DOCOSAHEXAENOIC ACID PROMOTES NEUROGENESIS IN VITRO AND IN VIVO

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Abstract—Docosahexaenoic acid (22:6n-3), one of the main structural lipids in the mammalian brain, plays crucial roles in the development and function of brain neurons. We examined the effect of docosahexaenoic acid on neuronal differentiation of neural stem cells in vitro and in vivo. Neural stem cells obtained from 15.5-day-old rat embryos were propagated as neurospheres and cultured under differential conditions with or without docosahexaenoic acid for 4 and 7 days. Docosahexaenoic acid significantly increased the number of Tuji-positive neurons compared with the control on both culture days, and the newborn neurons in the docosahexaenoic acid group were morphologically more mature than in the control. Docosahexaenoic acid significantly decreased the incorporation ratio of 5-bromo-2'-deoxyuridine, the mitotic division marker, during the first 24 h period; it also significantly decreased the number of pyknotic cells on day 7. Thus, docosahexaenoic acid promotes the differentiation of neural stem cells into neurons by promoting cell cycle exit and suppressing cell death. Furthermore, dietary administration of docosahexaenoic acid significantly increased the number of 5-bromo-2'-deoxyuridine(+)/NeuN(+) newborn neurons in the granule cell layer of the dentate gyrus in adult rats. These results demonstrate that docosahexaenoic acid effectively promotes neurogenesis both in vitro and in vivo, suggesting that it has the new property of modulating hippocampal function regulated by neurogenesis. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: docosahexaenoic acid, neural stem cells, neuronal differentiation, neurogenesis, adult rats.

Neural stem cells (NSCs) are defined as immature cells with self-renewing and multipotent abilities. In the mammalian brain, most neurons are generated by NSCs in the later embryonic period. In two specific brain regions, the dentate gyrus (DG) of the hippocampus and the olfactory bulb (OB), however, new neurons are generated by NSCs throughout life (Eriksson et al., 1998; Bedard and Parent, 2004). In the DG, newborn neurons behave like functional granule neurons with electrophysiologic properties (van Praag et al., 2002) and extend axonal projections to the hippocampal CA3 regions. Also, the levels of hippocampal neurogenesis correlate with the performance of memory tasks (Shors et al., 2001; Drapeau et al., 2003). These findings indicate that adult hippocampal neurogenesis is associated with the formation and function of memory and learning ability.

Docosahexaenoic acid (DHA), one of the predominant polyunsaturated fatty acids in the brain, is essential for normal neural development, and deficiency of DHA induces loss of discriminative learning ability (Green and Yavin, 1998). Chronic administration of DHA enhances long-term memory in young and aged rats (Gamoh et al., 1999, 2001), and dietary DHA has preventive and ameliorative effects on the impairment of spatial cognition learning ability in amyloid-β-infused rats (Hashimoto et al., 2002, 2005). These findings suggest that DHA plays crucial roles in the development and function of the CNS throughout life. Interestingly, a reduction in DHA concentrations in the brain impairs the performance of spatial learning tasks regulated by the OB (Greiner et al., 1999), where neurogenesis occurs in adulthood. These findings imply some relation between DHA and adult neurogenesis, although it is still unclear whether DHA affects the potential of NSCs for differentiating into neurons. This study examines the effect of DHA on neural differentiation.

EXPERIMENTAL PROCEDURES

Cell culture and differentiation into neural lineage

NSCs were cultured by the neurosphere method as described (Reynolds and Weiss, 1992). Rat embryos on embryonic day 15.5 (E15.5, with the morning of plug detection designated as E0.5) were dissected out into PBSG (0.6% glucose in PBS (→) containing 1 U/mL penicillin/streptomycin), and the telencephalons were isolated. Tissues were mechanically disrupted into single cells by repeated trituration in a serum-free conditioned medium (N2 medium) comprising DMEM/F12 1:1, 0.6% glucose, sodium bicarbonate, 5 mM HEPES, 100 μM human transferrin, 20 nM progesterone, 30 nM sodium selenite, 60 μM putrescine, and 25 μg/mL insulin. The dissociated cells were plated onto cell culture dishes at a density of 1×10⁵ cells/mL in N2 medium with basic fibroblast growth factor (bFGF; 20 ng/mL, R&D Systems, Minneapolis, MN, USA) and heparin. All experimental procedures were carried out in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, and the number of rats used and their suffering were minimized.

The differentiation into neurons was induced by bFGF withdrawal from the culture medium. Dispersed cells were plated at a density of 2×10⁵ cells/well onto poly-L-ornithine (15 μg/mL) -coated 24-well plates with or without DHA (10 μM) in a complex with 0.1% fatty acid-free bovine serum albumin and cultured for 4 and 7 days. The culture medium was changed on days 2 and 4.

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Differentiation was assayed with four independent cell lines obtained from four pregnant rats.

Immunohistochemistry and data analysis

For fluorescent staining, cells were fixed with 4% paraformaldehyde for 15 min and washed for 10 min three times with 0.1 M Tris-buffered saline, pH 7.5 (TBS). After blocking with 3% goat serum in TBS containing 0.3% Triton X-100 for 1 h, the cells were incubated with primary antibodies at 4 °C overnight. The primary antibodies used were mouse anti-Tuj1 (1:1000, R&D Systems), mouse anti-MAP2ab (1:1000, Chemicon, Temecula, CA, USA), mouse anti-nestin (1:2000, Chemicon), and rabbit anti-GFAP (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After washing in TBS, the FITC- or Cy5-conjugated secondary antibody (1:500, Southern Biotechnology, Birmingham, AL, USA and Chemicon) was added, and the cells were incubated for 1 h. To estimate their total number, the cells were treated with propidium iodide (PI; 2 μg/mL, Dojin Kagaku, Mashiki, Japan) for 30 min at room temperature. Fluorescent signals were then visualized by the confocal laser microscope system (CLSM FV300, Olympus, Tokyo, Japan) and processed by Adobe Photoshop (Adobe Systems, Mountain View, CA, USA) or NIH image version 1.63. The number of Tuj1 immunoreactive cells was counted in each of seven random fields per well. Data are indicated as the means ± S.E.M. Statistical significance was evaluated by Student’s t-test; a level of P<0.05 was considered significant.

Proliferation study

The proliferative activity of NSCs was investigated by treating cells with 10 μM of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO, USA), a thymidine analog incorporated into genetic material during the S phase of mitotic division, for 24 h with or without DHA. Cells were treated with 2 M HCl for 10 min at 37 °C to denature DNA and with 0.1 M borate buffer (NaB₃O₃, pH 8.5) for 5 min. Cells were treated with mouse anti-BrdU antibody (1:1000, Chemicon) then with FITC-conjugated secondary antibody. Seven random fields were captured in each well, and BrdU-incorporated cells were counted. The ratio of BrdU total cells was calculated, and statistical significance was evaluated by Student’s t-test.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL staining was carried out with Red In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer’s protocol. Cells cultured for 7 days under differential conditions with or without DHA were fixed with 4% paraformaldehyde, and incubated for 1 h at 37 °C with digoxigenin-dUTP in the presence of TdT. TUNEL labeled cells were visualized by Rhodamine-conjugated anti-digoxigenin antibody and observed under a confocal laser microscope.

DHA administration and BrdU injection into animals

Eighteen-month-old male Wistar rats fed a fish-oil deficient diet (F-1R; Funabashi Farm Co., Funabashi, Japan) over three generations were used in this study. To estimate the effects of DHA on hippocampal neurogenesis, the rats were orally fed ethyl-ester 4,7,10,13,16,19-docosahexaenoate (Harima Chemicals, Tokyo, Japan) emulsified in 5% gum arabic solution at 300 mg/kg/day; controls were fed a similar volume of only the 5% gum arabic solution. DHA or vehicle supplementation was continued for seven weeks. Two weeks after the start of DHA administration, the rats received one daily i.p. injection of 50 mg/kg BrdU dissolved in PBS (–) for 5 days.

Tissue preparation and immunohistochemistry

Four weeks after the last BrdU injection, the rats were perfused transcardially with PBS (–) and fixed with 4% paraformaldehyde. The brain was removed and post-fixed for 24 h in paraformaldehyde, and replaced by the 25% sucrose. Coronal sections (40 μm) were cut on a microtome and subjected to immunohistochemical analysis.

BrdU-labeled nuclei were visualized as described (Liu et al., 1998). Free-floating brain sections were treated with 50% formamide–2× SSC (0.3 M NaCl and 0.03 M sodium citrate) for 2 h at 65 °C and incubated for 30 min at 37 °C in 2 M HCl. The sections were treated with 0.1 M boric acid (pH 8.5) and incubated for 24 h in rat anti-BrdU antibody (Oxford Biotechnology Ltd., Oxfordshire, UK) with 3% goat serum in TBS containing 0.3% Triton X-100. After washing with TBS, Cy5-conjugated secondary antibodies were added, and the brain sections were incubated for 1 h at 25 °C. To estimate the number of newborn neurons, the brain sections were treated with mouse anti-NeuN antibody (Chemicon) then with FITC-conjugated secondary antibody. Finally, fluorescent signals were visualized under a confocal laser microscope as described above.

Stereoity

Every sixth section (240 μm apart) was selected throughout the rostrocaudal extent of the granule cell layer in the complete DG, and BrdU–NeuN-colocalized cells were counted in the granule cell layer and the subgranular zone (the inner region lining the granule cell layer). To avoid over- or undersampling of BrdU–NeuN-positive nuclei because of size differences, every sixth adjacent section was stained with 2 μg/mL PI. The reference volume of the granule cell layer was determined by summing the granule cell areas for each section multiplied by the distance between sections sampled. Total BrdU–NeuN positive cells were expressed as the means ± S.E.M for each hemisphere; statistical significance was analyzed by Student’s t-test. Differences of P<0.05 were considered significant.

RESULTS

Effects of DHA on neuronal differentiation of NSCs in vitro

Cells isolated from E15.5 embryos generated neurospheres in the presence of bFGF, and numerous cells were immunoreactive to nestin (data not shown). Under differential conditions, some of the cells were immunoreactive to neural markers Tuj1, MAP2ab or to mature astrocyte marker GFAP (data not shown) and consequently identified as NSCs.

To evaluate the effect of DHA on NSCs, the cells were cultured for 4 and 7 days with or without 10 μM DHA (Fig. 1). DHA is effective in a narrow range (i.e. 2–10 μM); higher concentrations induce neuronal cell death (Insua et al., 2003). On day 4, the ratio of Tuj1 (+) neurons/total cells increased 1.47-fold in the presence of DHA (Fig. 1C; control: 9.3±0.4%, n=5; DHA: 13.0±0.7%, n=5; *P=0.0004). Also on day 7, the ratio of Tuj1 (+)/total cells increased 1.46-fold in the presence of DHA (Fig. 1C; control: 11.5±0.8%, n=6; DHA: 16.8±0.9%, n=6; *P=0.0003). No difference in the total number of cells was observed between the control and the DHA-treated group on both culture days (day 4: control 2198±192, DHA: 2155±248 cells/well, P=0.950; day 7: control 2496±244, DHA 2450±248 cells/well, P=0.986).
Influence of DHA on the proliferative activity of NSCs in vitro

BrdU was added to the NSC culture during the first 24 h of the differentiation period, and BrdU incorporated into the cells was visualized immunohistochemically. The number of BrdU-immunoreactive cells decreased significantly in the presence of DHA (Fig. 2 A; control: 23.6 ± 2.2%, n = 6; DHA: 16.3 ± 1.0%, n = 6; P < 0.02). No difference in the number of total cells was observed between the control and the DHA-treated group (control: 1807 ± 159, DHA: 1640 ± 86 cells/well, P = 0.125), indicating that DHA inhibits the proliferation of NSCs in the early stages of differentiation.

The preventive effects of DHA on cell death in vitro

The nuclei of differentiated NSCs were stained with PI, and the pyknotic cells were quantified. The pyknotic cells

Fig. 1. (A) Confocal images of Tuj1 immunostaining in control and DHA groups on day 7, Tuj1 (green), PI (red). Scale bar = 100 μm. (B) Quantification of Tuj1 immunoreactive cells in control and DHA groups. Data are shown as the means ± S.E.M. obtained from five to six independent cultures. Seven random fields were counted in each culture. **P < 0.0005.

Fig. 2. (A) Quantification of BrdU immunoreactive cells in control and DHA groups during a first 24 h period. Data are shown as the means ± S.E.M. obtained from six independent cultures. Seven random fields were counted in each culture. **P < 0.05. (B) Quantitative analysis of pyknotic cells in control and DHA groups on day 7. Data are shown as the means ± S.E.M. obtained from six independent cultures. Seven random fields were counted in each culture **P < 0.01.
showed characteristic features of dense nuclei and shrunken cell-bodies, whereas the surviving cells had large cell-bodies, non-fragmented and weak PI-stained images (data not shown). Stained by TUNEL, the labeling of specifically the 3’-hydroxyl terminal of DNA strands broken by apoptosis, pyknotic cells was strongly positive for TUNEL, whereas the cells with unshrunken cell-bodies were negative (data not shown). Thus, cells with pyknotic PI images were regarded as apoptotic.

The quantitative analysis of pyknotic cells revealed that DHA supplementation significantly decreased the ratio of pyknotic cells/total cells on day 7 (Fig. 2B; control: 60.0±3.2%, n=6; DHA: 54.3±3.7%, n=6; P=0.01). No difference in the number of total cells was observed between the control and the DHA-treated group (control: 2496±244, DHA: 2500±248 cells/well, P=0.996), indicating that DHA prevents cell death of differentiating NSCs.

Effects of DHA on neurite growth of neurons differentiated from NSCs in vitro

To examine whether DHA accelerates the maturation of neurons differentiated from NSCs, we compared the morphological appearance of neurons between the control and the DHA group on day 7. In the control, most neurons had bipolar neurites and few branches characteristic of immature neurons (Fig. 3A, arrows), whereas in the DHA group, each neuron had characteristic features of more mature and longer neurons, multipolar neurites and more branches (Fig. 3B, arrows), suggesting that DHA accelerates neurite growth and promotes the maturation of neurons differentiated from NSCs.

Effects of DHA on hippocampal neurogenesis in adult rats

To examine whether neurogenesis in the DG of adult rats can be activated by DHA administration, the dividing cells were labeled with BrdU in the DG, and the newborn neurons were visualized by using specific antibodies of BrdU and neuron-specific marker NeuN as described (Liu et al., 1998; Cameron and Mackay, 1999).

Four weeks after the last BrdU injection, most BrdU-labeled cells distributed throughout the entire granule cell layer and their nuclei were morphologically large and round, characteristics of mature granule neurons (Kuhn et al., 1996). Furthermore, most BrdU (+) cells also expressed NeuN (Fig. 4A, B and C) in the control and the DHA groups, suggesting that most of the cells that incorporated BrdU in the granule cell layer differentiated into mature neurons, which is in agreement with previous reports (van Praag et al., 1999; Yagita et al., 2001).

The number of newborn neurons (BrdU/NeuN double immunoreactive cells) in the entire granule cell layer of DG increased significantly, 1.6-fold in DHA-administrated rats (Fig. 4D-a; control: 538±60.2, DHA: 878±72.3, six hemispheres from three animals, P=0.005). This difference was not due to the difference in the size of the granule cell layer, especially that the reference volume of the analyzed area did not differ between the control and the DHA groups (Fig. 4D-b; control: 0.919±0.0515 mm³, DHA: 0.920±0.0547 mm³, P=0.993), suggesting that the dietary administration of DHA promotes hippocampal neurogenesis in adult rats.

DISCUSSION

NSCs are identified in many regions of the embryonic CNS (cerebral cortex, hippocampus, spinal cord, and cerebellum; Temple 2001), where they generate the neural and glial cell lineage during the developmental period. The levels of DHA in the rat brain increase in the period between E14 and E17, which coincides with the peak of neurogenesis (Green et al., 1999). Therefore, NSCs isolated from E15.5 rats, used in this study, are fitting for examining the effects of DHA on developmental neurogenesis in vitro. DHA supplementation significantly increased the number of TuJ1 immunoreactive cells in vitro (Fig. 1); the increase was not due to any difference in the neural progenitors of the control and DHA groups, especially that most NSCs, isolated and purified from the E15.5 rat embryonic brain, expressed nestin (>90%, data not shown), but few expressed TuJ1 (<1%, data not shown).

Several mechanisms may be involved in the induction of neural differentiation by DHA. One suggests that DHA promotes the proliferative activity of neural stem/progenitor cells and increases the number of newborn neurons. To
elucidate this possibility, our examination of proliferative activity of NSCs in the presence of DHA revealed that DHA significantly decreased BrdU incorporation into NSCs during the first 24 h (Fig. 2A), which is consistent with a report demonstrating that DHA promotes cell cycle exit and decreases the expression of nestin in retinal neural progenitor cells (Insua et al., 2003); it also reduces mitosis in tumor cells by inhibiting S phase entry (Chen and Iistfan, 2001). Thus, the negative effect that DHA exerts on the proliferative activity of cells is not likely to increase the number of NSCs/neural progenitor cells. In general, however, NSCs proceed to mitotic division during the undifferentiated phase; when they transit into the differentiation phase, they stop dividing and begin differentiating into neurons. Therefore, DHA may promote the cell cycle exit of NSCs and, consequently, accelerate their transition from the undifferentiated to the differentiated phase into neurons.

In this study, DHA significantly decreased the number of pyknotic cells in the culture of differentiated NSCs on day 7 (Fig. 2B). Chronic administration of DHA decreases the levels of lipid peroxide in the cerebral cortex and hippocampus (Gamoh et al., 1999; Hashimoto et al., 2002, 2005), and prevents an increase in neuronal apoptotic products induced by the infusion of amyloid-β into the rat cerebral ventricle (Hashimoto et al., 2002). Membrane lipid peroxidation increases the production of 4-hydroxy-2,3-nonenal, which induces apoptotic cell death in cortical
neurons through caspase activation (Camandola et al., 2000). DHA also prevents the apoptosis of retinal photoreceptors by regulating pro- and anti-apoptotic molecules Bax and Bcl-2 (Rotstein et al., 2003). Furthermore, DHA activates Akt signaling, which inactivates pro-apoptotic factors such as BAD, caspase 9, FKHR1 and blocks cell death induced by these mediators (Namikawa et al., 2000; Akbar et al., 2005). Thus, DHA may prevent apoptotic cell death and increase the survivability of newborn neurons.

Interestingly, the neurons in the DHA group demonstrated more mature morphology than the controls (Fig. 3). Several studies have described the beneficial effects of DHA on neural maturation: It stimulates phospholipid synthesis and promotes neurite growth induced by NGF in PC12 cells (Ikemoto et al., 1997, 1999); it also promotes neurite growth in the culture of embryonic hippocampal neurons in vitro, and reduced DHA in the embryonic hippocampus in vivo inhibits neurite growth in the same culture (Calderon and Kim, 2004). Thus, DHA may enhance neurite growth of newborn neurons differentiated from NSCs, suggesting that it plays important roles in the developmental neurogenesis of CNS.

NSCs in the adult hippocampus including those in rat and humans generate new neurons in adulthood (Schinder and Gage, 2004). The above results raise the question: Does DHA promote hippocampal neurogenesis in adult animals? To elucidate this issue, we administered dietary DHA to adult rats and examined the effect on active hippocampal neurogenesis. Indeed, dietary administration of DHA significantly increased the number of newborn neurons (Fig. 4D), demonstrating that DHA promotes neurogenesis not only in cultured embryonic NSCs but also in the hippocampus of adult rats, and suggesting that DHA also modulates the generation of new neurons in the adult brain.

The physiological function of adult hippocampal neurogenesis remains unclear. Recent growing evidence strongly suggests, however, that newborn neurons participate in the formation of learning and memory (Schinder and Gage, 2004). Newly generated neurons are functionally integrated into hippocampal circuits and can survive for several months (Song et al., 2002). They have the unique membrane properties that facilitate synaptic plasticity, and it is suggested that their increase causes the enhancement in long-term potentiation (LTP) (Schmidt-Hieber et al., 2004; Schinder and Gage, 2004). The mouse housed with a running wheel demonstrates increases of neurogenesis in the hippocampus, improved learning ability in the Morris water maze and selectively enhanced LTP in the DG (van Praag et al., 1999). Moreover, a significant relation between the number of newborn neurons and cognitive learning performance in the Morris water maze task has been demonstrated (Drapeau et al., 2003; Kempermann et al., 1997). Conversely, blocking NSCs proliferation in the hippocampus with antimitosis drugs or by X-ray irradiation impairs hippocampal-dependent learning tasks (Shors et al., 2001; Madsen et al., 2003). Based on these evidences, it is highly probable that the endogenous neurogenesis modulates learning and memory functions.

DHA is crucial for inducing of LTP in the CA1 region of rat hippocampus slices and for enhancing the potassium chloride-evoked release of acetylcholine in the rat hippocampus (Fujita et al., 2001; Aid et al., 2005). Moreover, dietary administration of DHA increases the levels of DHA in the hippocampus and improves learning and memory performance in the eight-arm radial maze task (Gamoh et al., 1999, 2001). In addition to these findings, the data in the present study suggest that DHA increases newborn neurons and imparts beneficial properties to the function of learning and memory. We therefore hypothesized that DHA modulates both the generation of new functional neurons and the already existing neural function, as well as improves the hippocampal function of learning and behavior.

If endogenous NSCs could be activated by specific manipulations, they may contribute to the generation of new neurons and repair injured CNS function. The beneficial effects of DHA on transient forebrain ischemia and Alzheimer’s disease (Okada et al., 1996; Calon et al., 2004; Hashimoto et al., 2005) focus mainly on the protective effect of DHA on cell-death damage in existing neurons and on accelerating their function. In addition to these, the present study proposes that DHA has a new property that offers an appropriate neurogenic environment and helps in the recovery of injured CNS function. To restore injured CNS function, newborn neurons need to be integrated into existing networks in order to establish the new connections and synaptic plasticity, and to ameliorate the pathological conditions at all levels in the whole animal (Schinder and Gage, 2004). Although, whether DHA induces new neurons to meet these criteria remains unclear in this study, further investigations aimed at clarifying these issues would provide insights into the significance of DHA in neuronal replacement therapy.

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