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Lecithinized brain-derived neurotrophic factor promotes the differentiation of embryonic stem cells in vitro and in vivo

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Abstract

The addition of lecithin molecules to brain-derived neurotrophic factor (BDNF) has been reported to markedly enhance its pharmacological effect in vivo. In the current study, we show that lecithinized BDNF (PC-BDNF) has a higher affinity than BDNF for neural precursor cells. Although BDNF only slightly increased the expression of the genes for Mash-1, p35, 68 kDa neurofilament, and TrkB receptor, PC-BDNF caused a significant increase in their expression. PC-BDNF also increased the level of neurofilament protein and dramatically increased TrkB mRNA gene expression, which was followed by a sustained activation of the p42/p44 extracellular-regulated kinases. Finally, transplantation of PC-BDNF-treated cells was more effective than BDNF-treated cells at improving impaired motor function caused by spinal cord injury. These findings showed that PC-BDNF has a better potential than BDNF for promoting neural differentiation, partly due to a higher cellular affinity. Furthermore, PC-BDNF-treated cells could be useful for transplantation therapy for central nervous system injuries.

Keywords: BDNF; Lecithinized-BDNF; Differentiation; Embryonic stem cells; Spinal cord injury

Brain-derived neurotrophic factor (BDNF) is a multifunctional neurotrophic factor that plays a key role in cell survival and differentiation in the central nervous system (CNS) [1–3]. BDNF is also implicated in axonal regeneration, synaptic formation [4,5], and synaptic plasticity [6,7]. Following CNS injury, exogenous or endogenous BDNF can rescue neurons from damage and promote reorganization of the neuronal network [8]. Moreover, BDNF can enhance the differentiation of neural precursor cells [9–11]. Therefore, BDNF may be useful as a therapeutic agent for CNS injuries, such as spinal cord injury (SCI), by protecting neurons and inducing differentiation of neural stem cells at sites of damage.

Transplantation of neural cells to sites of injury is expected to be useful for treatment of CNS injuries. These neural cells can be derived from embryonic stem (ES) cells, which can be preferentially differentiated into neural precursor cells when cultured in conditions that favor their genesis, survival, and enrichment. The transplantation of ES-derived neural precursor cells has been shown to allow recovery from SCI in the rat [12]. BDNF may be a useful tool in this regard because it can induce the differentiation of ES cells into neural cells.

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To improve the residence of BDNF at target sites, it can be derivatized with three molecules of lecithin (PC-BDNF). We have previously shown that PC-BDNF reduces plasma glucose level, food intake, and body weight in C57BL/KsJ-db/db diabetic mice and that it is 20-fold more potent than BDNF itself [13]. In addition, PC-BDNF induces the sustained activation of p42/44 extracellular-regulated kinases (ERKs) in PC-12 cells when expressed along with the BDNF receptor, which is also known as TrkB.

Based on this background, we suspected that PC-BDNF can be used to promote the differentiation of neural precursor cells, which could then be used for transplantation. Therefore, in the current study, we examined whether PC-BDNF could cause the differentiation of retinoic acid-treated neural precursor cells and whether transplantation of these cells could enhance recovery from impaired motor function as a result of SCI.

Materials and methods

Animals. All animals were housed at a constant temperature (23 ± 1 °C) and humidity (50–60%) with free access to a standard diet and water. The animal room had a 12-h light/dark cycle. All of the experimental procedures were in accordance with the St. Marianna University guidelines for the welfare of animals.

Synthesis of PC-BDNF. BDNF was kindly provided by Sumitomo Pharmaceutical (Osaka, Japan). PC-BDNF was synthesized according to the method of Igarashi et al. [13]. Analysis by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager DE mass spectrometer (PE Biosystems, Foster city, USA) confirmed that each molecule of PC-BDNF was derivatized with an average of three lecithin molecules.

Differentiation of ES cells. Undifferentiated mouse R-CMTI-1 ES cells (Dainippon Pharmaceutical, Osaka, Japan; passages 12–18) were propagated in the presence of leukemia inhibitory factor (Chemicon International, Temecula, CA, USA). Differentiation of these cells was induced as previously described [14,15]. Briefly, cells were cultured as embryonic bodies in the absence of leukemia inhibitory factor for 4 days, and 1 μM all-trans RA (Sigma–Aldrich, St. Louis, MO, USA) was added on the fourth and sixth days. The treatment of ES cells with RA has been reported to induce neural differentiation [16]. About 80% of the retinoic acid-treated cells became NCAM-positive [17]. On the eighth day, embryonic bodies were trypsinized to a single cell suspension, which was then used for studies of morphology, mRNA and protein expression, and transplantation in vivo.

Morphology. Cells were grown in Dulbecco's modified essential medium supplemented with 20% fetal calf serum, seeded at 1×10^6/well in 10-cm diameter petri dishes, and cultured at 37 °C in an atmosphere of 5% CO_2. The cells were observed by phase contrast microscopy at a magnification of 200×. Neurites extending from the cell bodies were counted if longer than 25 μm.

Determination of BDNF and PC-BDNF binding affinity. The affinity of ES cells for BDNF or PC-BDNF was examined using [^{125}I]-labeled BDNF or PC-BDNF, prepared using the Chloramine T method [18]. Cells (3×10^5/well) were incubated with [^{125}I]-labeled PC-BDNF (4.9 kBq/μg) or BDNF (20.4 kBq/μg) in an atmosphere of 5% CO_2 for 2 h at 4 °C. The cells were then washed three times with PBS, and bound radioactivity was determined using a γ-counter (Aloka Accu-FLEX 7000, Tokyo, Japan). After measurement of the bound radioactivity, the cells were further incubated for 2 h at 4 °C with a 100-fold excess of unlabeled BDNF, and the radioactivity was again determined after washing four times with PBS.

Cellular affinity for immunostaining for BDNF. ES cells (2×10^5) were grown on BIOCOAT coverslips (Becton–Dickinson Labware, Franklin Lakes, NJ, USA). The cells were incubated in the presence or absence of 30 μg/ml BDNF or PC-BDNF for 2 h. The cells were fixed with 4% formaldehyde and stained using rabbit anti-BDNF (Chemicon International, Temecula, CA, USA) followed by goat fluorescein isothiocyanate-labeled anti-rabbit IgG (American Qualex Antibodies, San Clemente, CA, USA). Fluorescence images were acquired using a conventional microscope equipped with epifluorescence optics (model IX71/CoolSNAP-HQ, Olympus, Melville, NY, USA).

Cell proliferation and viability. Cells were grown in Dulbecco's modified essential medium containing N2 supplement and 5 μg/ml fibronectin (Invitrogen Life Technologies, Carlsbad, CA, USA), and seeded in 96-well plates at 1×10^4/100 μl/well. After the cells were incubated for 24 h with BDNF or PC-BDNF, bromodeoxyuridine (BrdU) incorporation was assayed using a Cell Proliferation ELISA System (Amersham Biosciences, Piscataway, NJ, USA). Cell viability was assayed using mitochondrial conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H tetrazolium bromide (MTT) and measurement of the absorbance at 595/655 nm.

Reverse transcription-PCR. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) and then treated with RNase-free DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA). One-step Reverse transcription (RT)-PCR was performed with 2.5 μg of total RNA, Ready-to-Go RT-PCR Beads (Amersham Biosciences, Piscataway, NJ, USA), and the following primers (product size in parentheses): for Mash-1 (244 bp) 5′-AAGTCAGCGGCCAAGCAGG ATAAG-3′ (forward) and 5′-CGCAGCGTCTCCACCTTGCTCA TCT-3′ (reverse); for p35 (923 bp) 5′-CGGCACGGTGCTGTCC CTGTCT-3′ (forward) and 5′-TCACCGATCCAGGCCTAGGAG-3′ (reverse); for 68 kDa neurofilament (NF68) (327 bp) 5′-TGGAGA ATGAGCTGAGAAGC-3′ (forward) and 5′-TTCGTAGCCTCAA TGGTCTC-3′ (reverse); for TrkB (320 bp) 5′- TGCGCTTCAG TGGTTCTACAA-3′ (forward) and 5′-CCGTGGAGGGGATTTC ATTAC-3′ (reverse); and glyceraldehyde 3′-phosphate dehydrogenase (G3PDH) (452 bp) 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). PCR was carried out with the following cycling conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The cycle was repeated 30 times for Mash-1, 35 times for P35, 20 times for NF68, 28 times for TrkB, and 23 times for G3PDH. PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and then detected with a BioDoc-It System UV Transilluminator (UVP, Upland, CA, USA).

Western blotting. Cells were washed three times with ice-cold Tris-buffered saline(20 mM Tris–HCl, pH 7.4 and 0.5 M NaCl) and lysed with 250 μl lysis buffer (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, and 5 μg/ml aprotinin). Aliquots of the lysates were centrifuged for 20 min at 15,000 rpm and separated by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL, USA). Proteins were transferred to Clearblot P polyvinylidene difluoride membranes (ATTO, Tokyo, Japan) and were reacted with anti-NF200 rabbit IgG (Sigma–Aldrich), anti-phospho-p44/42 Map Kinase rabbit IgG (Cell Signaling Technology, Beverly, MA, USA), or anti-β actin rabbit IgG (Abcam, Cambridgeshire, UK). The membranes were then reacted with horseradish peroxidase-labeled anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK). Finally, the membrane was reacted with ECL Plus Western Blotting Detection Reagents (Amersham Biosciences), and the signal was detected using No. 667 Polaroid film.

SCI and cell transplantation. Adult female SD rats weighing 210–230 g were used for the SCI model. Under anesthesia, a dorsal lami-