Effects of High Cholesterol Diet on Newly Generated Cells in the Dentate Gyrus of C57BL/6N and C3H/HeN Mice

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ABSTRACT. In this study, we observed and compared the effects of a high cholesterol diet (HCD) on cell proliferation and differentiation in the subgranular zone of the dentate gyrus of C57BL/6N (B6, susceptible strain) and C3H/HeN (C3H, resistant strain) mice. Ki67 (a marker for cell proliferation) positive cells were significantly decreased in HCD-fed B6 mice compared to those in B6 (49.7%) and C3H mice fed a low cholesterol diet (LCD). In addition, doublecortin (DCX, a marker for cell differentiation or neuroblasts)-immunoreactive cells in HCD-fed B6 mice were significantly decreased compared to those in LCD-fed B6 and C3H mice. These results suggest that B6 strains are sensitive to HCD, which impairs cell proliferation and differentiation.

KEY WORDS: C3H/HeN, C57BL/6N, doublecortin, high cholesterol diet, Ki67.

Cholesterol is a lipid found in cell membranes, and about 25% of unesterified cholesterol is concentrated in the central nervous system (CNS) [6]. In this regard, unesterified cholesterol must be supplied to neurons by de novo synthesis within neurons or by specific ligands and membrane transporters from the extracellular environment [26]. Changes in cholesterol homeostasis directly or indirectly alter sterol balance across the CNS and contribute to the development of neurodegeneration [21, 35, 36].

The hippocampus, which is involved in memory formation, is one of the brain regions most vulnerable to Alzheimer’s disease. Granule cells are capable of replacing themselves throughout adulthood by neurogenesis from neural progenitors located in the subgranular zone of the hippocampal dentate gyrus [11, 14] and likely contribute to the normal hippocampal function [15, 33]. In this region, actively dividing cells are easily detected by Ki67 and doublecortin (DCX), which is an endogenous marker for proliferation expressed during the late G1, S, M, and G2 phases of the cell cycle [5] and for neuroblasts [2, 9, 13], respectively.

Although high calorie diets impair hippocampal synaptic plasticity and neurogenesis in mice [8, 20], no study has examined the effects of high cholesterol diets (HCD) on cell proliferation and differentiation in C57BL/6N (B6) and C3H/HeN (C3H) mice. In this study, we chose these two strains because lipopolysaccharide (LPS) increased the activity of hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, in B6 strain, not C3H strain [1] and finally B6 and C3H mice are sensitive and resistant to diet-induced hypercholesterolemia, respectively [19, 22]. Therefore, in this study, we investigated the effects of HCD on cell proliferation and neuroblasts using Ki67 and DCX, respectively.

MATERIALS AND METHODS

Experimental animals and diets: Ten female male B6 and C3H mice (both 8-week-old) were purchased from Orient Bio (South Korea). They were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and free access to food and water. The animals were adapted to a chow diet for 1 week, and animals were fed commercially available HCD (D12336, Research Diets, NJ, U.S.A.) and low cholesterol diet (LCD) (D12337, Research Diets) with the same calorie for 12 weeks. The procedures for handling and caring for the animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, revised 1996). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Tissue processing for histology: For the histological analysis, 5 animals at each group were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 hr. The brain tissues were cryoprotected by infiltration.
with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

**Immunohistochemistry for Ki67 and DCX:** Immunohistochemistry was performed under the same conditions in each group in order to examine whether the degree of immunohistochemical staining was the accurate. The sections were sequentially treated with 0.3% hydrogen peroxide (H$_2$O$_2$) in PBS for 30 min and 10% normal goat or rabbit serum in 0.05 M PBS for 30 min. They were then incubated with diluted rabbit anti-Ki67 antibody (1:1,000, Abcam, Cambridge, UK) or goat anti-DCX antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit or rabbit anti-goat IgG and streptavidin peroxidase complex (diluted 1:200, Vector, Burlingame, CA). They were then visualized by staining with 3,3′-diaminobenzidine in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The sections were mounted in Canada Balsam (Kanto, Tokyo, Japan) following dehydration. A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in any structures.

**Quantification of data and statistical analysis:** All measurements were performed in order to ensure objectivity in blind conditions, by 2 observers for each experiment, carrying out the measures of experimental samples under the same conditions.

To elucidate the effects of HCD on cell proliferation and differentiation in mice, the number of Ki67 and DCX positive cells in all groups was counted in the dentate gyrus in 15 sections/each animal using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). The studied tissue sections were selected according to anatomical landmarks corresponding to Bregma –1.46 ~ –2.46 mm of mouse brain atlas [10]. Cell counts were obtained by averaging the counts from the sections taken from each animal: A ratio of the count was calibrated as %. In addition, dendritic complexity of DCX positive cells was analyzed using the accompanying software (NeuroExplore, MicroBrightField, Inc., VT) calculating complexity including dendritic length and number of branches.

**Statistical analysis:** Data are expressed as the mean ± SEM. The data were elevated by one-way ANOVA SPSS program and the means assessed using Duncan’s multiple-range test. Statistical significance was considered at $P<0.05$.

**RESULTS**

**Effects of HCD on cell proliferation:** In all groups, Ki67-immunoreactive nuclei were detected in the subgranular zone of the dentate gyrus and the pattern of Ki67 staining was similar between groups (Fig. 1A-1D). However, there were significant differences in the number of Ki67-immunoreactive cells between groups (Fig. 1E). In the HCD-fed B6 mice, Ki67-immunoreactive cells were significantly decreased by 49.7% compared to those in the LCD-fed mice (Fig. 1A, 1B and 1E). In the LCD-fed C3H mice, Ki67-immunoreactive nuclei were slightly decreased compared to those in the LCD-fed B6 mice. However, in the HCD-fed B6 mice, the number of Ki67-immunoreactive nuclei was significantly decreased by 79% and 64.2% compared to those in the LCD-fed C3H mice and B6 mice (Fig. 1C, 1D and 1E). In the HCD-fed C3H mice, the number of Ki67-immunoreactive nuclei was abundant than in the B6-HCD group (Fig. 1E).

**Effects of HCD on cell differentiation:** Like Ki67-immunoreactive cells, DCX-immunoreactive cells were mainly detected in the subgranular zone in the dentate gyrus in all groups (Figs. 2–4). However, there were significant differences in the number of DCX-immunoreactive cells and fibers between groups (Fig. 2A-2D). In both the LCD-fed B6 and C3H mice, DCX-immunoreactive fibers extended into two-thirds of the molecular layer of the dentate gyrus (Figs. 2A, 2C, 3A and 3C). In the HCD-fed B6 mice, the number of DCX-immunoreactive cells was significantly decreased in the subgranular zone of the dentate gyrus by 72.2% compared to those in the LCD-fed B6 mice (Fig. 4). Especially, the number of DCX-positive cells with tertiary dendrites in the HCD-fed B6 mice was prominently decreased compared to that in the LCD-fed B6 mice (Figs. 2B, 3B and 4). In the LCD-fed C3H mice, the number of DCX-positive cells with or without tertiary dendrites was slightly abundant compared to that in the LCD-fed B6 mice (Figs. 2C, 3C and 4). In the HCD-fed C3H mice, the number of DCX-immunoreactive cells with tertiary dendrites was significantly decreased in the subgranular zone compared to that in the other groups. However, the number of DCX-positive cells without tertiary dendrites was most abundant in this group (Figs. 2D, 3D and 4).

**DISCUSSION**

A link between abnormal cholesterol metabolism and Alzheimer’s disease pathogenesis has been suggested [3, 27, 31]. We observed the effects of HCD for 12 weeks on cell proliferation and differentiation in B6 and C3H mice. In this study, we used B6 mice as susceptible strain and C3H mice as resistant strain to atherogenic diets. In our previous study, levels of total cholesterol and triglyceride in the B6 mice were much lower than those in the C3H mice when they were fed atherogenic diets [24]. In addition, there was no increase of high density lipoprotein cholesterol (HDLc) in B6 ApoE$^{-/-}$ strain, while in C3H ApoE$^{-/-}$ strain HDLc increased significantly in the C3H ApoE$^{-/-}$ strain [23, 24, 32]. This change in HDLc is suggested as a contributing factor for resistance of C3H strain to diet-induced atherosclerosis [25].

In this study, we used Ki67 as a marker for cell prolifera-
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The total number of Ki67-positive cells reflects cellular proliferation in a manner consistent with BrdU labeling and both methods quantitatively yield similar measures of ischemia- and radiation-induced changes in cellular proliferation [18].

In this study, the number of Ki67- and DCX-immunoreactive cells was significantly decreased in the HCD-fed B6 mice compared to that in the LCD-fed B6, C3H and HCD-fed C3H mice. This result suggests that HCD may induce the reduction of cell proliferation and differentiation in the subgranular zone of the dentate gyrus in B6 mice. In this study, we also observed that the number of DCX-positive cells with tertiary dendrites was significantly decreased in the HCD-fed C3H mice compared to that in the LCD-fed B6, C3H and HCD-fed B6 mice. However, the number of DCX-positive cells without tertiary dendrites was significantly increased in the HCD-fed C3H mice. It has been reported that neurons with horizontally orientated or basal dendrites show an immature feature of granule cells in rodents [30], while neurons with vertically orientated dendrites are relatively mature neurons among the population of DCX+ neurons in the DG [29]. Considering this, the significant reduction of dendrites in the DCX positive neuroblasts were suppressed by HCD although C3H mice are relatively resistant to HCD compared to other strains. It has been reported that knockdown of DCX via RNAi transfection reduces branch points, total length and complexity of dendrites. In contrast, overexpression of DCX resulted in an increase in branch points and complexity of dendrites [4].

Hypercholesterolemia can damage endothelial cells of arteries and capillary vessels, decrease blood flow, impair metabolism, and decrease nutritive and oxygen levels in the...

Fig. 1. The photomicrograph of Ki67 immunohistochemistry in the dentate gyrus of LCD-fed (A and C) and HCD-fed (B and D) B6 (A and B) and C3H mice (C and D) after 12 weeks. Note that Ki67-immunoreactive cells are fewer in HCD-fed B6 mice than in other mice. Bar=100 μm. E. Relative number of Ki67-immunoreactive cells in LCD-fed B6 mice. Differences between means are analyzed by one-way analysis of variance followed by the Tukey test (n=5 per group; \( p < 0.05 \), significantly different from LCD-fed B6 mice, \( b \) \( p < 0.05 \), significantly different from HCD-fed B6 mice, \( c \) \( p < 0.05 \), significantly different from LCD-fed C3H mice). Bars indicate means ± SEM.
In addition, disturbances in cholesterol homeostasis may contribute to the etiology of Alzheimer’s disease by promoting Aβ generation [12]. However, there are also some conflicting data on the relationship between the level of cholesterol circulating in the plasma and the development of CNS disorders [7, 34].

Fig. 2. Low magnification of DCX immunohistochemistry in the dentate gyrus of LCD-fed (A and C) and HCD-fed (B and D) B6 (A and B) and C3H mice (C and D) after 12 weeks. DCX-immunoreactive cells are detected in the subgranular zone of the dentate gyrus. Note that DCX-immunoreactive cells are significantly lower in HCD-fed B6 mice than in other mice. Bar=100 μm.

Fig. 3. High photomicrograph of DCX-immunoreactive cells in the dentate gyrus in LCD-fed (A and C) and HCD-fed (B and D) B6 (A and B) and C3H (C and D) mice after 12 weeks. Note that DCX-immunoreactive fibers are abundant in all mice (arrows) except HCD-fed C3H mice. Bar=25 μm.
The reduction of cell proliferation and differentiation is not associated with atherosclerotic lesions in the carotid arteries or hepatic fibrosis because atherosclerotic lesions do not develop in the carotid arteries even when animals are fed a HCD containing high fat/cholesterol and cholate (the Paigen diet) for up to 1 year [17, 23, 28]. In addition, we used equal calorie diets (HCD and LCD) to rule out high calorie-induced impairment of hippocampal functions as high calorie diets have been shown to impair hippocampal synaptic plasticity and neurogenesis [8, 20].

In conclusion, HCD significantly reduced the absolute number of Ki67- and DCX-immunoreactive cells in B6 mice compared to that in C3H mice. In addition, HCD in C3H mice also reduced the development of dendrites in neuroblasts.

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REFERENCES


