Biochemical and Biophysical Research Communications **256,** 198–203 (1999)
Article ID bbrc.1998.9497, available online at http://www.idealibrary.com on IDEAL®

Cholestanol Induces Apoptosis of Cerebellar Neuronal Cells

Kenji Inoue, Shunichiro Kubota, and Yousuke Seyama[1]
Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Received September 14, 1998

Cerebrotendinous xanthomatosis (CTX) is a hereditary lipid storage disease characterized by hypercholestanolemia, cerebellar ataxia, xanthoma, and cataract. We hypothesized that cholestanol in serum of CTX patients might induce neuronal cell death in the cerebellum and eventually lead to cerebellar ataxia. To gain support for this hypothesis we developed hyper-cholestanolemia rats by feeding cholestanol. Neuronal cells, especially Purkinje cells in the cerebellum were stained by Sudan black B only in the cholestanol-fed rats, indicating the deposit of cholestanol in cerebellum. To examine effects of cholestanol *in vitro,* cerebellar neuronal cells were cultured with cholestanol. The cholestanol concentration increased and the viability decreased in cells cultured with cholestanol. Apoptosis was evident in cells cultured with cholestanol more frequently than in control cells, determined using the terminal deoxynucleotidyl transferase (TdT) dUTP nick end-labeling (TUNEL) method. As activities of interleukin-1β-converting enzyme (ICE) and CPP32 protease were increased in cells cultured with cholestanol, all these data taken together suggest that cholestanol induced apoptosis of cerebellar neuronal cells. Our observations may explain the mechanism of cerebellar ataxia of CTX patients.

Cerebrotendinous xanthomatosis (CTX) is a hereditary lipid storage disease characterized by hypercholestanolemia, Achilles tendon xanthomas, cerebellar ataxia, dementia, atherosclerosis and juvenile cataract (1–3). CTX is an autosomal recessive disease caused by mutations in the sterol 27-hydroxylase gene (4–7). In 1968, Menkes *et al.* discovered an accumulation of cholestanol, which resembled cholesterol in structure, in the cerebrum and cerebellum of CTX patients (8). Cholestanol is a saturated sterol and differs from cholesterol by replacement of the 5 and 6 double bond with hydrogen atoms. The amount of cholestanol in human serum is much lower than that of cholesterol. Normally, the major part of cholestanol is synthesized in the liver directly from cholesterol with 4-cholesten-3-one as an intermediate (9–12). Philippart and van Bogaert reported abnormal elevation of the concentration of cholestanol in serum, tendon xanthomas and cerebellum of CTX patients (13). In contrast, the concentration of cholesterol in serum of CTX patients was usually normal or even lower than that in healthy subjects.

In our previous study we observed increased cholestanol levels in cerebellum of cholestanol-fed mice (14), a finding which led to the hypothesis that cholestanol in serum of CTX patients might induce neuronal cell death in cerebellum and eventually cerebellar ataxia would occur. In the present study, we developed hyper-cholestanolemia rats and examined the effects of cholestanol on death of cerebellar neuronal cells.

MATERIALS AND METHODS

Animals and diet. Thirty male Wistar rats (5-week-old) weighing from 90 to 110 g were obtained from Nippon SLC Inc. (Hamamatsu, Japan), and were fed 3 different diets (standard diet, 1% cholesterol diet, 1% cholestanol diet) for 15 weeks. The standard rats diet (CE-2) contained 24.8% protein, 4.4% fat, 3.5% fiber, all necessary vitamins and minerals at recommended levels, 0.018% (w/w) cholesterol and trace amounts (below 0.003%) of cholestanol. These diets were prepared by Clea Japan Inc. (Tokyo, Japan). The rats were provided diets *ad libitum* for 15 weeks. Body weight was monitored every week.

Collection of blood and body tissues. Rats were anesthetized with ether, and blood samples were withdrawn from the carotid artery, and was centrifuged at 2,000 g for 10 min to obtain the serum. The cerebellum, liver and lens were excised and weighed. Aqueous humor was aspirated from the anterior chamber of each eye. Serum, cerebellum, liver, lens, and aqueous humor samples were stored at −80°C until analysis.

[1] Corresponding author. Fax: 81-3-5689-2704. E-mail: yousuke@m.u-tokyo.ac.jp.

Abbreviations used: CTX, cerebrotendinous xanthomatosis; HPLC, high-performance liquid chromatography; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end-labeling; ICE, interleukin-1β-converting enzyme.

0006-291X/99 $30.00

Biochemical analysis. Cholesterol (5-cholesten-3β-ol), cholestanol (5α-cholestan-3β-ol) and epicoprostanol (5β-cholestan-3α-ol) as an internal standard were purchased from Sigma Chemical (St. Louis, MO). All other chemicals and solvents used were the highest grade. Sample preparation and analysis of sterols by high performance liquid chromatography (HPLC) were performed as described (15).

Histopathology. The eyes, livers and cerebellums of affected and control rats were sectioned at 4 μm and stained with hematoxylin and eosin (HE) and Sudan black B solution. These samples were examined by light microscopy. Stainings specific for cholesterol were performed according to the methods described by Schultz (16) or Adams (17).

Cell culture and treatments. Dissociated cerebellar neuronal culture was prepared from brains of 20-21-day-old fetuses of Wistar rats as described (18, 19), but with slight modification. Cerebellums were incubated at 37°C for 9 min in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 0.25% trypsin and were triturated in Ca^{2+}/Mg^{2+}-free HBSS containing 0.05% DNase I and 12 mM $MgSO_4$ with a pasteur pipette. The cells collected by centrifugation were suspended in Dulbecco's Modified Eagle's medium (DMEM)/F-12 (1:1) (GIBCO BRL, Grand Island, NY) containing 10% fetal calf serum and grown on poly-L-ornithine (100 μg/ml)-coated dishes at a density of 0.5-1.0 $\times$ 10^7 cells/ml in a 5% CO_2-humidified incubator. The serum-free culture medium was added to each dish after 3 h. The serum-free medium was composed of DMEM/F-12 supplemented with bovine insulin (20 μg/ml), bovine serum albumin (100 μg/ml), penicillin G (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), glutamine (200 μg/ml), human apotransferrin (200 μg/ml), progesterone (40 nM), putrescine (100 μM), sodium selenite (30 nM), triiodothyronine (0.5 ng/ml), and basic fibroblast growth factor (10 ng/ml). The medium was replaced with experimental medium (DMEM/F-12 containing 10 μg/ml cholestanol in 1% ethanol, 10 μg/ml cholesterol in 1% ethanol, or 1% ethanol) and incubated for 3 or 6 days.

Detection of apoptosis. DNA breaks were detected in situ by nick end labeling using the TUNEL method (20). As cerebellar granule and Purkinje cells were co-cultured, Purkinje cells were stained with anti-rat CD3 monoclonal antibody (Pharmingen, San Diego). Cells were trypsinized, collected by centrifugation, rinsed with PBS, fixed with a freshly prepared paraformaldehyde solution (4% in PBS; pH 7.4) for 30 min at room temperature, rinsed with PBS and incubated in permeabilization solution (0.1% triton X-100 in 0.1% sodium citrate) for 2 min on ice. The cells were rinsed twice with PBS, added 50 μl TUNEL reaction mixture, incubated in a humidified chamber for 60 min at 37°C, rinsed 3 times with PBS and analyzed immediately using an anchored cell analysis and sorting (ACAS) 570 scanning laser microscope (Meridian Instrument, Okemos, MI). Fluorescence intensity of fluorescein isothiocyanate (FITC) was measured at 530 nm with excitation at 488 nm.

Activities of ICE and CPP32 proteases. ICE and CPP32 protease activities were measured using a modified procedure of Walker *et al.* (21). Protein concentration was measured by Bradford's method (22). Cells were trypsinized, collected by centrifugation and rinsed with PBS. Cells were suspended in lysis buffer (50 mM Tris-HCl; pH 7.5, 0.2% triton X-100, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and incubated at 37°C for 10 min. Lysates were added to reaction buffer (50 mM Tris-HCl; pH 7.5, 2 mM DTT, 1 mM EDTA, and 40% glycerol) and incubated with 10 mM of enzyme substrate Ac-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA) or Ac-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA) (Peptide Institute Inc, Osaka, Japan) at 37°C for 1 hr. Amino-4-methylcoumarin (AMC) release was measured by spectrofluorometry (Corona Electric, Ibaragi, Japan) at an excitation wavelength at 365 nm and an emission wavelength of 450 nm. AMC concentration was determined from a standard curve.

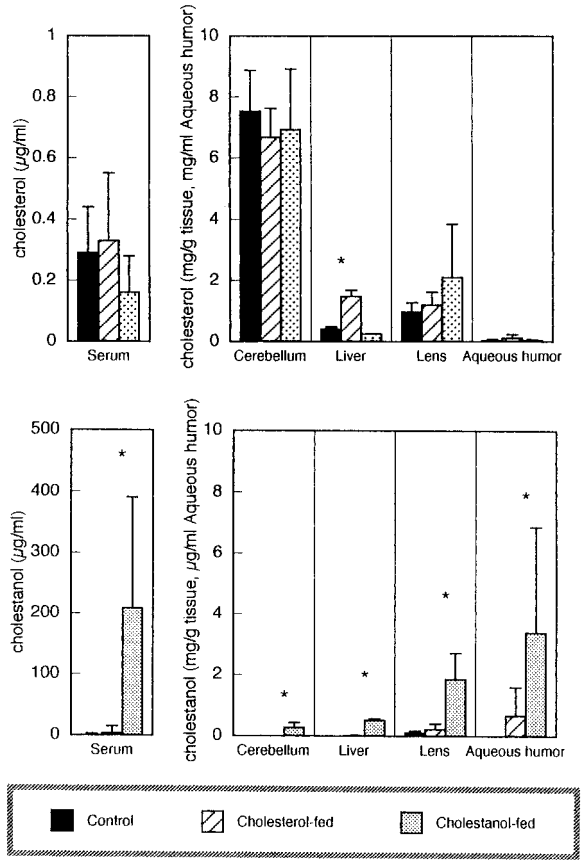

FIG. 1. Contents of cholesterol (upper panel) and cholestanol (lower panel) in serum and several tissues of rats as measured by HPLC. The serum (0.1 ml), cerebellum (120–300 mg), liver (1.2-4.0 g), lens (14-40 mg), and aqueous humor (50-100 μl) were hydrolyzed with 1 ml of 1 M ethanolic KOH and processed by the same procedure as described in Materials and Methods. Values are means $\pm$ S.D. from duplicate assays. Significantly different from control values: *$p < 0.01$ by Fisher's PLSD test.

Statistical analysis. Data were presented as mean $\pm$ S.D. unless otherwise indicated. Statistical analysis was made with ANOVA, with comparison of different groups by Fisher's partial least-squares difference (PLSD) test, or chi-square test (Statview II; Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Thirty male Wistar rats were fed a standard diet, a diet containing 1% cholesterol or 1% cholestanol for 15 weeks. There was no difference in the animals' body weight gain among three dietary groups. Figure 1 shows the contents of sterols in various tissues. The concentration of cholestanol is shown in the lower panel of Fig. 1. In cholestanol-fed rats, the contents of cholestanol in serum (209 $\pm$ 182 μg/ml), cerebellum (0.27 $\pm$ 0.16 mg/g tissue), liver (0.51 $\pm$ 0.05 mg/g tissue) and lens (1.85 $\pm$ 0.86 mg/g tissue) were significantly increased as compared with those of cholestanol in serum (0.8 $\pm$ 1.7 μg/ml), cerebellum (undetectable

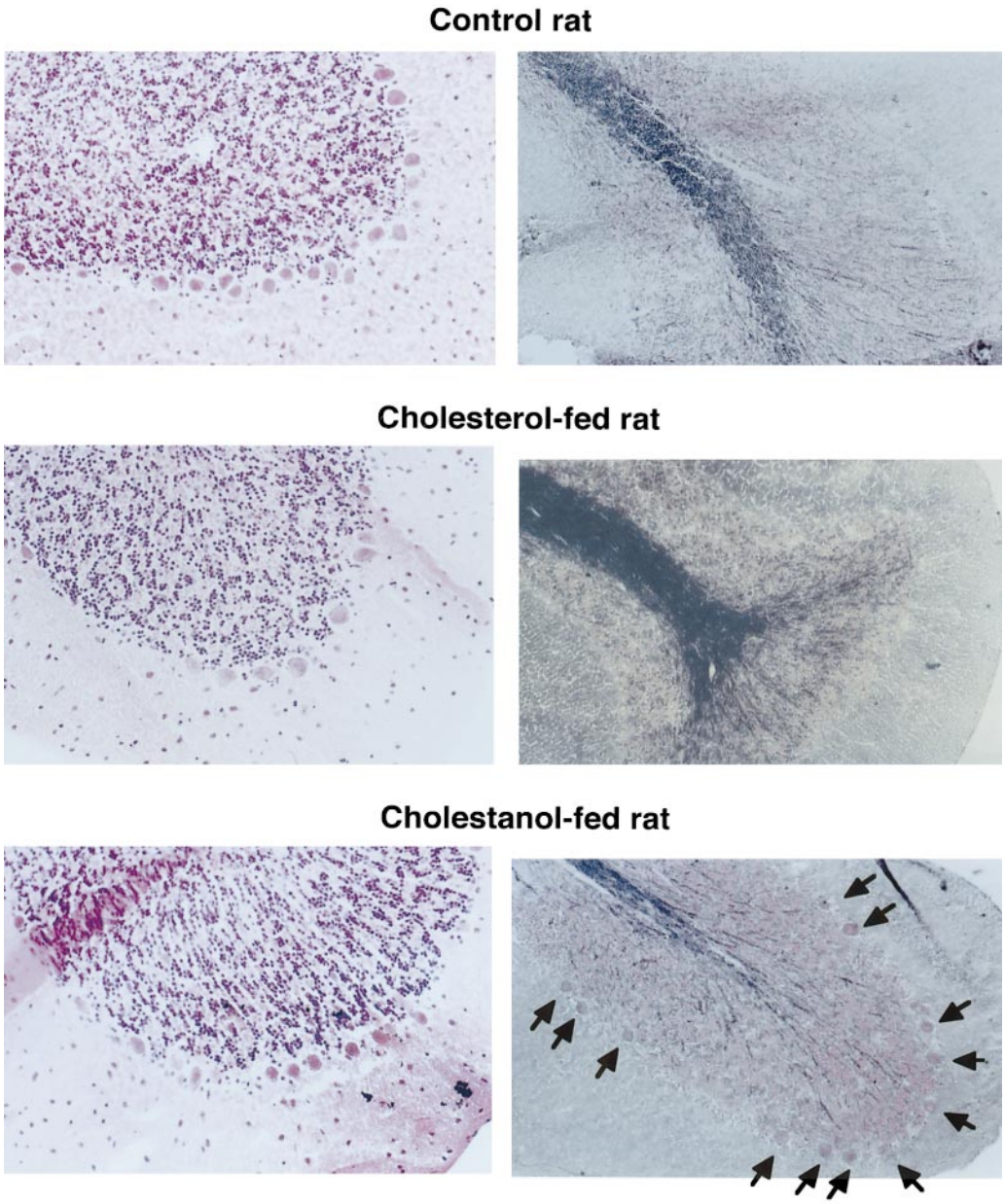

FIG. 2. Histopathology of cerebellums of rats. Cerebellums isolated from rats fed a standard diet (control), cholesterol diet or cholestanol diet for 6 weeks were sectioned at 4 μm and stained with hematoxylin and eosin (left panel) and Sudan black B solution (right panel), and examined by light microscopy ($\times$ 200). Black arrows show the deposition of neutral fat.

level), liver (undetectable level) and lens (0.10 $\pm$ 0.06 mg/g tissue) of standard diet rats ($P < 0.01$), respectively. The concentration in aqueous humor of cholestanol was significantly increased in the cholestanol-fed group (3.38 $\pm$ 3.49 μg/ml), as compared to findings in the control group (undetectable level) ($P < 0.01$). On the other hand, levels of cholesterol in the cerebellum, lens and aqueous humor did not significantly differ among the three groups (Fig. 1, upper panel). The level of cholesterol in the liver of cholesterol-fed group (1.47 $\pm$ 0.20 mg/g tissue) was significantly higher than that in the control group (0.41 $\pm$ 0.07 mg/g tissue) and cholestanol-fed group (0.24 $\pm$ 0.02 mg/g tissue) ($P < 0.01$).

The eyes, livers and cerebellums of affected and control rats fed for 6 weeks were sectioned and stained with HE and Sudan black B solution, and examined using light microscopy. In eyes and livers, no morphological change was observed among the three groups (data not shown). As shown in Fig. 2 (left panel), cerebellar neuronal cells, including Purkinje cells, were stained with HE solution equally among the three groups. On the contrary, as shown in the right panel of Fig. 2, neuronal cells, including Purkinje cells, were

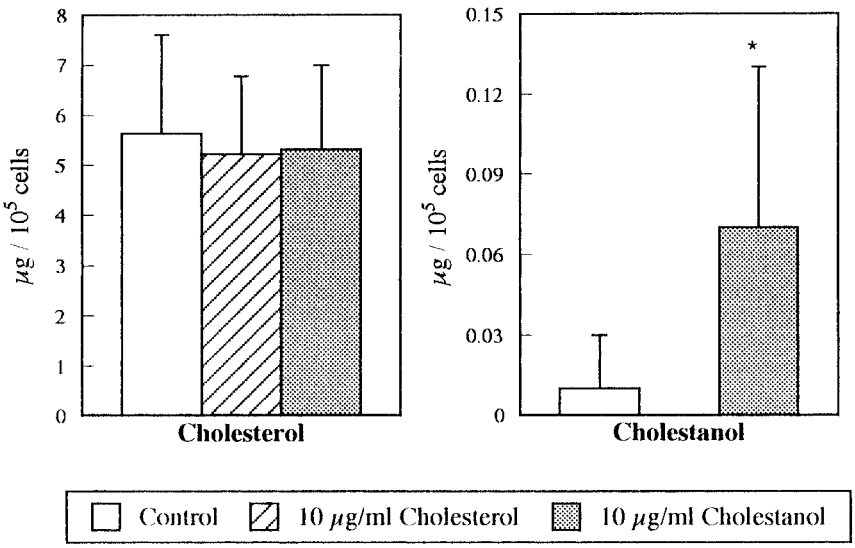

FIG. 3. Contents of sterols in cerebellar neuronal cells. Cerebellar neuronal cells prepared from brains of 20-21-day-old fetuses of Wistar rats were cultured in DMEM/F12 containing 10 μg/ml cholestanol in 1% ethanol, 10 μg/ml cholesterol in 1% ethanol, or 1% ethanol (control) for 3 days. The contents of sterols in cerebellar neuronal cells in vitro were measured by HPLC, as described in Materials and Methods. Values are means from duplicate assays. Significantly different from control values: *$p < 0.01$ by Fisher's PLSD test.

stained with Sudan black B solution only in cholestanol-fed group. Purkinje cells were stained more strongly than other neuronal cells. The staining was attributed to deposition of neutral fats in neuronal cells, especially Purkinje cells. On the other hand, Purkinje cells from cholesterol-fed group and control group did not stain with Sudan black B. Purkinje cells in frozen sectioned tissue in the cholestanol-fed group did not stain using Schultz's methods which is specific for cholesterol staining (data not shown). Therefore, cholestanol was deposited in cerebellar neuronal cells, especially in Purkinje cells, only in the cholestanol-fed group.

We next studied the effects of cholestanol on Purkinje cell death in vitro. Because cerebellar Purkinje cells were difficult to isolate, Purkinje cells were cultured together with cerebellar granule cells. When cholestanol was added to the culture medium, it was taken up into the cells. Fig. 3 shows the contents of sterols in co-cultured cerebellar neuronal cells. The concentration of cholestanol (0.07 ± 0.06 μg/10^5 cells) in cells cultured with 10 μg/ml cholestanol for three days was significantly increased compared to that in control cells (0.01 ± 0.02 μg/10^5 cells) ($p < 0.05$). The level of cholesterol was not significantly different. As shown in Fig. 4, the viability of the cells cultured with 10 μg/ml cholestanol for 3 ($75.2 \pm 1.3\%$) and 6 days ($69.5 \pm 0.9\%$) was significantly lower than that for control cells for 3 ($86.2 \pm 0.8\%$) ($p < 0.01$) and 6 days ($77.6 \pm 1.3\%$) ($p < 0.05$), respectively. There was no significant difference in cell viability between cells cultured with cholesterol and controls.

We next asked if cholestanol induced apoptosis of cerebellar Purkinje cells. Because neuronal cells were co-cultured, Purkinje cells were identified by staining with anti-CD3 antibody (data not shown). Every 3 days Purkinje cells were stained using the TUNEL method and analyzed using the ACAS system. As shown in Fig. 5, the TUNEL method clearly demonstrated the distinct pattern of staining in cells cultured with cholestanol. The number of Purkinje cells stained were counted, microscopically. As shown in Fig. 6, the per-

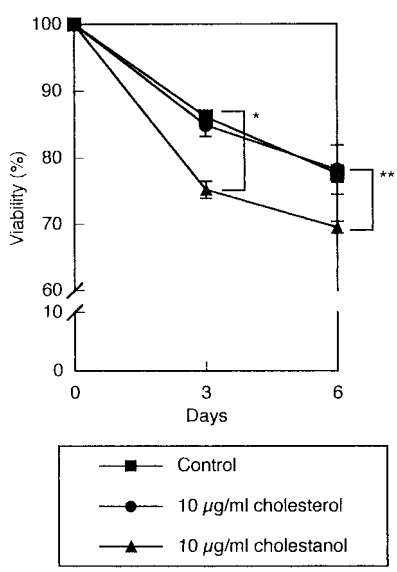

FIG. 4. Viability of cerebellar neuronal cells. Cell viability was measured by trypan blue staining. Cells cultured with 10 μg/ml cholesterol in ethanol, 10 μg/ml cholestanol in ethanol, or 1% ethanol (control) for 3 and 6 days were stained with trypan blue solution. Values are means of duplicate assays. Significantly different from control values: *$p < 0.01$, **$p < 0.05$, by Fisher's PLSD test.

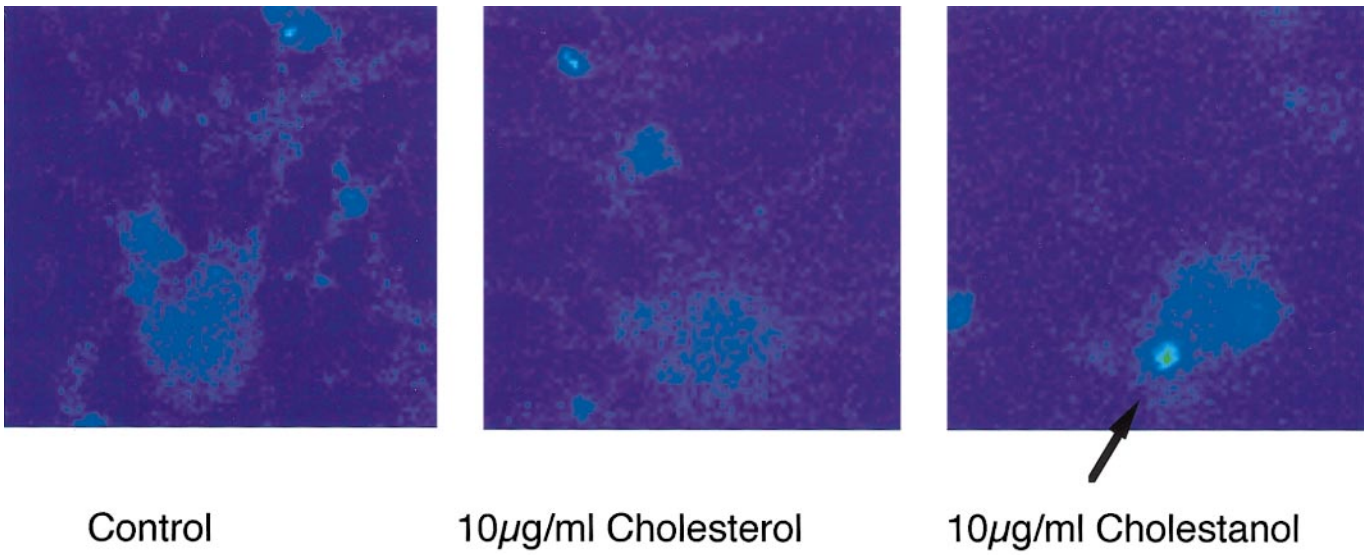

FIG. 5. Apoptosis of cerebellar Purkinje cells induced by cholestanol. Cerebellar neuronal cells cultured with 10 μg/ml cholesterol, 10 μg/ml cholestanol, or 1% ethanol (control) for 3 days were stained using the TUNEL method and analyzed using the ACAS system, as described in Materials and Methods. Fluorescence intensity of fluorescein isothiocyanate (FITC) was measured at 530 nm with excitation at 488 nm. The nuclei of Purkinje cells cultured in medium containing 10 μg/ml cholestanol were clearly visible (black arrow).

centage of apoptosis was significantly prominent in cells cultured with cholestanol for 3 and 6 days ($p < 0.05$), compared to that of control cells and cells cultured with cholesterol. As ICE and CPP32 proteases are thought to play important roles in apoptosis, ICE and CPP32 activities were measured. The ICE protease activity of cerebellar neuronal cells cultured with cholestanol for 6 days was 8.96 ± 0.58 units/mg protein, a value significantly higher than that for control cells (5.74 ± 0.48 units/mg protein) ($p < 0.01$), and that for cells cultured with cholesterol (5.50 ± 0.48 units/mg protein) ($p < 0.01$) (Fig. 7, left panel). The CPP32 protease activity of cerebellar neuronal cells cultured with cholestanol for 6 days was 14.8 ± 2.14 units/mg protein, a value significantly higher than that for control cells (5.85 ± 0.53 units/mg protein) ($p < 0.01$) and that for cells cultured with cholesterol (8.72 ± 0.56 units/mg protein) ($p < 0.05$) (Fig. 7, right panel). This seems to be the first evidence that cholestanol induces activation of ICE and CPP32 proteases with concomitant induction of neuronal cell apoptosis.

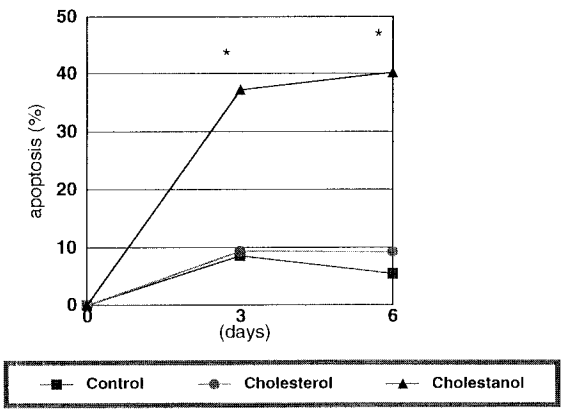

FIG. 6. Percentage of apoptosis of cerebellar Purkinje cells induced by cholestanol. Cerebellar neuronal cells cultured with 10 μg/ml cholesterol, 10 μg/ml cholestanol, or 1% ethanol (control) for 3 and 6 days were stained using the TUNEL method and analyzed using the ACAS system, as described in Materials and Methods. The number of Purkinje cells stained using the TUNEL method was counted microscopically. Significantly different from controls: *$p < 0.01$ by χ^2 test.

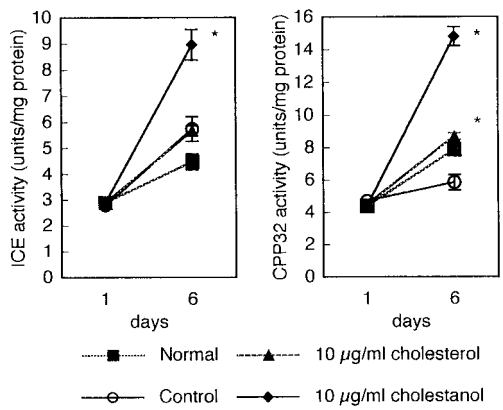

FIG. 7. Effects of cholestanol on ICE and CPP32 protease activities. ICE and CPP32 protease activities were measured in cerebellar neuronal cells which had been cultured with 10 μg/ml cholesterol, 10 μg/ml cholestanol, or 1% ethanol (control) for 6 days. Amino-4-methylcoumarin (AMC) release was measured by spectrofluorometry at an excitation wavelength at 365 nm and an emission wavelength of 450 nm. Significantly different: *$p < 0.01$, by Fisher's PLSD test.

DISCUSSION

We developed hyper-cholestanolemia rats by feeding animals with cholestanol. Cholestanol was deposited in various tissues, especially in cerebellar Purkinje cells. Purkinje cells are considered to be the central functional unit of the cerebellar cortex. In Purkinje cell degeneration (*pcd*) mice, the major clinical sign was a moderate ataxia of gait (23). In shaker mutant rats, Purkinje cells were degenerated and rats showed ataxia and tremor (24). In CTX patients the level of cholestanol in various tissues increases and deposition of cholestanol finally leads to tendon xanthoma, cataract, atherosclerosis and neurological disease. Philippart and van Bogaert autopsied CTX patients and found that the dentate and fastigial nuclei in the cerebellum were completely destroyed and a few Purkinje cells were missing (13).

Apoptotic cell death in the nervous system occurs during the progression of certain neurodegenerative diseases (25), such as amyotrophic lateral sclerosis (ALS) (26), Alzheimer's disease (27), Parkinson's disease (28), and Huntington's disease (29). It was reported that free radicals (30) and X-rays (31) induced apoptosis of Purkinje cells. It was also reported that in the Lurcher mouse which had a mutation of lurcher gene apoptosis was induced in Purkinje cells (23). In the present study, we hypothesized that cholestanol might induce neuronal cell death (apoptosis), especially Purkinje cell death. To prove this hypothesis primary culture of cerebellar Purkinje cells was done, and we found that cholestanol induced apoptosis of cerebellar neuronal cells, especially Purkinje cells. Further, we have shown that cholestanol induced ICE and CPP32 protease activities. Such cell death may eventually induce cerebellar ataxia in CTX patients.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, and the International Scientific Research Program.

REFERENCES

1. Björkhem, I. and Skrede, S. (1989) *in* The Metabolic Basis of Inherited Disease, 6th ed. (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.), pp. 1283–1302, McGraw-Hill, New York.
2. Van Bogaert, L., Scherer, H. J. and Epstein, E. (1937) *in* Une forme cérébrale de la cholesterinose généralisée, pp. 1–159. Masson et Cie., Paris, France.
3. Schimschock, J. R., Alvord, E. C., Jr., and Swanson, P. D. (1968) *Arch. Neurol.* **18,** 688–698.
4. Cali, J. J., Hsieh, C-L., Francke, U., and Russell, D. W. (1991) *J. Biol. Chem.* **266,** 7779–7783.
5. Kim, K-S., Kubota, S., Kuriyama, M., Fujiyama, J., Björkhem, I., Eggertsen, G., and Seyama, Y. (1994) *J. Lipid Res.* **35,** 1031–1039.
6. Chen, W., Kubota, S., Nishimura, Y., Nozaki, S., Yamashita, S., Nakagawa, T., Kameda-Takemura, K., Menju, M., Matsuzawa, Y., Björkhem, I., Eggertsen, G., and Seyama, Y. (1996) *Biochim. Biophys. Acta.* **1317,** 119–126.
7. Chen, W., Kubota, S., Kim, K. S., Cheng, J., Kuriyama, M., Eggertsen, G., Björkhem, I., and Seyama, Y. (1997) *J. Lipid Res.* **38,** 870–879.
8. Menkes, J. H., Schimschock, J. R., and Swanson, P. D. (1968) *Arch. Neurol.* **19,** 47–53.
9. Anker, H. S., and Bloch, K. (1949) *J. Biol. Chem.* **178,** 971–976.
10. Rosenfeld, R. S., Zumoff, B., and Hellman, L. (1967) *J. Lipid Res.* **8,** 16–23.
11. Björkhem, I., and Karmar, K. E. (1974) *Biochim. Biophys. Acta* **337,** 129–131.
12. Skrede, S., Björkhem, I., Buchmann, M. S., and Midtvedt, T. (1985) *J. Biol. Chem.* **260,** 77–81.
13. Philippart, M., and van Bogaert, L. (1969) *Arch. Neurol.* **21,** 603–610.
14. Byun, D-S, Kasama, T., Shimizu, T., Yorifuji, H., and Seyama, Y. (1988) *J. Biochem.* **103,** 375–379.
15. Kasama, T., Byun, D. S., and Seyama, Y. (1987) *J. Chromatogr.* **400,** 241–246.
16. Glick, D. (1949) Techniques of Histo- and Cytochemistry, p. 41, Interscience, London.
17. Adams, C. W. M. (1961) *Nature* **192,** 331–332.
18. Fischer, G. (1982) *Neurosci. Lett.* **28,** 329–335.
19. Furuya, S., Ono, K., and Hirabayashi, Y. (1995) *J. Neurochem.* **65,** 1551–1561.
20. Gavrieli, Y., Sherman, Y., and Ben-Sasson, SA. (1992) *J. Cell. Biol.* **119,** 493–501.
21. Walker, N. P., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., and Hammill, L. D. (1994) *Cell* **78,** 343–352.
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
23. Mullen, R. J., Eicher, E. M., and Sidman, R. L. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 208–212.
24. Tolbert, D. L., Ewald, M., Gutting, J., and LaRegina, M. C. (1995) *J. Comp. Neurol.* **355,** 490–507.
25. Arends, M. J., and Wyllie, A. H. (1991) *Int. Rev. Exp. Pathol.* **32,** 223–254.
26. Rabizadeh, S., Gralla, E. B., Borchelt, D. R., Gwinn, R., Valentine, J. S., Sisodia, S., Wong, P., Lee, M., Hahn, H., and Bredesen, D. E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3024–3028.
27. Thompson, C. B. (1995) *Science* **267,** 1456–1462.
28. Hartley, A., Stone, J. M., Heron, C., Cooper, J. M., and Schapira, A. H. V. (1994) *J. Neurochem.* **63,** 1987–1990.
29. Portera-Cailiau, C., Hedreen, J. C., Price, D. L., and Koliatsos, V. E. (1995) *J. Neurosci.* **15,** 3775–3787.
30. Wood, K. A., and Youle, R. J. (1995) *J. Neurosci.* **15,** 5851–5857.
31. Ferrer, I., Serrano, T., Rivera, R., Olive, M., Zujar, M. J., and Graus, F. (1993) *Acta Neuropath. (Berlin)* **86,** 491–500.