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Nerve growth factor facilitates perivascular innervation in neovasculatures of mice

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ABSTRACT

It is well known that blood vessels including arterioles have a perivascular innervation. It is also widely accepted that perivascular nerves maintain vascular tone and regulate blood flow. Although there are currently prevailing opinions, unified views on the innervation of microcirculation in any organs have not been established. The present study was designed to investigate whether there are perivascular nerves innervated in microvessels and neovessels. Furthermore, we examined whether nerve growth factor (NGF) can exert a promotional effect on perivascular nerve innervation in neovessels of Matrigel plugs. A Matrigel was subcutaneously implanted in mouse. The presence of perivascular nerves in Matrigel on Day 7–21 after the implantation was immunohistochemically studied. NGF or saline was subcutaneously administered by an osmotic mini-pump for a period of 3–14 days. The immunostaining of neovasculatures in Matrigel showed the presence of perivascular nerves on Day 21 after Matrigel injection. Perivascular nerve innervation of neovessels within Matrigel implanted in NGF-treated mice was observed in Day 17 after Matrigel implantation. However, NGF treatment did not increase numbers of neovessels in Matrigel. These results suggest that perivascular nerves innervate neovessels as neovasculatures mature and that NGF accelerates the innervation of perivascular nerves in neovessels.

1. Introduction

It is well recognized that blood vessels including arterioles, except capillary vessels, have a perivascular innervation. It is also widely accepted that perivascular nerves maintain vascular tone and regulate organ and tissue blood flow. With few exceptions, the postganglionic adrenergic nerves constitute the most significant efferent neural pathway to the blood vessels and maintain the tone (1). Although there are currently prevailing opinions, there are no unified views on the innervation of the microcirculation in any organs. There is a great diversity in the distribution and density of the microvascular adrenergic innervation (2,3). The degree that adrenergic nerves influence the control of microcirculation depends, in part, on the pattern of distribution of these nerves to the various vessels, which form the microvascular network (4). The functional significance of adrenergic neural control of the microcirculation varies among different organs and between different segments of the microvascular network in any given tissue. Since very few systematic studies have described the adrenergic innervation of the microcirculation in any organ (2,3), the present study was designed to investigate whether there are perivascular nerves innervated in microvessels and neovessels.

Perivascular nerves play an important role in maintenance and regulation of vascular tone and regional blood flow along

Abbreviations: α-SMA, α-smooth muscle actin; bFGF, basic fibroblast growth factor; CGRP, calcitonin gene-related peptide; LI, like immunoreactivity; NE, norepinephrine; NGF, nerve growth factor; NPY, neuropeptide Y; TH, tyrosine hydroxylase; PBS, phosphate-buffered saline; PGP 9.5, Protein Gene Product 9.5.

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with endothelial cells. Resistant vessels, such as the mesenteric artery, are innervated by various perivascular nerves including sympathetic adrenergic nerves and non-adrenergic non-cholinergic nerves, which include calcitonin gene-related peptide (CGRP) containing nerves (CGRPergic nerves) (1) and nitric oxide-containing nerves (5). Recently, we demonstrated that innervation of CGRPergic nerves and neuropeptide Y (NPY)-containing sympathetic adrenergic nerves in rat mesenteric resistance arteries was markedly reduced by topical application of phenol *in vivo* and that nerve growth factor (NGF) facilitates reinnervation of both types of nerves (6). Some reports have shown that peripheral nerves such as the chorda tympani, lingual nerves (7) and motor neurons in hypoglossal nerves (8) could regenerate with new processes if they are supplied with growth-promoting substrates. There are a few reports that adrenergic neurons and sensory neurons are involved in angiogenesis (9,10). However, it is still unknown whether neovessels resulted from angiogenesis have innervation of perivascular nerves, and it remains unclear whether NGF has neurotrophic effects on peripheral nerves of neovessels.

Therefore, the present study was designed to investigate whether there are perivascular nerves, which are innervated in microvessels of the mouse adipose tissue and neovessels derived by Matrigel technique. Furthermore, we examined whether NGF have an ability to exert a promotive effect on perivascular nerve innervation in neovessels of Matrigel plugs.

2. Materials and methods

2.1. Experimental animals

Five-week-old BALB/c Cr Slc mouse (purchased from Shimizu Experimental Animals, Shizuoka, Japan) were used in this study. The animals were given food and water *ad libitum*. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22 °C with 50 ± 10% relative humidity and with a 12-h light/12-h dark cycle (lights on at 8:00 AM). This study was carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law No. 115 and Japanese Government Notification on Feeding and Safekeeping of Animals No. 6. Every effort was made to minimize the number of animals used and their suffering.

2.2. Animal treatments and experimental protocols

The Matrigel, which is a basement membrane component mixture, has been highly useful in various studies including *in vivo* angiogenesis assays, 3D cell culture and cell invasion and migration assays (11–15). In this study, therefore, Growth Factor Reduced (GFR) Corning Matrigel Matrix (Cat. Nos. 354230) with adding basic fibroblast growth factor (bFGF) and heparin as an angiogenic growth factor was employed to derive neovessels and investigate the effects of NGF on innervation of neovessels. Under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally), 500 μL of GFR Matrigel, which was supplemented with bFGF (1 ng/mL) and heparin (50 units/mL) in liquid form at 4 °C, was bilaterally injected into the flank subcutaneous tissue of each mouse. After the injections of Matrigel, the animals were transferred into individual cages in the temperature-controlled room, and the animals were killed by deep anesthesia on Day 7, 10, 14, 17 and 21 for use in the experiments described below.

2.3. Tissue processing

The animals treated were anesthetized with a large dose of sodium pentobarbital (50 mg/kg, intraperitoneally). The dorsal subcutaneous adipose tissue including Matrigel was surgically removed and immersion-fixed in the Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer) for 48 h. After fixation, the specimens were repeatedly rinsed in phosphate-buffered saline (PBS) and prepared for immunohistochemical study as either whole mount or paraffin sections.

2.4. Immunohistochemical study of whole mount tissue

After fixation, the dorsal subcutaneous adipose tissue was repeatedly rinsed in phosphate-buffered saline (PBS), immersed in PBS containing 0.5% TritonX-100 overnight, and incubated with PBS containing normal goat serum (1:100) for 60 min. The tissue was then incubated in rabbit polyclonal anti-PGP 9.5 IgG (NeoMarkers Inc., Fremont, CA) at a dilution of 1:100 or rabbit polyclonal anti-CGRP antiserum (Biogenesis Ltd., Oxford, UK) at a dilution of 1:300 or rabbit polyclonal anti-neuropeptide Y (NPY) antiserum (Phoenix Pharmaceuticals Inc., Belmont, CA) at a dilution of 1:300 or rabbit polyclonal anti-tyrosine hydroxylase (TH) IgG (Chemicon international, Inc., Temecula, CA) at a dilution of 1:200 for 72 h at 4 °C. After the incubation, the adipose tissue was washed in PBS and the sites of antigen–antibody reaction were detected by incubation with fluorescein-5-isothiocyanate (FITC)-labeled goat anti-rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, OH) at a dilution of 1:100 for 60 min at 4 °C. Thereafter, the tissue was thoroughly washed in PBS, mounted on a slide, cover-slipped with glycerol/PBS (2: 1 v/v) and observed under a confocal laser scanning microscope (CLSM510, Carl Zeiss, Tokyo, Japan) in Okayama University Medical School Central Research Laboratory.

2.5. Immunostaining of paraffin sections

The Matrigel were dehydrated, embedded in paraffin and cut thin sections (50 μm thick). After deparaffinization and rehydration, the sections were incubated with PBS containing normal goat serum (1:100) for 60 min. The sections were then incubated in rabbit polyclonal anti-PGP 9.5 IgG (NeoMarkers Inc., Fremont, CA) at a dilution of 1:100 or rabbit polyclonal anti-CGRP antiserum (Biogenesis Ltd., Oxford, UK) at a dilution of 1:300 or rabbit polyclonal anti-NPY antiserum (Phoenix Pharmaceuticals Inc., Belmont, CA) at a dilution of 1:300 or rabbit polyclonal anti-TH IgG (Chemicon international, Inc., Temecula, CA) and rabbit monoclonal anti-α-smooth muscle actin (α-SMA) FITC conjugate IgG (Sigma Aldrich Japan, Tokyo, Japan) at a dilution of 1:300 for 72 h at 4 °C. After the incubation, the sections were washed in PBS and the sites of antigen–antibody reaction were detected by incubation with fluorescein-5-isothiocyanate (FITC)-labeled goat anti-rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, OH) at a dilution of 1:100 for 60 min at 4 °C. Thereafter, the sections were thoroughly washed in PBS, mounted on a slide, cover-slipped with glycerol/PBS (2: 1 v/v) and observed under a confocal laser scanning microscope (CLSM510, Carl Zeiss, Tokyo, Japan) in Okayama University Medical School Central Research Laboratory.

The perivascular nerve length and microvessel area were analyzed with a computer using the Image J 1.49v software program (National Institutes of Health, USA). The perivascular nerve length per microvessel area in 3 different fields was analyzed for each sample.