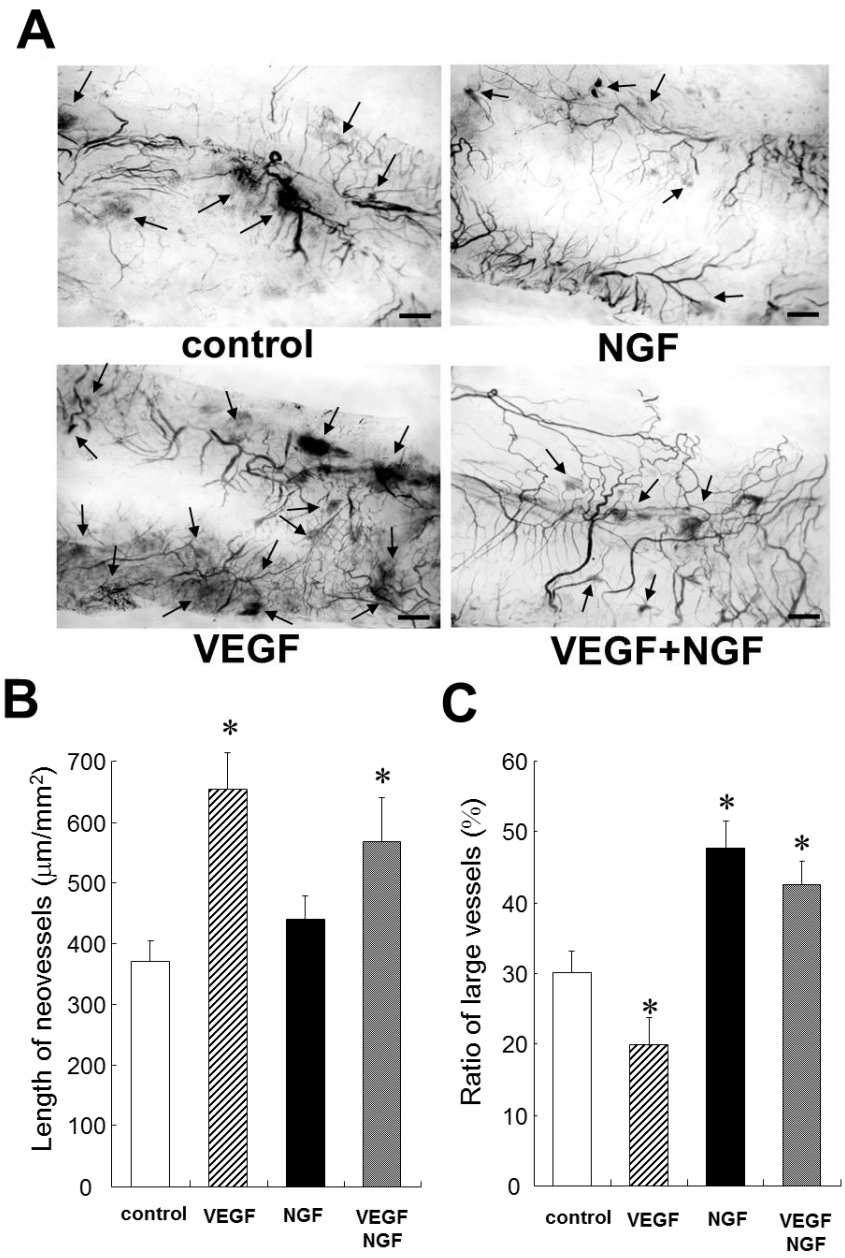

Figure 2 Effects of NGF on the angiogenesis of injured arterial walls. Matrigel containing VEGF and/or NGF was placed around the CCT and injured artery. Two weeks later, neovessels in the CCT-membrane were traced (**A**) and divided into two groups: large mature (≥20 μm in diameter) and small immature microvessels (<20 μm). Blood leakage from microvessels was also observed (arrow). The total length of neovessels (**B**) and the ratio of large microvessels among total neovessels (**C**) were calculated. Data are the means ± SEM (*$p < 0.05$ vs. control, $n = 7$). Bars indicate 100 μm.

Figure 3

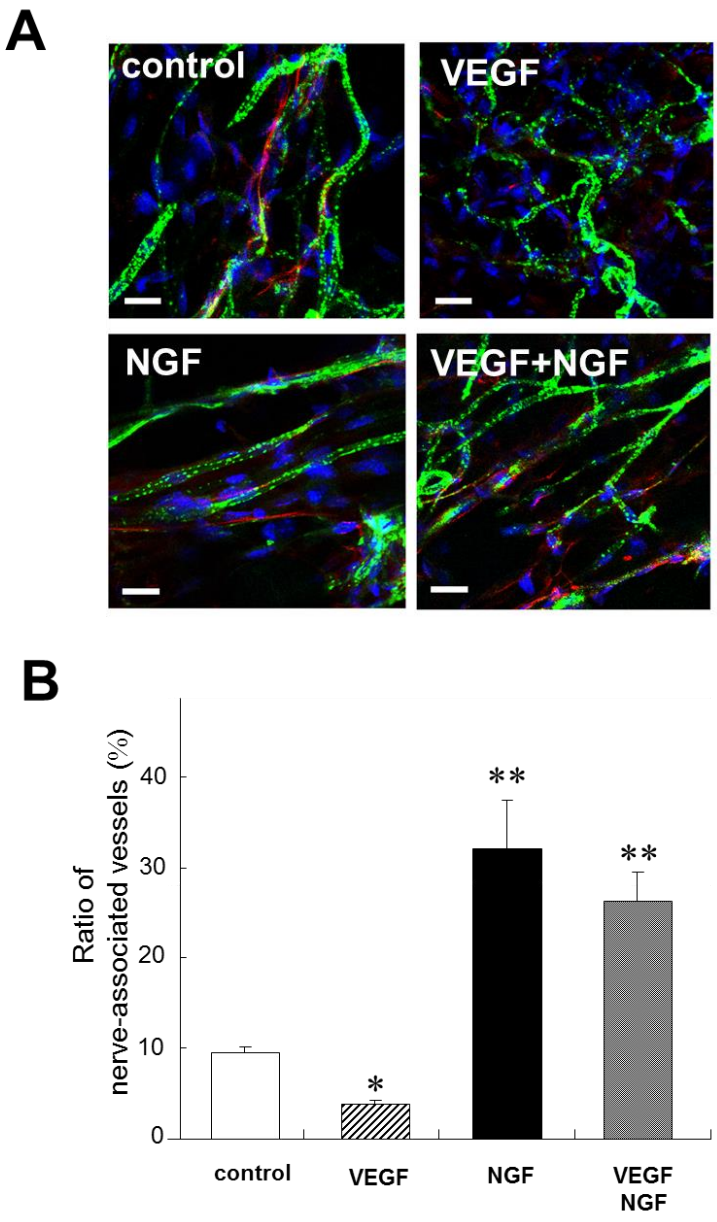

Figure 3 NGF stimulated innervation by nerve fibers around microvessels. Peripheral nerves within the CCT-membrane were immunostained using anti-PGP antibody. Representative confocal-laser micrographs in each group are shown (**A**). PGP-positive nerve fibers (red) were associated with lectin-stained microvessels (green). The lengths of both total microvessels and nerve fiber-associated vessels were measured, and the ratio of innervated vessels per total microvessels was calculated (**B**). Data are the means ± SEM (*$p < 0.05$, **$p < 0.01$ vs. control, n = 6-7). Bars indicate 20 μm.

Figure 4

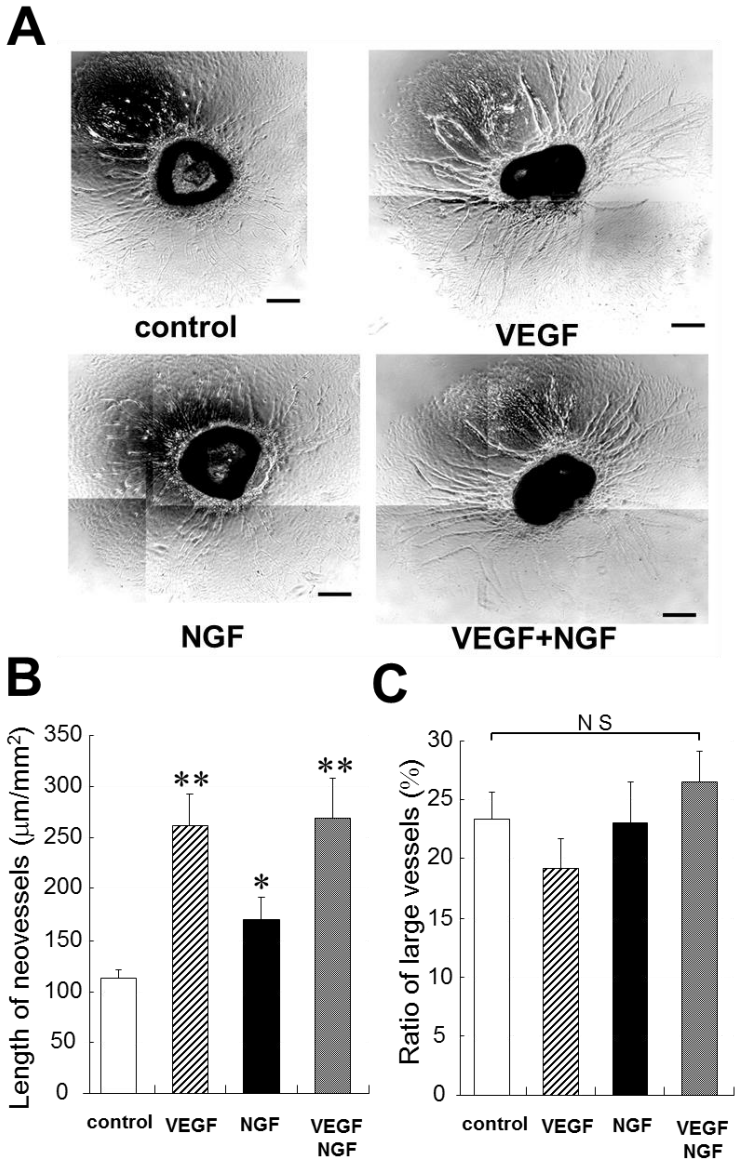

Figure 4 Effects of NGF on *ex vivo* angiogenesis. The angiogenesis assay using isolated aorta rings was performed in the presence of the indicated growth factors. After incubation for 7 days, microvessel-like tubes sprouting from aorta tissues were traced (**A**) and divided into two groups: large mature (≥10 μm in diameter) and small immature microvessels (<10 μm). The total length of sprouting vessels (**B**) and the ratio of enlarged vessels per aorta ring (**C**) were calculated. Data are the means ± SEM (*$p < 0.05$, **$p < 0.01$ vs. control, $n = 10$). Bars indicate 250 μm.