学 位 論 文 の 要 旨

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学　位　論　文　名	*Cis*-9, *trans*-11-Conjugated Linoleic Acid Promotes Neuronal Differentiation Through Regulation of Hes6 mRNA and Cell Cycle in Cultured Neural Stem Cells
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論 文 内 容 の 要 旨

INTRODUCTION

Conjugated linoleic acids, a group of polyunsaturated fatty acids (PUFAs), are positional and geometrical isomers of linoleic acid (LA). *Cis*-9, *trans*-11-conjugated linoleic acid (CLA) is produced directly by bacterial hydrogenation of LA in ruminants or by Δ9 desaturation of vaccenic acid in most mammalian tissues. CLA is the primary isomer naturally present in milk, dairy and ruminant meat products. Since CLA is actively incorporated into the brain and metabolized, suggesting some biological effects on the central nervous system, we hypothesized that CLA also has beneficial effect on the brain.

Neural stem cells (NSCs) are self-renewing and multipotent progenitor cells that differentiate into neuronal or glial cells. Since the stages of neural development proceed in the order of proliferation of NSCs, neuronal differentiation, and glial differentiation and these stages are maintained in cultured NSCs, we used cultured NSCs to investigate the effects of CLA on neural development *in vitro*. Basic helix-loop-helix (bHLH) transcription factors play important roles in the regulation of proliferation and differentiation of NSCs. Neuronal differentiation is promoted by activator-type bHLH factors such as Mash1 and NeuroD, whereas repressor-type bHLH factors such as Hes1, inhibit neuronal differentiation and promote proliferation. The balance of activity among these factors is thought to determine cell fate.

Regulation of the cell cycle affects the proliferation, differentiation, and apoptosis of NSCs. Cell differentiation is usually accompanied by irreversible cell cycle exit that is arrested at the G1/S-phase and enters the G0-phase without passing the G1 to S-phase restriction point. G1 regulatory molecules such as $p21^{cip1}$ and $p27^{kip1}$, have been shown as exquisitely regulated

during the differentiation process.

Here, we investigated whether LA and CLA affect neuronal differentiation and modulate the expression level of bHLH transcription factors, cyclin-dependent kinase inhibitors, and the cell cycle in NSCs.

MATERIALS AND METHODS

NSCs were cultured by the neurosphere method. Forebrain isolated from 14.5-day embryonic rats were mechanically disrupted into single cells by repeated pipetting in a serum-free conditioned medium (N2 medium) with basic fibroblast growth factor (bFGF) and heparin. Three to five days after incubation NSCs were grown as neurospheres. Neurospheres were collected and dissociated to single cells, which were then used for experiments.

For differentiation, the neurospheres (passage 2) were mechanically dissociated and seeded onto poly-*L*-ornithine (15 μg/mL)-coated 24-well plates at a density of 2×10^5 cells/well in N2 medium without bFGF or heparin. The cultures were then treated with LA (1.0 and 10 μM) or CLA (0, 0.001, 0.01, 0.1, 1.0, and 10 μM) and dissolved in N2 medium containing 1.0% fatty acid-free bovine serum albumin (BSA). 0.01% BSA was used as the control, and the culture medium was changed every other day.

Cultured cells were fixed with 4% paraformaldehyde, blocked with 3% normal goat serum in TBS containing 0.3% Triton X-100 and incubated with mouse anti-neuron-specific class III beta-tubulin (Tuj-1) antibody. The cells were incubated with Alexa Fluor 488-conjugated secondary antibody and the cells were counterstained with propidium iodide. Visualized under a fluorescent laser microscope (CLMS FV300) and processed using Image J software. The number of Tuj-1-positive cells and of total cells was counted in each of seven random fields per well.

The NSCs were allowed to differentiate for 6, 12, 24, and 96 h in differentiation medium in the presence of LA or CLA. Total RNA was isolated, cDNA was synthesized and amplified by the ABI prism 7000 sequence detection system with gene specific primer sets for Hes1 and 6, Mash1, NeuroD, MAP2, $p21^{cip1}$ and $p27^{kip1}$.

After incubating the cells for 11 h, 5-bromo-2'-deoxyuridine (BrdU, 10 μM) was added to the culture medium and the cells were allowed to incubate for another 1 h. Cell cycle was analyzed with a BrdU Flow Kit using Becton Dickinson FACS Calibur cytometer. Samples were acquired and analyzed with the use of CELLQuest 3.3 software, and the percentage of cells in G0/G1-, S-, and G2/M-phases was determined.

RESULTS AND DISCUSSION

To assess the effect of CLA on the neuronal differentiation of NSCs, NSCs were treated with different dose of CLA or 0.01% BSA as control for 7 days and number of Tuj-1-positive

cells (neurons) was counted. CLA dose-dependently increased the percentage of Tuj-1-positive cells by a maximum of 18.3% at 1.0 μM. These results suggest that CLA induces neuronal differentiation of NSCs. To confirm that this result is specifically observed by CLA, NSCs were treated with LA, structural analogue of CLA, and counted Tuj-1 positive cells. The number of Tuj-1 positive cells in LA treatment (1.0 and 10 μM) was not different from that of the control, whereas cells treated with CLA (1.0 and 10 μM) increased significantly by 134.5% and 121.7%, respectively, compared with those of the control. These data indicate that CLA, but not LA, specifically enhances neuronal differentiation of NSCs.

To assess the mechanisms of CLA-induced neuronal differentiation of NSCs, mRNA expression of bHLH transcription factors were measured. Compared with the control, a 96 h-treatment of NSCs with CLA significantly increased the mRNA expression level of Mash1, an activator-type bHLH transcription factor. Similarly, a 24 h-treatment significantly increased the expression level of Hes6, an activator-type bHLH transcription factor, and MAP2, neuron specific gene, by 50% and 80%, respectively. In contrast, the mRNA expression level of NeuroD, an activator-type bHLH transcription factor, and Hes1, a repressor-type bHLH transcription factor, demonstrated no significant difference. Treatment with LA did not affect the mRNA expression level of any of the bHLH transcription factors. It has been reported that Hes6 suppressed Hes1 from inhibiting Mash1-E47 heterodimer and Hes6 enables Mash1 and E47 to up-regulate transcription such as MAP2 in the presence of Hes1. These results suggest that CLA may inhibit Hes1 action by increasing the expression level of Hes6 and stimulate neuronal differentiation by altering the balance of bHLH transcription factors.

Since Hes1 directly regulates cell cycle through $p21^{cip1}$ and $p27^{kip1}$ down-regulation and cell cycle affects the differentiation of NSCs, $p21^{cip1}$ and $p27^{kip1}$ mRNA levels and cell cycle were measured. Treatment with CLA increased the mRNA expression levels of $p21^{cip1}$ and $p27^{kip1}$, while treatment with LA had no effect. Treatment with CLA significantly increased the percentage of G0/G1-phase and reduced that of S-phase of NSCs. On the other hand, treatment with LA did not affect cell cycle distribution in NSCs compared with the control. Treatment with CLA increased $p21^{cip1}$ and $p27^{kip1}$ mRNA levels, while Hes1 mRNA expression level did not affect, indicating that CLA inhibits Hes1 action by increasing Hes6 mRNA level.

CONCLUSION

CLA increases the expression of Hes6 mRNA and promotes neuronal differentiation by activating $p21^{cip1}$ and $p27^{kip1}$ to arrest cell cycle. Therefore, CLA is speculated to control cell fate and to be useful in regenerative therapies for neurodegenerative diseases such as Alzheimer's and Parkinson's disease, particularly by controlling differentiation of NSCs pre- and post-transplantation.