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Vascular endothelial cells promote cortical neurite outgrowth via an integrin β3-dependent mechanism

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ABSTRACT

The interaction of neurons with their non-neuronal milieu plays a crucial role in the formation of neural networks, and wide variety of cell-contact-dependent signals that promote neurite elongation have been identified. In this study, we found that vascular endothelial cells promote neurite elongation in an integrin β3-dependent manner. Vascular endothelial cells from the cerebral cortex promoted neurite elongation of cortical neurons in a cell contact-dependent manner. This effect was mediated by arginine–glycine–aspartic acid (RGD), a major recognition sequence for integrins. Pharmacological blockade of integrin β3 abolished the neurite elongation effect induced by the endothelial cells. Immunocytochemical analysis revealed that integrin β3 was expressed on cultured cortical neurons. These results demonstrate that the neurite elongation promoted by vascular endothelial cells requires integrin β3. Vascular endothelial cells may therefore play a role in the development and repair of neural networks in the central nervous system.

1. Introduction

Neural circuits are established with a high degree of precision and reproducibility in the developing and regenerating nervous system. This is accomplished by the directed growth of axonal growth cones to their synaptic target cells. Diffusible and substrate-bound guidance cues act either at a distance to outline the target, or locally at choice points to specify the pathway taken by axons [1]. Further, contact interactions with their surroundings, specific cell–cell recognitions, and adhesion interactions are likely to instruct an axon to grow.

The vascular and nervous systems display parallelism in their architectural patterning and functional organization. One explanation for their coupling is that neurons and the endothelium are affected by the same guidance cues, or that one regulates the other's formation. The former hypothesis was supported by findings that axon guidance cues control vessel branching and, *vice versa*, that angiogenic factors modify axon projection [2,3]. Recent studies have focused on the latter possibility and provide evidence that factor(s) secreted from the endothelium have a potential for axon growth during development and repair [4,5]. Although neurovascular congruence is considered to be regulated by the interdependence of neurons and blood vessels, unassailable evidence that neural circuits are regulated by contact with blood vessel has not yet been obtained.

Integrins are members of a large family of transmembrane glycoproteins, composed of two associated chains, α and β. There are 16 α subunits and 8 β subunits, and they dimerize in multiple combinations to form over 20 different integrin receptors [6]. Integrins are activated by ligands on adjacent cells or in the extracellular matrix (ECM) and modulate intracellular signal pathways by virtue of their subunit dependency, thereby regulating cellular morphology, differentiation, and proliferation [7]. Here, we show that vascular endothelial cells promote neurite elongation by a mechanism dependent on integrin β3.

2. Materials and methods

2.1. Animals

Wistar rats were purchased from Japan SLC (Hamamatsu, Japan). All the experiments were conducted in accordance with

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the guidelines laid down by the animal welfare committees of Osaka University.

2.2. Primary culture of cortical neurons

Cortices were dissected from postnatal-day-1 Wistar rats. The tissue was then incubated in 0.25% trypsin (Gibco Carlsbad, CA, USA) in phosphate-buffered-saline (PBS) for 15 min at 37 °C followed by resuspension in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco) and trituration. The isolated cells were plated on collagen-type IV- (Sigma, St. Louis, MO, USA) and fibronectin- (Sigma) precoated Lab-Tek 4-well chamber slides (Nalge Nunc International, Rochester, NY, USA) at a density of 2×10^5 cells/well in DMEM containing 10% FBS. In co-culture experiments, cortical neurons were cultured on chamber slides that had previously cultured vascular endothelial cells after 48 h plating.

The following pharmacological agents were used in the cultures: 200 μm RGD (Arg-Gly-Asp) peptides (Enzo Life Sciences, Farmingdale, NY, USA), 200 μm control for RGD peptide (Enzo Life Sciences), 20 μg/ml anti-integrin-β1 antibody (BD Bioscience, San Jose, CA, USA), 20 μg/ml anti-integrin-β3 antibody (Biolegend, San Diego, CA, USA), 20 μg/ml control hamster IgM (BD Bioscience), and 20 μg/ml control hamster IgG (BD Bioscience).

2.3. Isolation of brain microvessels and primary culture of endothelial cells

Primary cultures of rat brain capillary endothelial cells were prepared from 3-week-old Wistar rats. Cerebral cortices that were free of meninges by dissection were dissociated in a mixture of 1 mg/ml collagenase type 2 (Worthington, Lakewood, NJ) and 6.7 μg/ml DNase in DMEM for 1.5 h at 37 °C. The cell pellet was separated by centrifugation in 20% bovine serum albumin (BSA; minimum 98% electrophoresis grade, Sigma)-DMEM (1000g, 10 min), and was then incubated for another 45 min at 37 °C with a mixture of 1 mg/ml collagenase–dispase (Roche, Basel, Switzerland) and 6.7 μg/ml DNase in DMEM at 37 °C. Microvessel endothelial cell clusters were separated on a 33% continuous Percoll (Pharmacia, Uppsala, Sweden) gradient, collected, and plated on culture dishes coated with 0.1 mg/ml collagen type IV and 0.1 mg/ml fibronectin. Endothelial cells were grown in DMEM supplemented with 10% FBS, 1 mM Hepes (Sigma), 2 mM glutamine (Gibco), 2 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA), 4 μm hydrocortisone (Sigma), and 4 μg/ml puromycin (Sigma). Endothelial cell culture was confirmed by immunostaining of the endothelial cell marker CD31/PECAM-1 (BD Bioscience).

2.4. Immunostaining and observation

After culturing, cells were fixed with 4% paraformaldehyde for 20 min at 37 °C followed by permeabilizaion using 0.1% Triton-X-PBS for 1 h at room temperature, and blocking with PBS containing 5% BSA. The samples were subsequently incubated with primary antibodies in PBS containing 5% BSA overnight at room temperature, followed by incubation with Alexa Fluor 488, and 568-conjugated secondary antibodies for 1 h at room temperature. Anti-β-tubulin (Tuj1) antibody (1:100; Covance, Princeton, NJ, USA), and anti-integrin-β3 antibody (H-96) (1:100; Santa Cruz Biotechnology, CA, USA) were used as the primary antibodies. The images were taken with an upright microscope (Olympus, Tokyo, Japan) and analyzed using a DP-controller image system (Olympus). The length of the longest neurite per neuron was measured using ImageJ. The experiment was replicated three times.

2.5. Statistical analysis

Data are presented as mean ± SEM. Statistics were analyzed using analysis of variance with Sheffe's multiple comparison test. *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. Vascular endothelial cells enhance neurite outgrowth in cortical neurons

We employed a co-culture of cortical neurons obtained from postnatal day 1 rat cerebral cortex and cortical vascular endothelial cells obtained from 3-week-old rat brain. Dissociated cortical neurons were plated on vascular endothelial cells and were visualized with anti-Tuj1 antibody, a neuronal marker (Fig. 1A). After 48 h of culturing, the neurite length of cortical neurons remarkably increased on the vascular endothelial cells compared with the neurons cultured without endothelial cells (control, 109.43 ± 13.74 μm; co-culture, 182.2 ± 8.68 μm; Fig. 1B). These results suggest that some factors on the surface of vascular endothelial cells enhanced neurite elongation.

3.2. The arginyl–glycyl–aspartic acid (RGD) sequence is required for the promotion of neurite elongation by vascular endothelial cells

We next investigated the involvement of adhesion molecules in vascular endothelial cell-induced neurite elongation. Many adhesive proteins present the RGD sequence as their cell recognition site. To confirm the requirement of the RGD sequence for the effects of vascular endothelial cells, synthetic peptides containing the RGD sequence were tested for their ability to inhibit vascular endothelial cell-dependent neurite outgrowth. We treated the co-culture with either an RGD-blocking or control peptide. The RGD blocking peptides abrogated the effect of endothelial cells on neurite growth (endothelial cells + control peptide, 171.3 ± 14 μm; endothelial cells + RGD peptide, 132.5 ± 12.6 μm; Fig. 2A and B). In the culture grown in the absence of vascular endothelial cells, neither RGD peptide nor control peptide significantly affected the length of neurite (control + control peptide, 79.24 ± 4.72 μm; control + RGD peptide, 80.62 ± 6.45 μm). These results show that cell adhesion molecules, containing the RGD sequence, may promote neurite elongation in cortical neurons.

3.3. Integrin β3 is a key molecule in vascular endothelial cell-mediated neurite elongation

We next tried to identify the key molecule containing the RGD sequence for endothelial cell-mediated neurite elongation. β1 and β3 integrins are emerging as key regulators of neuronal development, including synapse formation [8–10], spine morphology [11], and neurite elongation [12]. To determine whether and which integrin β subunits were involved in neurite elongation by vascular endothelial cells, antibodies that recognize and inhibit the activity of each integrin subunit were tested for their effects on vascular endothelial cell-stimulated neurite elongation. Cortical neurons were cultured on vascular endothelial cells in the presence of antibodies against β3 integrin. Treatment with antibodies for β3 integrin resulted in the reduction of neurite outgrowth in co-culture compared to control antibody (control + control antibody: 139.6 ± 3.39 μm; control + β3 integrin antibody: 131.62 ± 4.89 μm; endothelial cell + control antibody: 193.1 ± 5.25 μm; endothelial cell + β3 integrin antibody: 163.7 ± 4.54 μm; Fig. 3A and B). Meanwhile, neither antibody for β1 integrin nor isotype-matched control antibody significantly affected the endothelial cell-induced neurite

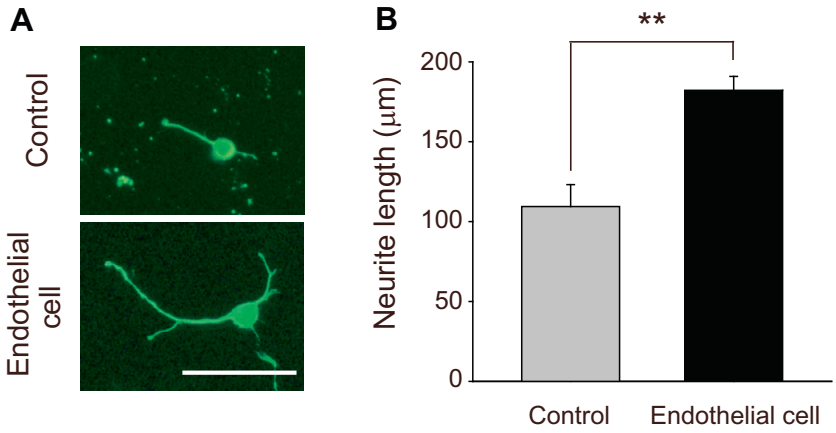

Fig. 1. Vascular endothelial cells promote cortical neurite elongation. (A) Representative images of cultured cortical neurons stained with Tuj1 antibody. In neurons cultured on vascular endothelial cells, the endothelial cells promote neurite extension. Control: neurons cultured without endothelial cells. Bar, 50 μm. (B) The graph shows the neurite length of cultured neurons. Values represent the mean ± SEM (n = 90 neurons). $^{**}P < 0.01$ using Student's t-test.

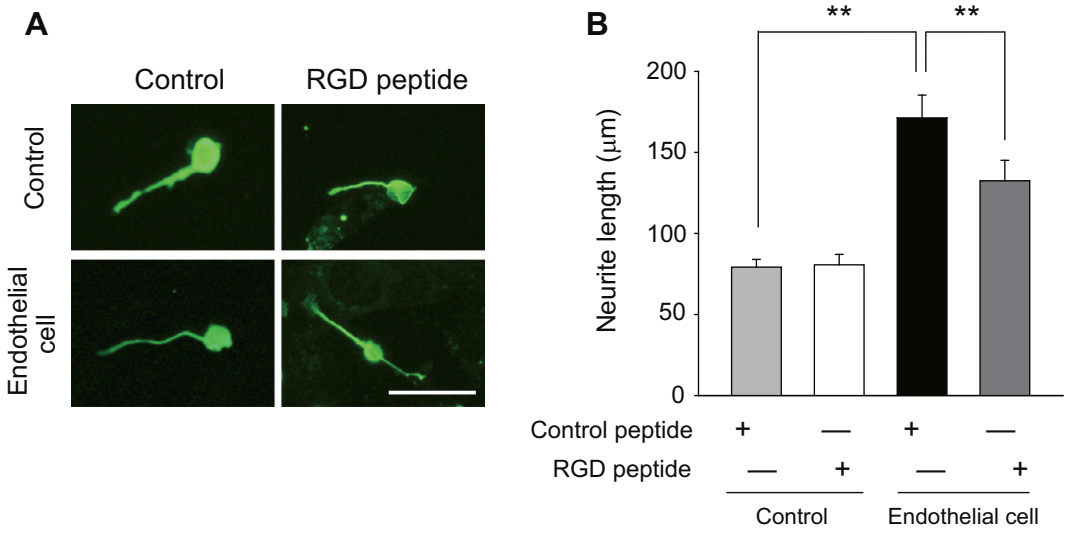

Fig. 2. The RGD motif is required for vascular endothelial cell-induced neurite elongation. (A) Representative images of the neurons stained with Tuj1 antibody under the indicated conditions. Bar, 50 μm. (B) The graph shows the neurite length of cultured neurons. Treatment with RGD blocking peptides inhibited the promotion of neurite elongation by vascular endothelial cells. Values represent the mean ± SEM (n = 90 neurons). $^{**}P < 0.01$ using Tukey's test.

elongation (control + control antibody: 100.38 ± 2.95 μm; control + β1 integrin antibody: 96.2 ± 3.14 μm; endothelial cell + control antibody: 137.85 ± 3.72 μm; endothelial cell + β1 integrin antibody: 127.15 ± 3.56 μm; Fig. 3C and D). We immunocytochemically confirmed the expression of integrin β3 subunits in cultured cortical neurons (Fig. 4). Taken together, our findings demonstrated that vascular endothelial cells promote neurite elongation by a mechanism dependent on integrin β3 expressed on the neuron.

4. Discussion

In the normal adult CNS, a vascular niche provides trophic support for neural stem cells, and regulates cell growth, survival, and fate destination [13]. Moreover, neurogenic cells that migrate from the subventricular zone to the ischemic area in a mouse stroke model are observed to be closely associated with blood vessels [14]. Newly generated neurons differentiate into mature neurons in the ischemic striatum, suggesting neuronal replenishment from endogenous precursors in the adult brain after stroke. Although the fact that the vascular network allows for neural cell generation is well accepted, the role of the vascular system in the formation of neuronal networks in the CNS has not been fully elucidated. Although one study showed that regenerating axons were closely associated with vasculature around the lesion after spinal cord injury, with unknown significance [15], the function of blood vessels that run along with neurites remains to be clarified. This study uncovered a new role of blood vessels in the scaffold for neuronal wiring.

Ligand binding to integrins leads to integrin clustering, and promotes recruitment of actin filaments [16]. Actin polymerization by environmental stimuli regulates growth cone behavior and neurite elongation [17]. Thus, vascular endothelial cell-evoked actin polymerization may be involved in neurite elongation in cortical neurons. In addition, our observation that cortical neurons constantly express the integrin β3 subunit suggests that cortical neurons may have the potential to receive vascular endothelial signaling constitutively, leading to constitutive actin assembly. Actin is the most prominent cytoskeletal protein at the synapse and contributes to cytoarchitectural changes that are associated with synaptic plasticity in mature neurons [8–11]. Our findings demonstrate the

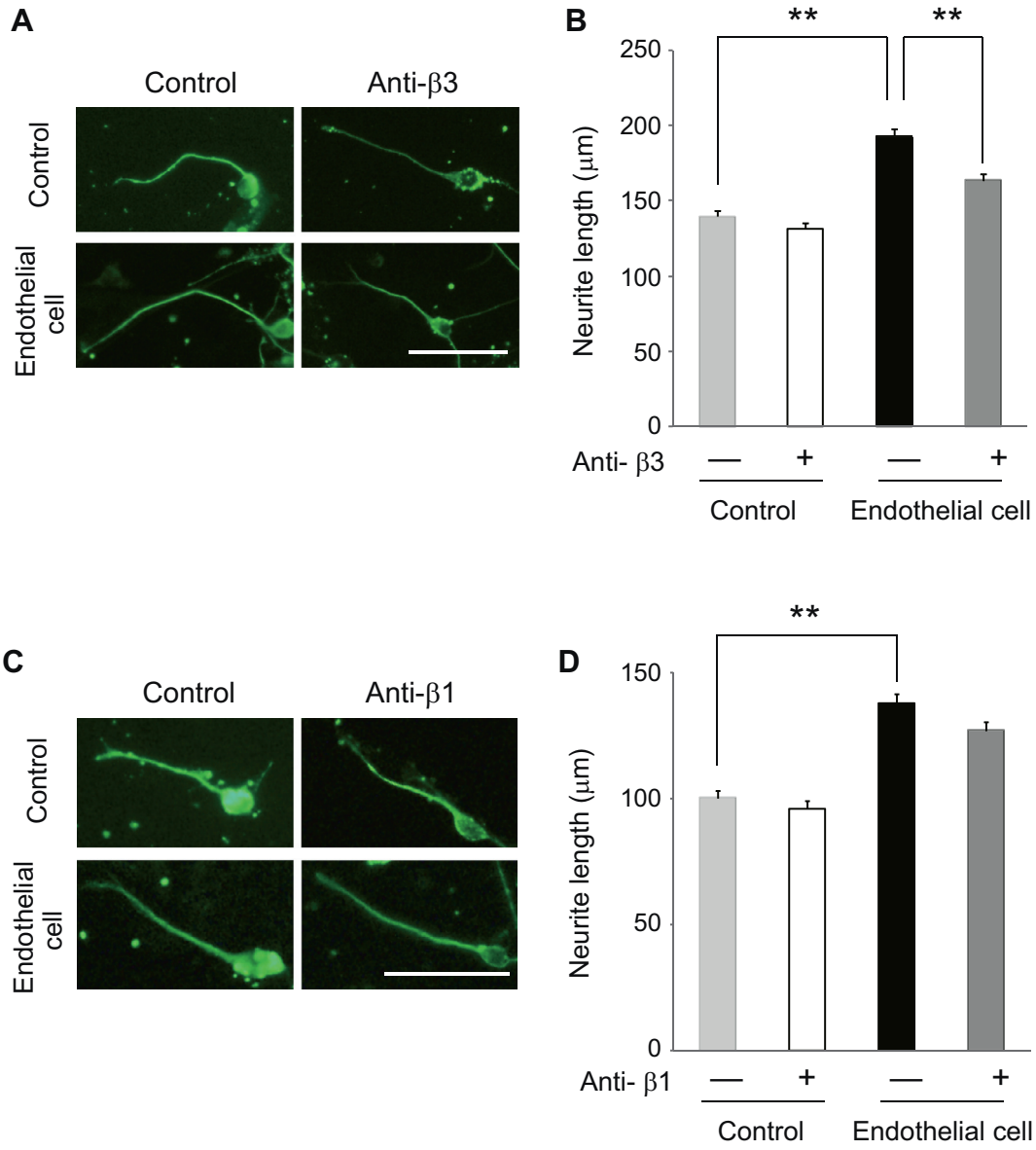

Fig. 3. Integrin β3 is involved in vascular endothelial cell-induced neurite elongation. (A) and (C) Representative images of the neurons stained with Tuj1 antibody under the indicated conditions. Bar, 50 μm. (B) and (D) The graphs are a quantification of the neurite length. Anti-integrin β3 antibodies (B), but not anti-integrin β1 antibodies (D), prevented the promotion of neurite elongation by vascular endothelial cells. Values represent the mean ± SEM ($n = 90$ neurons). $^{**}P < 0.01$ using Tukey's test.

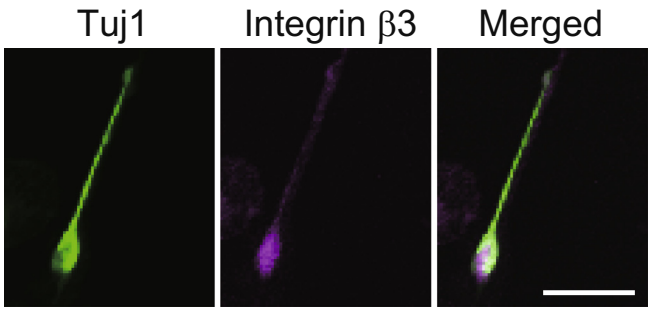

Fig. 4. Expression of integrin β3 in cultured cortical neurons. Immunoreactivity for integrin β3 was observed in Tuj1-positive neurons. Bar, 50 μm.

possibility that the vascular system undergoes continuous plastic changes in the adult CNS. Focusing on neuro-vascular communication may offer new perspectives on physiological and pathological neuronal events in the CNS.

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