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# Glial Cell Line-Derived Neurotrophic Factor Induces Barrier Function of Endothelial Cells Forming the Blood–Brain Barrier

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**Since a deep involvement of astrocytes, a kind of glial cells, in differentiation of the blood–brain barrier (BBB) has been suggested, we examined the relation of glial cell line-derived neurotrophic factor (GDNF) to the BBB. First, immunohistochemical examination of the cerebral cortex of rats revealed that glial cell line-derived neurotrophic factor receptor (GFR$\alpha$1) was preferentially expressed on the cell membranes of capillary endothelial cells. Second, to elucidate the effects of GDNF on the BBB, capillary endothelial cells isolated from the porcine cerebral cortex were cultured and then changes in tight junction function of the endothelial cells were examined after addition of GDNF, in terms of transendothelial electrical resistance (TER) and permeability. GDNF at concentrations of 0.1 and 1 ng/ml significantly activated the barrier function of the endothelial cells in the presence of cAMP. Since GDNF is secreted from astrocytes sheathing capillary endothelial cells in the brain cortex, our results strongly suggest that GDNF enhances the barrier function of tight junctions of the BBB on the one hand, and also supports the survival of neurons on the other hand.** 

The blood–brain barrier (BBB) is known as a highly selective barrier between the blood and the central nervous system, playing an essential role in maintaining homeostasis of the system. It has become clear that endothelial cells of the capillaries of the brain cortex are mostly responsible for the formation of the BBB, in which highly impermeable tight junctions are the most important cellular apparatus for performing the paracellular barrier function (1). However, the molecular mechanisms of development of the BBB have not yet been fully clarified.

Astrocytes, a kind of glial cells forming perivascular ensheathment in the brain in vivo, have been shown to contribute to development of the BBB presumably by their secretion of unknown factors that differentiate capillaries to the BBB type, in terms of developing far less impermeable tight junctions (2–6). Recently, glial cells were reported to secrete glial-cell-derived-neurotrophic factor (GDNF) to maintain dopaminergic (7) and motor (8) neurons in vivo. GDNF is a distant member of the TGF-$\beta$ family and binds to a receptor on the cell surface, GFR$\alpha$1, which interacts with c-ret (9–12). Since glial cells secrete GDNF (7), the effects of GDNF on paracellular barrier function of endothelial cells of the porcine cerebral cortex were elucidated in the present study, as a candidate for an inducer of highly functional endothelial cells of the BBB.

## MATERIALS AND METHODS

*Isolation and culture of porcine brain capillary endothelial cells.* Porcine brain capillary endothelial cells were purified by the procedure of Abbott *et al.* (13) with slight modification (14). Briefly, after peeling the meninges and choroid plexus off of brains obtained from miniature pigs weighing about 20 kg, cortical gray matter was carefully resected and minced with scissors into small pieces and digested in 0.25% dispase (Godo Shusei, Tokyo) and 0.12% collagenase (Yakult, Tokyo) in Ca, Mg-free Hanks' balanced saline solution (HBSS) at 39°C for 60 min. During the enzyme digestion, the solution containing the tissues was bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. After extensive pipetting, the capillaries were sepa-

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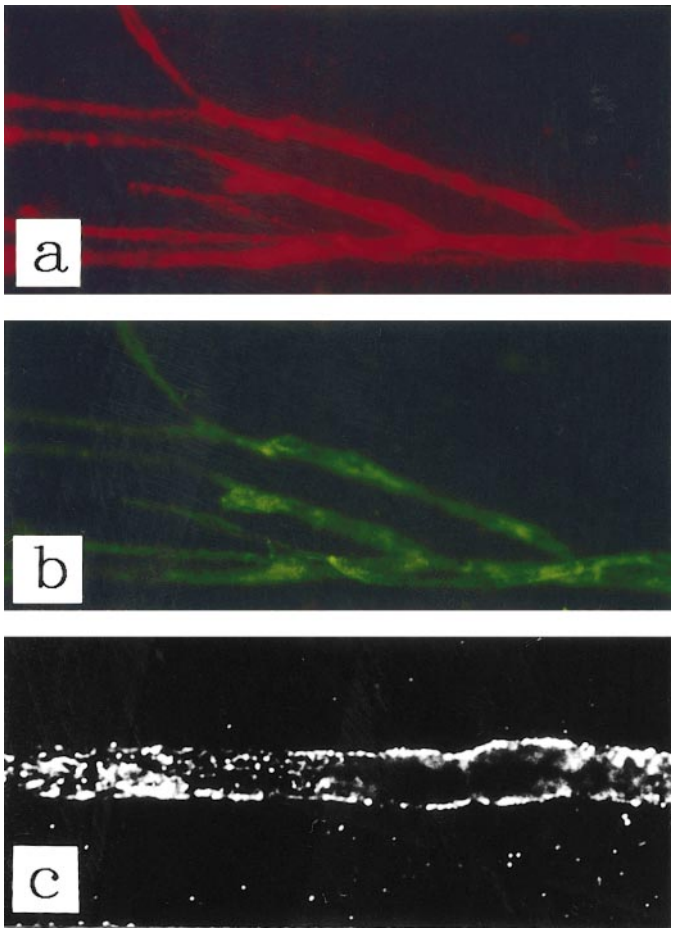


**FIG. 1.** Double immunohistochemistry for GFR$\alpha$1 and von Willebrand Factor on the cerebral cortex of rats: (a) von Willebrand Factor, ×200; (b) GFR$\alpha$1, ×200; (c) confocal image of GFR$\alpha$1, ×800.

rated from the remaining slurry by centrifugation at 1000g for 15 min in phosphate-buffered saline (PBS) containing 25% bovine serum albumin. After several rinses by centrifugation, fragments of capillaries were seeded onto 12-well tissue-culture plates coated with type IV collagen (Nitta Gelatin, Osaka) in Dulbecco's modified Eagle's medium (DMEM) 1:1 with Ham's F-12 nutrient (D/F12) mixture (Kyokuto), supplemented with 15% heat-inactivated fetal bovine serum (FBS; Moregate), 75 $\mu$g/ml endothelial growth supplement (Sigma, St Louis, MO), 80 $\mu$g/ml heparin (Sigma), 5 $\mu$g/ml insulin (Collaborative Biomedical, Bedford, MA), 5 $\mu$g/ml transferrin (Collaborative Biomedical), 5 ng/ml selenous acid (Collaborative Biomedical), 100 U/ml and antibiotics. At day 1 after plating, 60 nM vincristin (Sigma) was added to the cultures to eliminate undesirable cells until the cell density reached sub-confluence (15). The medium was renewed every other day. When the endothelial cells reached sub-confluence, they were released by 0.25% trypsin-EDTA (Gibco, Grand Island, NY) and seeded at 25,000 cells/filter on 0.33 $cm^2$ rat tail collagen-coated polycarbonate Coaster Transwell filters (0.4 $\mu$m pore size; Coaster Corp., Cambridge, MA). Four-day cultivation without vincristin after passage, the medium of the endothelial cells was changed to medium containing GDNF at various concentrations, 125 $\mu$M 8-(4-chlorophenylthio) cAMP (CPT-cAMP; Sigma) and 17.5 $\mu$M phosphodiesterase inhibitor, RO20-1724 (RBI, Natick, MA).

*RNA extraction, reverse transcription (RT)-PCR, and Southern blotting.* RNA preparation, RT-PCR and Southern blotting were performed according to the standard protocols (16). Briefly, total RNA was isolated from the kidneys of male Sprague-Dawley rats using the single-step thiocyanate-phenol-chloroform extraction method as modified by Xie and Rothblum (17). RT-PCR was performed using an RT-PCR kit supplied by Perkin-Elmer Co. (Branchburg, NJ) according to the manufacture's recommendations. The primers for rat GFR$\alpha$1 (9) were 5′-TCTAGAACCATGTTCCTAGCCACTCTGTAC-3′ (nucleotides [nt] 302 to 322 linked with the Xba I site) and 5′-TCTAGACTACGACGTTTCTGCCAACGATAC-3′ (nt 1685 to 1708 plus the Xba I site). The PCR products were subcloned into T-vector (pB II-rGFR$\alpha$1), and the entire amplified insert was sequenced to check for any misamplification. To confirm expression of the GFR$\alpha$1 transcripts in capillaries of the cerebral cortex, total RNA was extracted from capillary endothelial cells derived from the porcine cerebral cortex and rat lung endothelial cells (RLE), as well as from the rat kidney tissues as a control, followed by RT-PCR using one $\mu$g the total RNA as a template. The primers for GFR$\alpha$1 (9) were 5′-CTGCGTATCTACTGGAGCATGTACCAG-3′ (nt 599 to 625) and 5′-TTCTTCATAGGAGCACACGGGGACGAT-3′ (nt 986 to 1012). To make sure that amplifications were in the linear range, PCR was performed using 20, 25, 30 and 35 cycles. Aliquots of PCR products (10 $\mu$l) were loaded onto 1% agarose gel, electrophoresed, and transferred to GeneScreen plus membranes (NEN, Boston, MA). The blots were subsequently probed with the corresponding random primed $^{32}$P-labeled cDNA. Hybridization was in 50% formamide at 42°C overnight. The blots were washed twice in 2 × SSC at room temperature for 30 min, and were washed in 0.1% SDS and 2 × SSC at 50°C at least for15 min, followed by exposure for autoradiography.

*Immunohistochemistry of GFR$\alpha$1.* Slices of cerebral cortex were put between glass slides, and then the slides were pressed hard against each other, pulled apart, and immediately immersed in a cold mixture of acetone and ethanol (1:1) for 5 min. After rinsing with PBS, the slides were incubated with a goat polyclonal anti-rat GFRa1 antibody (Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti-human von Willebrand Factor (DAKO, Copenhagen) at 4°C overnight. The tissues were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG and rhodamine-conjugated anti-rabbit IgG (DAKO) at room temperature for 1 hr. All samples were examined with a Nikon Fx epifluorescence photomicroscope (Nikon, Tokyo) and/or a confocal laser scanning fluorescence imaging system (MRC-500J, Bio-Rad).

*Measurement of TER and permeability.* The transendothelial electrical resistance (TER) of the filter-grown endothelial cell monolayers in second culture was measured using an Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL) equipped with opposing circular disc current electrodes or with STX-2 Ag/AgCl

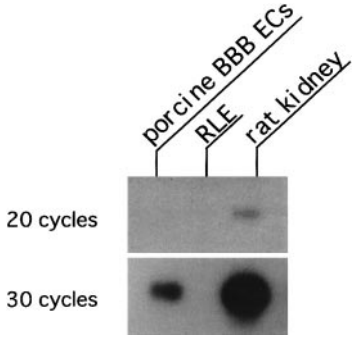


**FIG. 2.** Expression of GFR$\alpha$1 in endothelial cells forming porcine blood–brain barrier. After RT-PCR, Southern blotting was done (see Materials and Methods), to demonstrate expression of GFR$\alpha$1 using RNAs obtained from: porcine BBB ECs, 7-day cultured endothelial cells in primary culture derived porcine brain; RLE, rat lung endothelial cells; rat kidney.

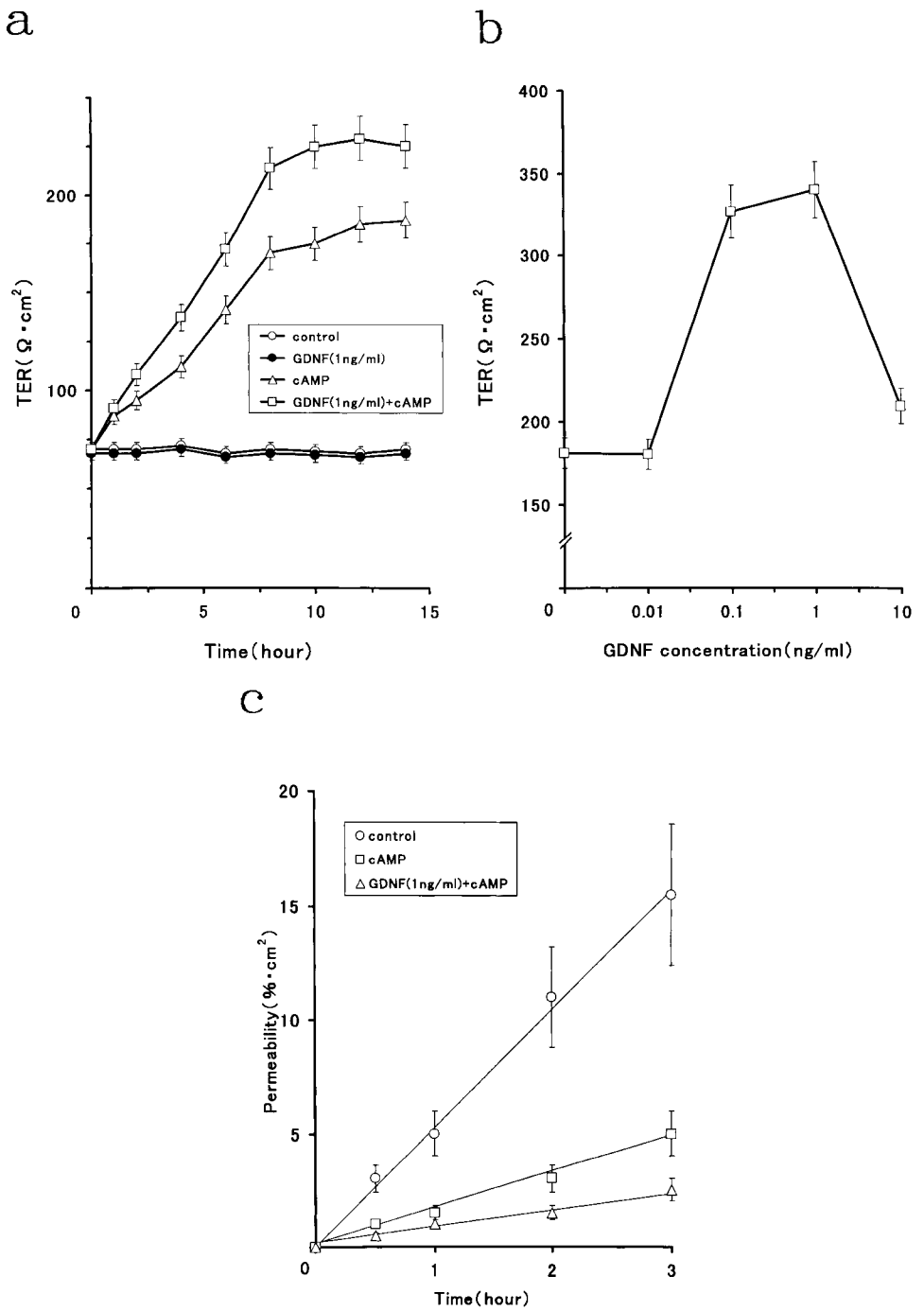


**FIG. 3.** Effects of GDNF on barrier function of endothelial cells forming the porcine BBB. (a) Effects of GDNF on TER of the cells. (b) Effects of GDNF concentrations on TER of the cells. (c) Effects of GDNF on permeability of the cells using mannitol as a tracer. The cells at a confluent density were cultured under various conditions for 8 hrs and then measured for permeability. GDNF concentration is 1 ng/ml. Each point in the figures is mean ± SD (n = 5 or 6).

electrodes (Endohm, World Precision Instruments). The measurements of TER were performed at 37°C on a thermal plate (Fine, Tokyo). TER was expressed in standard units of ohm · $cm^2$. For calculation of the resistance of endothelial cell monolayers, resistance of blank filters was subtracted from that of filters covered with cells. Each value was calculated from 5 or 6 cultures.

To determine the permeability of filter-grown endothelial cell monolayers to mannitol, 50 $\mu$l of medium containing $^{14}$C-labeled tracer was added to the inner chamber after 8-hr cultivation under the various conditions described above. Samples collected from the outer chamber at each time point were measured by scintillation counting. The results were expressed as clearance per centimeter squared per minute (%/$cm^2$/min). Each value was calculated from 5 or 6 cultures.

## RESULTS AND DISCUSSION

First, immunohistochemical examination of the cerebral cortex of rats using a confocal laser microscope clearly revealed that GFR$\alpha$1 preferentially localized on the cell membranes of capillary endothelial cells (Figs. 1a, 1b, and 1c), but not detected in the endothelial cells of hypophysis, lung and tongue (data not shown). This is the first demonstration that the capillaries of the brain cortex are positive for GFR$\alpha$1, though two reports using a in situ hybridization technique described that signals of GFR$\alpha$1 were weakly detected in the cerebral cortex, not in detail, at a low magnification (18, 19). Similarly, GDNF was reported to be weekly expressed in the cerebral cortex (18). These findings strongly suggest that endothelial cells forming BBB are target cells of GDNF. On the other hand, it was reported that the signal evoked by the binding of GDNF to GFR$\alpha$1 is transduced by c-ret (9–12). Immunohistochemically, however, we could not detect c-ret expression in capillaries of the cerebral cortex of adult rats. These observations mean that c-ret is not always necessary for the signal transduction of GDNF in endothelial cells of the cerebral cortex, as suggested by several researchers (20, 21).

Second, to clarify whether GDNF actually influences the barrier function of the BBB, endothelial cells obtained from porcine cerebral cortex were cultured and examined to determine the effect of GDNF on TER of the endothelial cells. As shown in Fig. 2, we demonstrated GFR$\alpha$1 expression in the endothelial cells in primary culture 7 days after isolation from the porcine cerebral cortex. Our culture medium contained 60 nM vincristin allows endothelial cells of BBB selectively to grow, eliminating contaminating cells (15). Changes in TER and permeability of the endothelial cells induced by GDNF with or without cAMP are shown in Figs. 3a and 3c. TER gradually increased by 8 hr after addition of both agents and then reached a plateau. However, GDNF alone did not increase TER. On the other hand, cAMP alone increased TER, which was significantly different from that obtained by the treatment with both GDNF and the agents. The results of permeability examination using mannitol were consistent with the results of TER measurements, as shown in Fig. 3c. These results clearly show that GDNF is a differentiation factor of endothelial cells of the BBB, in terms of enhancing tight junction function.

A phenomenon that medium conditioned by astrocytes in second culture significantly increases TER and permeability of capillary endothelial cells of cerebral cortex only when intracellular cAMP-elevating agents simultaneously existed was reported by Rubin *et al.* (3) and by us (14). The action of GDNF requires activation of cAMP-dependent pathways (22). The present experiments show that the presence of cAMP enhanced the effect of GDNF on TER and permeability of endothelial cells of cerebral cortex capillaries. Thus, GDNF is a strong candidate factor secreted from astrocytes in vitro to induce differentiation of the endothelial cells of the BBB.

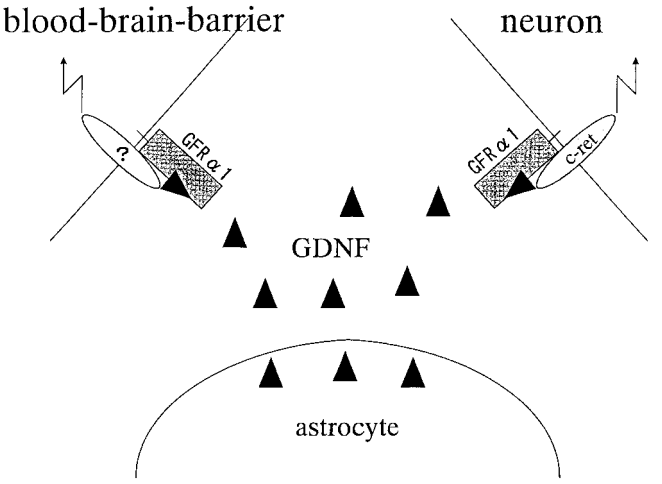


**FIG. 4.** Schematic diagram of GDNF action in the brain.

A dose-response relation between TER and the concentration of GDNF is demonstrated in Fig. 3b. The effect of GDNF on TER was observed at concentrations between 0.1 and 1 ng/ml in the presence of cAMP. Even at a concentration of 0.1 ng/ml, GDNF significantly increased TER of the endothelial cell monolayers. This concentration of GDNF exhibiting biological activity activating the barrier function was almost as low as that increasing the number of retinal cells in culture shown by Jing *et al.* (9).

GDNF was first found as a neurotrophic factor to that enhanced survival of dopaminergic and motor neurons from culture medium conditioned by a glial cell line (7). We herein demonstrate that GDNF directly acts on capillary endothelial cells of the cerebral cortex forming the BBB to activate the barrier function of the cells. These results suggest that GDNF enhances selective uptake of nutrients of endothelial cells by tightly sealing the paracellular pathway, on the one hand, and the survival of neurons, on the other hand, to maintain homeostasis of the central nervous system (Fig. 4).

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