cholesterol-poor HDL is generated by extracellular apoA-I [9]. The cholesterol content in the HDL generated by exogenous apolipoprotein is regulated by cholesterol–sphingomyelin interaction in "raft" domains of the astrocyte plasma membrane [10].

In the CNS and in the peripheral nerve system, apoE synthesis and secretion increase during their development or after their injury, and apoE accumulates in the damaged lesions [3,11–18]. Therefore, apoE has been proposed to play a major role in construction and regeneration of the nerve system. On the other hand, apoE4, one of the major three apoE isoforms, has been highlighted as it associates with Alzheimer's disease [19–21] and with poor prognosis after acute and chronic brain damage [22–24], though the specific mechanism is still unclear.

Some of the "apoE receptors" may function to uptake apoE-HDL so that the cholesterol-rich HDL with apoE would serve as a cholesterol delivery vehicle to the cells for their growth and regeneration. Those receptors include low density lipoprotein (LDL) receptor [25], very low density lipoprotein receptor [26], LDL receptor-related protein [26], or apoE receptor 2 [27], all of which belong to the LDL-receptor superfamily. The LDL receptor in neuron indeed seems to mediate cholesterol uptake from apoE-containing lipoprotein for regeneration of axons [28]. On the other hand, the large matrix protein Reelin, which regulates neuronal migration and positioning in the brain, binds to very low density lipoprotein receptor and apoE receptor 2, resulting in activation of tyrosine kinases via Dab 1 linked to its cytosolic domain and apoE receptor 2 [29,30]. Therefore, apoE may contribute to the neuronal activities in the brain by the control of the binding of Reelin to such apoE receptors.

The purpose of this study is to understand regulation of the synthesis and secretion of apoE in relation to cholesterol metabolism in astrocytes. If the upregulation of apoE-HDL secretion is required for development of brain or recovery from brain injury, extracellular factors may be involved in stimulation of apoE biosynthesis and apoE-HDL generation. In order to approach this problem, we investigated these parameters in a developmental model of astrocytes by employing long-term primary culture of the fetal rat brain cells. We demonstrate that apoE synthesis, HDL secretion and cholesterol biosynthesis were enhanced in rat astrocytes by an acidic fibroblast growth factor (aFGF)-like trophic factor secreted during the long-term primary culture of the brain cells.

2. Materials and methods

2.1. Preparation of fetal rat astrocytes

Astrocytes were prepared from the cerebrum of 17-day fetal Wistar rat according to the previous method [31,32]. After removal of the meninges, the cerebral hemisphere was cut into small pieces and treated with 0.1% trypsin solution in Dulbecco's phosphate buffered saline containing 0.15% glucose (0.1% trypsin/DPBS/G) for 5 min at room temperature. The cell pellets obtained by centrifugation at 1000 rpm for 5 min were cultured in F-10 medium containing 10% fetal calf serum (10% FCS/F-10) at 37 °C for 1 month (primary culture of rat brain, M-PC). The cells were then transferred to a 2.5-cm-diameter multiple tray for 1-week secondary culture (M/W) after treatment with the 0.1% trypsin/DPBS/G containing 1 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C for 5 min, and then centrifuged to obtain the cell pellets. Alternatively, astrocytes were prepared by a conventional method of 1-week primary and subsequent 1-week secondary cultures (W/W cells) [32].

2.2. Preparation of LDL containing ^{3}H-cholesteryl ester and labeling of astrocytes

LDL isolated by ultracentrifugation from fresh plasma of a healthy volunteer was incubated with lipid microemulsion containing [1,2-^{3}H]cholesteryl oleate (Amersham) and a plasma protein fraction of density >1.25 g/ml, and the labeled LDL was re-isolated by ultracentrifugation [33]. Astrocytes were washed three times with DPBS and incubated with 25 μg protein/ml of the labeled LDL in 0.02% bovine serum albumin (BSA)/F-10 for 24 h, followed by washing three times with DPBS. In order to examine the uptake of LDL and the labeling of the cells, lipid was extracted from the whole cells with hexane/isopropanol (3:2, v/v) and analyzed by thin layer chromatography (TLC).

2.3. De novo syntheses of mevalonic acid and cholesterol

Rat astrocytes at a confluent cell density were washed with DPBS four times and incubated in 0.1% BSA/F-10 for 24 h. To measure de novo syntheses of mevalonic acid and cholesterol, the cells were incubated with [^{3}H]acetate (20 μCi/ml, New England Nuclear) in a fresh 0.02% BSA/F-10. The cholesterol synthesis was measured also by using [^{3}H] mevalonolactone (5 μCi/100 nmol/ml) for the down stream of the step of hydroxymethylglutaryl-CoA (HMG-CoA) reductase in the biosynthesis pathway. After the cells were washed three times with cold DPBS, lipid was extracted from the cells with hexane/isopropanol (3:2, v/v) and analyzed by TLC for cholesterol biosynthesis measurement. To detect mevalonic acid synthesis, the cells were rinsed three times with cold DPBS, incubated with 0.25 N HCl at 37 °C for 15 min to allow lactonization of the [^{3}H]mevalonic acid, scraped with a rubber policeman and sonicated. The mevalonolactone was identified by TLC after extraction from the cell suspension with chloroform/methanol (2:1, v/v) [34].

2.4. HMG-CoA reductase assay

HMG-CoA reductase assay was carried out according to the method described elsewhere [35]. The cells were har-

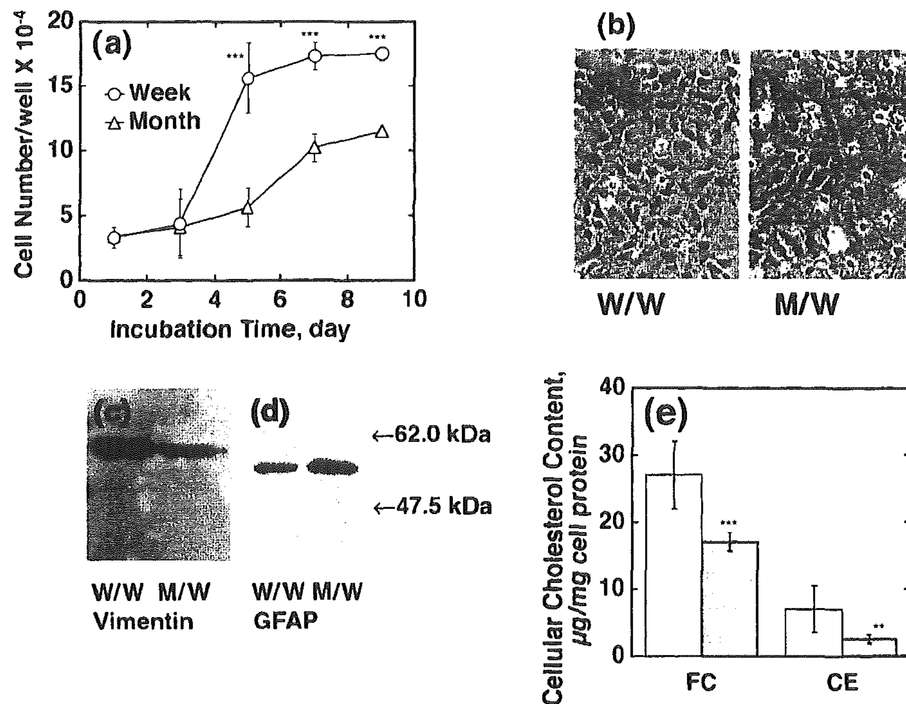

Fig. 1. Characterization of astrocytes after the primary culture of rat brain cells. (a) Growth curve of rat astrocytes. After 1-week (open circles) or 1-month primary culture (open triangles) of rat fetal brain cells, the cells were seeded at a density of 3.4×10^4 cells/well on 2.5-cm culture plates and maintained in 10% FCS/F-10 medium. Cell number was counted and the medium was replaced every 2 days. (b) Microscopic view of W/W and M/W cells—after 1-week secondary culture following 1-week and 1-month primary culture, respectively. (c and d) Expression of cellular marker proteins in the astrocytes. The cells were solubilized, and vimentin and GFAP was detected for W/W and M/W cells by immunoblotting analysis as described in Section 2. (e) Cholesterol content in astrocytes. Lipid was extracted from W/W and M/W cells with hexane/isopropanol, and free and total cholesterol mass were determined by enzymatic methods as described in Section 2. Cholesterol ester was calculated by subtracting free cholesterol from total cholesterol. ***: $P < 0.01$ from the 1-month primary culture in panel (a); ** and ***: $P < 0.05$ and $P < 0.01$ from W/W cells in panel (e).

vested by scraping with a rubber policeman and centrifuged at $1000 \times g$ for 10 min. The cell pellet was treated with buffer containing 50 mM of imidazole, 5 mM of EDTA, 200 mM of KCl and Brij 97, pH 7.4, at 37 °C for 10 min and centrifuged at $12{,}000 \times g$ for 15 min. The aliquot of detergent-solubilized extract was incubated with the buffer containing 0.2 M of potassium phosphate, 40 mM of glucose 6-phosphate, 5 mM of NADP, 8 mM of dithiothreitol, 20 unit/ml of glucose 6-phosphate dehydrogenase and DL-3-[3-^{14}C]-hydroxy-3-methylglutaryl CoA (0.1 Ci/ml) at 37 °C for 2 h. The reaction was terminated with 0.2 N HCl. The mixture was incubated at 37 °C for 15 min to allow lactonization of [^{14}C]mevalonic acid. The labeled mevalonolactone was identified by TLC by using acetone/benzene (1:1, v/v).

2.5. Analysis of cholesterol in the media

For standard measurement of cellular cholesterol release [33], astrocytes were labeled by incubating with 25 µg protein/ml of the ^{3}H-labeled LDL and the release of the labeled free cholesterol into the medium was measured during the incubation with the fresh medium for 8–24 h. Alternatively, the cells were labeled with [^{3}H]acetate (20–40 µCi/ml) for 12–24 h. After the cells were washed and incubated in the fresh 0.02% BSA/F-10 for 12 h, the medium was collected and centrifuged at 10,000 rpm for 1 h to remove the cell debris. Lipid was extracted from the medium and the whole cells separately with chloroform/methanol (2:1, v/v) and hexane/isopropanol (3:2, v/v), respectively, and analyzed by TLC to determine radioactivity of cholesterol. The mass of free and total cholesterol in the cells and media was determined by using enzymatic colorimetric assay kits purchased from Wako and from Sigma Diagnostics. Cholesterol ester was calculated by subtracting the amount of free cholesterol from total cholesterol. Cellular protein was measured by the method of Lowry using bovine serum albumin as a standard [36]. Other specific modifications are described in the figure legends.

Table 1
Cellular population in the astrocyte preparations

	GFAP-positive (astrocytes)	MBP-positive (oligodendroglias)	ED-1 antigen-positive (microglias)
W/W	95.0 ± 3.3	0.27 ± 0.10	2.6 ± 1.6
M/W	95.3 ± 4.3	0.36 ± 0.21	3.1 ± 2.2

The data indicate percentage to the total cells of the cells positively identified as astrocytes, oligodendroglia and microglia by immunostaining with an anti-GFAP antibody, an anti-myelin basic protein (MBP) and a monoclonal antibody against ED-1 that recognizes microglial antigen, respectively. The values represent average ± S.E. of three samples.

2.6. Analysis of lipoprotein produced by astrocytes

The astrocytes loaded with LDL (25 μg of protein/ml) for 24 h in a 10-cm culture dish were incubated with a fresh 0.02% BSA/F-10 without LDL for 24 h after washing. The medium (10 ml) was collected and cell debris was removed by centrifugation at 10,000 rpm for 1 h. The density of the medium was then adjusted to 1.20 g/ml with sucrose or the medium was overlayered on the layers of 1.20 g/ml and 1.07 g/ml of sucrose solutions (7.5 ml each), followed by centrifugation at 49,000 rpm for 48 h in a RP50T rotor (HITACHI). The sample was recovered into 12 equal fractions from the bottom. A 500 μL aliquot of each fraction was used for immunoblotting after the precipitation by the treatment with 10% trichloroacetic acid [9].

2.7. Analysis by western blotting

For Western blotting, the cells were treated with 2% Triton X-100 solution containing 0.1 N NaOH/1 mM EDTA. After the neutralization, the solubilized cell protein was subjected to 0.5% SDS/12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Sequi-BlotTM PVDF Membrane (Bio-Rad). For the analysis of the medium, the cells were

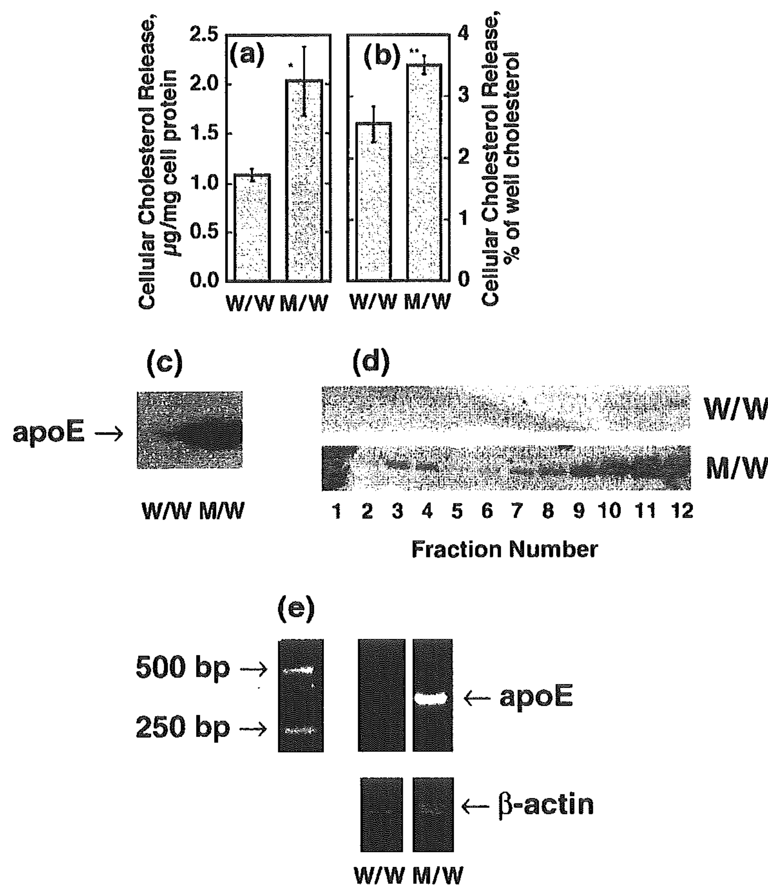

Fig. 2. Release of cholesterol and apoE, and apoE mRNA level in W/W and M/W cells. (a) Cholesterol mass measurement. The cells were incubated with fresh 0.02% BSA/F-10 medium for 24 h. Lipid was extracted from the medium with chloroform/methanol, and cholesterol was measured by an enzymatic method. (b) Radiolabeled cholesterol. The cellular cholesterol was labeled by incubating with LDL containing [^{3}H]-cholesterol ester for 24 h, and the release of the labeled cholesterol for 8 h was determined as described in the text. The uptake of LDL was estimated by incorporation of the radioactivity from LDL containing the radiolabeled cholesteryl ester and same between W/W and M/W cells. Cholesterol release was calculated as a percentage of free cholesterol count in the medium (esterified cholesterol in the medium was always negligible) against the total cholesterol count in the well (the total of free and esterified cholesterol in the cell plus medium). For panels (a) and (b), the data represent the average and S.E. of the triplicate samples, and * and ** indicate $P<0.05$ and $P<0.01$ from W/W cells. (c) Secretion of apoE by W/W and M/W cells. The cells at the confluent stage were incubated with LDL (25 mg of protein/ml) in 0.02% BSA/F-10 medium for 24 h, and then the release of apoE into the medium in the next 24 h was analyzed by Western blotting as described in Section 2. (d) Analysis of apoE in the conditioned medium of rat astrocytes by ultracentrifugation at a density of 1.20 g/ml. The media of W/W and M/W cells were fractionated from the bottom as described in Section 2. SDS-PAGE and Western blotting were carried out for each 12 fractions from the bottom to the top fraction after the 10% trichloroacetate treatment. (e) Expression of apoE mRNA in W/W and M/W cells. Total cellular RNA, 5 μg, was subjected to reverse transcription for 10 min at 25 °C, for 50 min at 50 °C, and then for 15 min at 70 °C, and 0.5 μg of cDNA product was amplified using apoE primer pairs (5′-GCGCACCTCCTCCATCTCCTC as a sense and 5′-AGGATCTATGCAACCGACTCG as an antisense) [73] with 30 cycles as described in Section 2. The PCR products for mRNA 356-747 were visualized as described in the text. The expression of the housekeeping gene β-actin was analyzed as a control.

incubated with fresh 0.02% BSA/F-10 medium for 24 h and the protein fraction of the medium was recovered as a pellet precipitated by centrifugation at 15,000 rpm for 15 min in 10% trichloroacetic acid. The aliquot (93.5 μg protein) of the pellet was analyzed by SDS-PAGE (12% polyacrylamide gel) and immunoblotting using a rabbit anti-rat apoE antibody. The membrane was immunostained with a mouse anti-vimentin monoclonal antibody (IF01, Oncogene Science, Inc.), a rabbit anti-cow glial fibrillary acidic protein (GFAP) antibody (Dakopatts) and a rabbit anti-rat apoE antibody, a generous gift from Dr. Jean Vance (University of Alberta). Density of apoE digital scanning by an image scanner (Epson GT 9500 ART) and semi-quantified by Adobe Photoshop was largely proportional to the amount of the protein when the medium was diluted in series.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from rat astrocytes by ISOGEN (Wako Life Science), and reverse-transcribed to cDNA using a Super Script Preamplification System (Gibco BRL). The resulting cDNA was subjected to PCR by using the DNA probes for rat apoE-mRNA, aFGF-mRNA, and actin-mRNA. After the electrophoresis of the products, agarose gel was stained with freshly prepared SYBR Gold nucleic acid gel stain solution (Molecular Probes, Inc.). The band was detected by a UV transilluminator, UVP NLM-20 E at 302 nm.

3. Results

3.1. Characterization of astrocytes

Fetal rat brain cells grown for 1 week or 1 month as a primary culture were seeded at 3.4×10^4 cells/well in a 2.5-cm-diameter multiple tray. The rate of cell growth and confluent cell density were both higher in the cells after 1-week primary culture than in those after 1-month primary culture (Fig. 1a). Since both reach the confluent stage at the day 7 of the secondary culture, the cells in this stage were used for further analysis as W/W and M/W cells, respectively. Relative cellular population was estimated in these two cell preparations by indirect immunohistochemical staining by using specific antibodies against GFAP and myelin basic protein and by an antibody ED-1 that recognizes microglia antigen [37,38], for positive identification of astrocytes, oligodendroglias and microglias, respectively. The result was similar for both preparations with respect to cellular composition (Table 1). Type-2 astrocyte-like cells were identified at 5–15% of the population of M/W cells

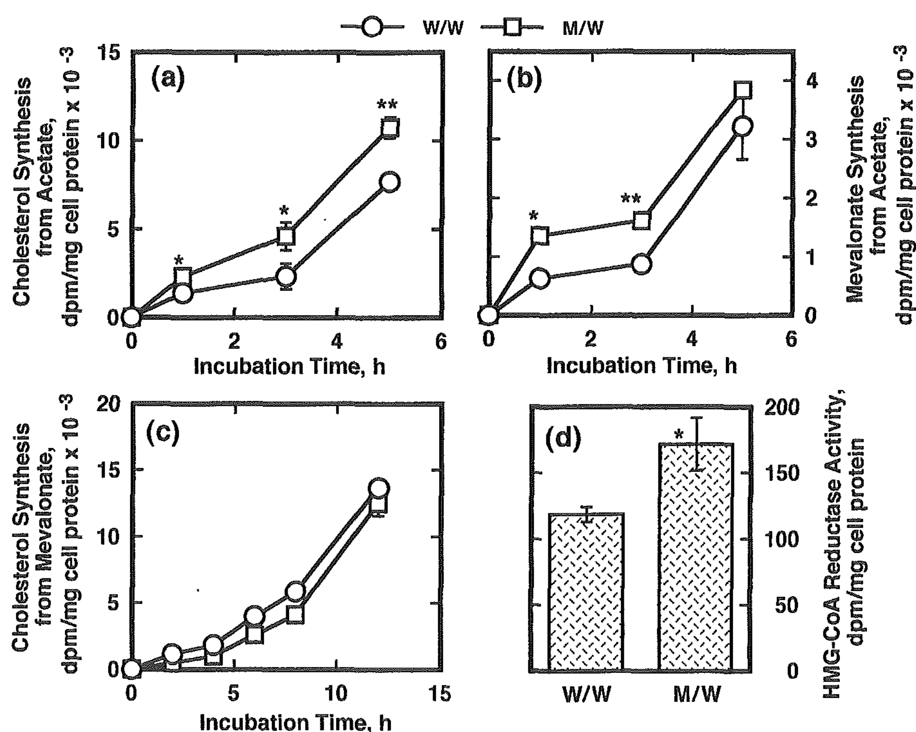

Fig. 3. Syntheses of cholesterol and mevalonic acid in rat astrocytes. (a and b) W/W, M/W and W/M cells were incubated with 20 μCi/ml of [^{3}H]-acetate, or (c) 5 μCi/ml of [^{3}H]-mevalonolactone in 0.02% BSA/F-10 for metabolic labeling of cholesterol in panels (a) and (c) and mevalonic acid in (b). Lipid was extracted according to the method described in Section 2. (d) HMG-CoA reductase activity of rat astrocytes was measured by using a substrate DL-[3-^{14}C] HMG-CoA as described also in the text. Each data represents the average and standard error of the triplicate samples, and * and ** indicate $P < 0.05$ and $P < 0.01$ from the W/W, respectively.

but not in W/W cells (Fig. 1b), indicating that the cells appeared to be activated. Neurons were hardly identified in either preparation.

The vimentin expression was lower in M/W cells than in W/W cells, and GFAP expression was higher in M/W cells than in W/W cells (Fig. 1c and d), indicating differentiation and/or activation of astrocytes during the long-time primary culture, being consistent with the morphological finding. As demonstrated in Fig. 1d, unesterified and esterified cholesterol levels were both lower in M/W cells than in W/W cells.

3.2. Release and synthesis of cholesterol and apoE by astrocytes

Cellular cholesterol release into the medium in 24 h was higher in M/W cells than W/W cells (Fig. 2a and b). M/W cells also secrete more apoE into the medium (Fig. 2c). Ultracentrifugal analysis of the media at the solvent density of 1.20 g/ml showed that most of apoE released from M/W cells was recovered in the lipoprotein fraction floated to the top (Fig. 2d), indicating that apoE is secreted to generate HDL with cellular lipid as previously reported [9]. Message of apoE markedly increased in M/W cells in comparison to W/W cells when examined by RT-PCR (Fig. 2e). Thus, the increased apoE biosynthesis and secretion appear to cause M/W cells to release more cholesterol as lipoprotein, resulting in the decrease of cellular cholesterol.

Syntheses of mevalonic acid and cholesterol from [^{3}H]acetate were more active in M/W cells than W/W cells (Fig. 3a and b) while cholesterol synthesis from mevalonate was not significantly different between M/W and W/W cells (Fig. 3c). The direct measurement of the HMG-CoA reductase activity showed that it was higher in M/W cells than W/W cells (Fig. 3d). The results thus showed that cholesterol biosynthesis in M/W cells was increased by upregulation of HMG-CoA reductase activity, perhaps due to the active HDL assembly by enhanced apoE synthesis and cholesterol release.

3.3. Effects of the conditioned medium and cytokines

In order to investigate the mechanism for this change, we examined involvement of a trophic factor(s) potentially secreted into the medium during the long-term primary culture of rat brain cells. The conditioned media of W/W cells and the 2-week and 1-month primary cultures (W/W-CM, 2W-CM and M-CM, respectively) were given to W/W cells and incubated for 2 weeks. Cholesterol release from W/W cells was increased by the pretreatment with M-CM (Fig. 4a and b). ApoE secretion from W/W cells was also stimulated by M-CM in the same manners (Fig. 4c and d). The effect of 2W-CM was inconclusive by showing apparent inconsistency between cholesterol release and apoE secretion, presumably due to technical limitation. These findings indicated that M-CM contains a trophic factor(s) to stimulate W/W cells to increase the release of apoE and

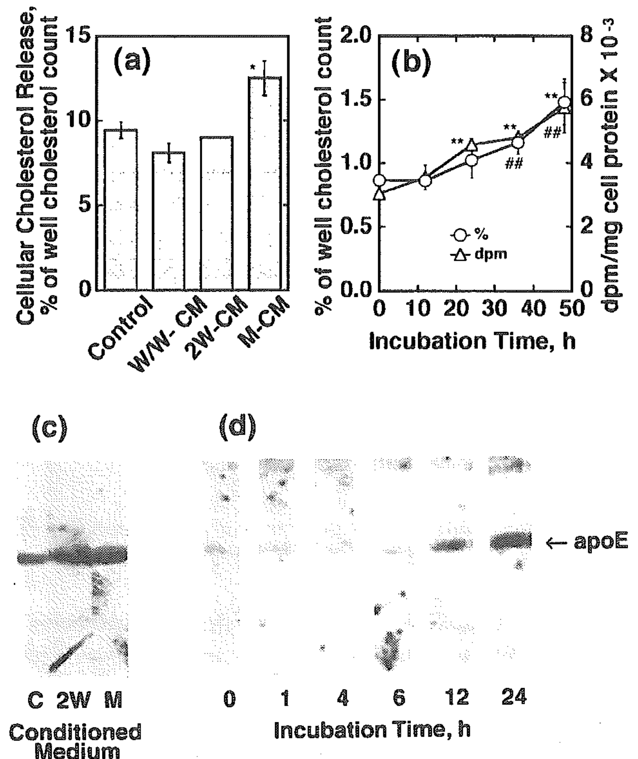

Fig. 4. Stimulation of cholesterol release from astrocytes by the conditioned medium of primary culture. (a) W/W cells were incubated for 2 weeks with 1 ml/2 ml of the conditioned medium prepared from the 2-week primary culture or the 1-month primary culture of rat brain cells (2W-CM and M-CM, respectively). The cells in the control experiment were incubated with 2 ml of 2% FCS/F-10 (control) or 1 ml/2 ml of the conditioned medium from the W/W cells (W/W-CM). The replacement of the stimulants in fresh 0.02% BSA/F-10 was carried out at every 2 days. After the treatment, the cells were washed with DPBS three times and cultured in 0.02% BSA/F-10 for 24 h. The cells were then incubated with 25 μg protein/ml of [^{3}H]-LDL in fresh medium for 24 h, followed by washing, replacement with the fresh medium and further 24-h incubation and then the release of the labeled cholesterol into the medium was analyzed. (b) The cells were incubated with M-CM (0.5 ml/ml) for the indicated time period. After washing and replacement with fresh 0.02% BSA/F-10, the cells were incubated with 40 μCi/ml of [^{3}H]-acetate for 12 h and washed three times. The cells were incubated for 12 h in the fresh 0.02% BSA/F-10 medium containing 1 mM acetate and release of the radioactive cholesterol in the medium was determined after separation by TLC. Cholesterol release was calculated as a percentage of free cholesterol in the medium against the free cholesterol radioactivity of the sum of the cell and medium, and * indicates $P < 0.05$ from control in panel (a). The release of cholesterol was shown by the same percentage and by the free cholesterol count released into the medium per cell protein, and ** and ## indicate $P < 0.01$ from control in the panel (b). The data represent the average and S.E. of the triplicate samples in the both panels. (c) Stimulation of apoE secretion from rat astrocytes by the conditioned medium of rat brain primary culture. W/W cells were incubated for 2 weeks with 2W-CM, M-CM or 2% FCS/F-10 as described in Fig. 2c. The astrocytes were washed with DPBS for four times and cultured in 0.02% BSA/F-10 for 24 h. The cells were washed again and further incubated for 24 h in the fresh 0.02% BSA/F-10 medium, and apoE in the medium was analyzed by Western blotting. The data represent one of the three experiments that all gave similar results, and the digital scanning indicated relative density of the apoE bands 1:2.4:3.2 for C/2W/M. (d) The medium was recovered from the 24-h cultured W/W cells after the stimulation by M-CM for the indicated period of time and washing, and apoE in the medium was analyzed by Western blotting.

cholesterol. The astrocytes appeared to be activated after the stimulation based on their morphological change.

We searched potential trophic factor(s) contained in M-CM to increase both apoE secretion and cholesterol release among several candidates that reportedly function in the brain (Fig. 5). Fig. 5a shows that among the examined trophic factors, aFGF, basic FGF, insulin-like growth factor 1, interleukin-1β and insulin, only aFGF enhanced the cholesterol release (Fig. 5a and b). Acidic FGF also increased syntheses of cholesterol and mevalonate in a dose-dependent manner (Fig. 5c and d). ApoE secretion by W/W cells and the message of apoE in W/W cells were both increased by aFGF (Fig. 5e and f). ApoE and cholesterol formed simultaneous peaks in density gradient analysis of the medium with and without stimulation by aFGF (Fig. 6), showing that aFGF increased the generation of HDL in the medium. Morphological evidence also indicated that the cells are activated by aFGF (data not shown). Thus, aFGF

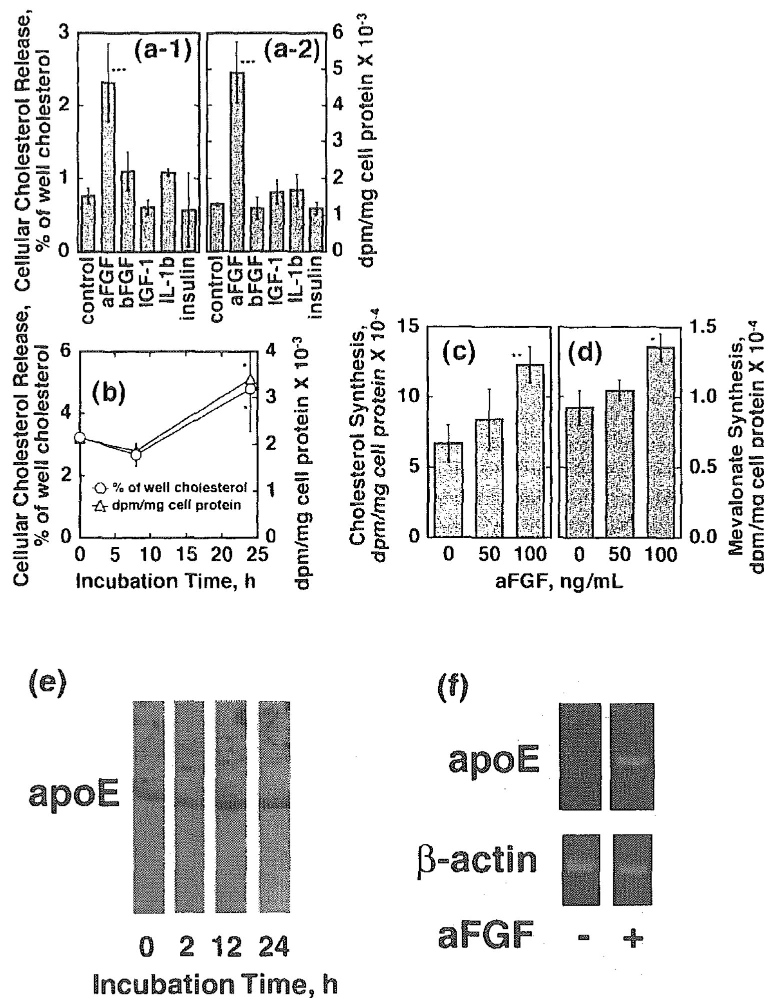

Fig. 5. Effect of growth factors on the synthesis and release of cholesterol of W/W cells. (a) After incubation in 0.02% BSA/F-10 for 24 h, W/W cells were treated with various growth factors such as aFGF, basic(b)FGF, insulin-like growth factor (IGF)-I, interleukin-1β (IL-1B), or insulin (each 100 ng/ml) for 24 h. After washing, the cells were labeled with 40 μCi/ml of [^{3}H]-acetate for another 12 h in the presence of each growth factors. The cells were washed and incubated for 8 h in the fresh medium containing 1 mM acetate and each growth factor. Lipid was extracted from the medium and cells and radioactivity in free cholesterol was analyzed by TLC. The results are expressed in both the percentage of the released cholesterol to the total well cholesterol (a-1) and the count of cholesterol per cell protein (a-2). (b) W/W cells were incubated with 100 ng/ml of aFGF for 0, 8 or 24 h, and labeled with 40 μCi/ml of [^{3}H]-acetate for 12 h after washing. The release of the labeled free cholesterol into the medium was determined for 12-h incubation in a fresh medium in the presence of 1 mM acetate. The results are shown in the two different ways again. (c and d) W/W cells were incubated with aFGF at the indicated concentration in 0.02% BSA/F-10 medium for 24 h and incubated with 20 μCi of [^{3}H]-acetate for 3 h in the fresh medium. The newly synthesized cholesterol (c) and mevalonic acid (d) in the cells were detected by counting radioactivity in each compound. Each data represents the average and standard error of the triplicate experiments, and ** and *** indicate $P<0.05$ and $P<0.01$ from control or time zero. Single asterisks in panels (b) and (d) indicate $P=0.056$ and $P=0.051$, respectively. (e) Stimulation of apoE secretion from W/W cells by aFGF and by the conditioned medium. W/W cells were incubated in 0.02% BSA/F-10 containing 50 ng/ml of aFGF for the indicated period of time. The cells were washed and incubated for further 24 h in the fresh medium, and the conditioned medium was used for immunoblotting analysis. The data represent one of the three experiments that all gave similar results, and the digital scanning indicated relative density of the apoE bands 1.0:1.3:1.7:2.0 for 0:2:12:24. (f) Messenger RNA in W/W cells treated with 100 ng/ml aFGF for 24 h. The experimental condition was the same as Fig. 2e.

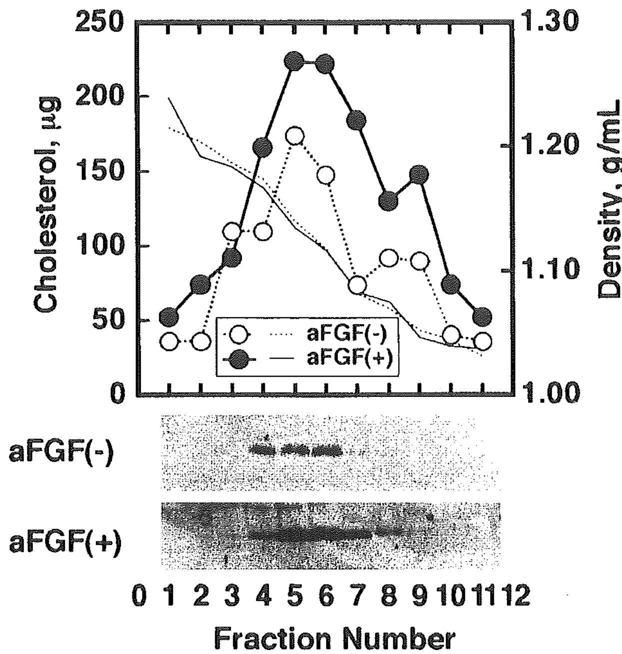

Fig. 6. Density gradient ultracentrifugation analysis of the culture medium of W/W cells treated with aFGF. After washing and preincubation in 0.1% BSA/F10 for 24 h, W/W cells in fresh 0.02% BSA/F10 medium were incubated with and without 100 ng/ml of aFGF for 24 h. The culture medium was analyzed by density gradient ultracentrifugation between the densities as described in the text. Each tube was fractionated from the bottom into 11 fractions. Cholesterol was measured by enzymatic colorimetric assay and apoE was analyzed by using an immunoblotting technique for each fraction. Lines without symbol indicate the density of each fraction.

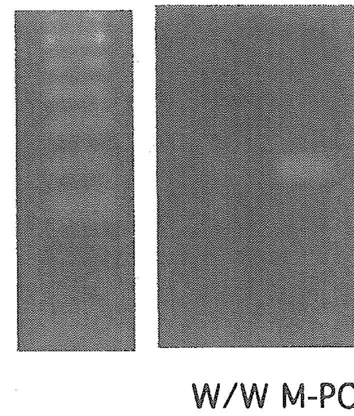

Fig. 8. Expression of aFGF mRNA in the rat brain cells of the 1-month-primary culture (M-PC). Total cellular RNA was extracted from the cells of M-PC and W/W cells as described in the text. RT-PCR was carried out by using aFGF primer pairs (5′-AAGCCCGTCGGTGTCCATGG and 5′-GATGGCACAGTGGATGGGAC) [74] with 30 cycles according to the methods as described in Fig. 2.

reproduced the effect of M-CM on W/W cells with respect to the increase of apoE biosynthesis and its release, and of the release and biosynthesis of cholesterol.

The M-CM was pretreated with an anti-aFGF antibody immobilized on Protein G-Sepharose in order to examine whether the stimulating effect of M-CM is related aFGF. The treatment resulted in the complete loss of the activity of M-CM to stimulate W/W cells to increase of the release of cholesterol and

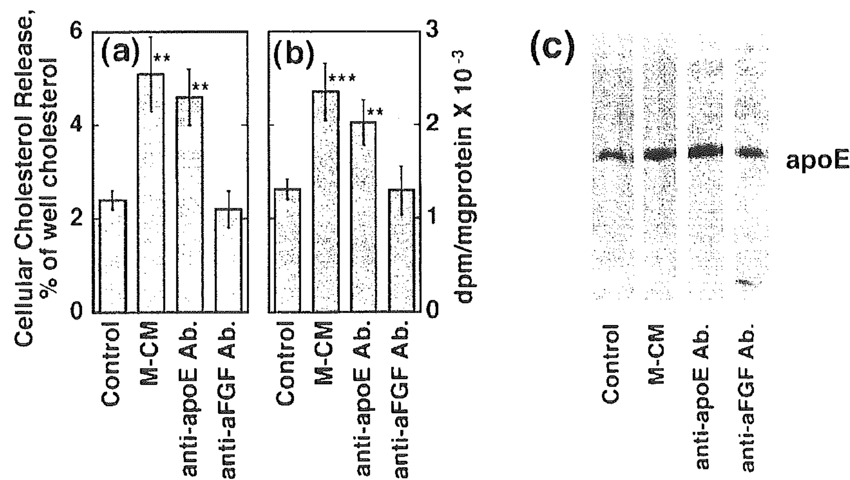

Fig. 7. Inhibition by an anti-aFGF antibody of the enhancement of cholesterol release and apoE secretion by the conditioned medium of the 1-month primary culture (M-CM). (a and b) The M-CM was treated with a goat anti-human aFGF antibody (Santa Cruz Biotech., Inc.) or a rabbit anti-rat apoE antibody conjugated on protein G-Sepharose (Amersham Pharmacia Biotech.) for 4 h at room temperature, and the gels were removed by centrifugation. W/W cells were incubated for 5 days with 0.5 ml/ml of M-CM, the medium pretreated by either antibody or 0.02% BSA/F-10 as a control. The cells were labeled with 30 μCi/ml of [^{3}H]-acetate for 12 h, washed three times with DPBS and incubated in 0.02% BSA/F-10 containing 1 mM acetate for further 12 h. The release of newly synthesized cholesterol into the medium was determined by counting the radioactivity in cholesterol. The results were expressed as percentage to total free cholesterol count in the well and as the count per cell protein. Each data represents the average and standard error of the triplicate experiments, and ** and *** indicate $P<0.05$ and $P<0.01$ from control in the panels. (c) Stimulation of apoE secretion from W/W cells by M-CM, and its inhibition by an anti-aFGF antibody. W/W cells were incubated for 5 days in the fresh 0.02% BSA/F-10 medium containing the indicated conditioned medium. The cells were washed and incubated for further 24 h, and the conditioned media was analyzed by immunoblotting. The data represent one of the three independent experiments.

apoE (Fig. 7a and b). Expression of aFGF mRNA was apparent in the cells after 1-month primary culture by RT-PCR while it was very faint in W/W cells (Fig. 8).

We thus concluded that an aFGF-like trophic factor(s) was released into the medium of the 1-month primary culture of rat brain cells and enhanced biosynthesis and secretion of apoE, release of cellular cholesterol, and perhaps subsequent decrease of cellular cholesterol level and increase of cholesterol biosynthesis, in rat astrocytes.

4. Discussion

Regulation of biosynthesis and secretion of apoE and generation of HDL particles with cellular lipid in the brain is one of the key factors for cholesterol homeostasis in the CNS, and plays an important role especially in development and recovery from injury [18] though it may not be an absolute requirement [6,17,39,40]. We investigated the mechanism for this regulation in astrocytes, a major site of the synthesis of apoE and generation of HDL. We focused on searching a potential trophic factor(s) involved in regulation of syntheses and secretion of apoE and HDL, by employing extended primary culture of the brain cells that might lead to a different stage of differentiation/activation of astrocytes. The findings are summarized as follows: (1) apoE biosynthesis, its secretion as HDL with cellular cholesterol and cholesterol biosynthesis were all increased in the astrocytes prepared after a month-long primary culture of rat brain cells (M/W cells) in comparison to the cells prepared by conventional 1-week primary and 1-week secondary cultures (W/W cells), and consequently, cellular cholesterol in M/W cells was found to decrease; (2) the conditioned medium of the 1-month-long primary culture (M-CM) stimulated W/W cells to gain the same properties of M/W cells with respect to the apoE and cholesterol metabolism; (3) aFGF stimulated W/W cells in the same manner as M-CM did, and the treatment of the medium with an anti-aFGF antibody abolished its stimulatory effects.

Decrease of vimentin, increase of GFAP and an appearance of type-2 astrocyte-like cells were observed in M/W cells. Vimentin decreases and GFAP increases in the brain during the CNS development [41]. In cultured astrocytes, glia maturation factor and cAMP increased GFAP and type-2 astrocytes [42,43]. Thus, low vimentin level and high GFAP level in M/W cells indicate activation or maturation of astrocytes during the long time primary culture. The factors secreted into the medium during this activation/maturation was examined with respect to stimulation of synthesis and secretion of apoE and cholesterol, in order to investigate whether an extracellular factor is involved in this change. M-CM was shown to induce the increase of these reactions in W/W cells. The results thus strongly indicated that the increase of apoE-HDL release by astrocyte after a long primary culture is a result of a process that involves stimulation of the cells by a trophic factor(s) released by the brain cells. The factor was identified as an aFGF-like factor(s) by the fact that stimulatory effects of M-CM were reproduced by aFGF and removed by an anti-aFGF antibody. Expression of aFGF mRNA was demonstrated in the cells after the 1-month primary culture by RT-PCR. Therefore, we conclude that an aFGF-like trophic factor(s), presumably produced and released into the medium during the primary culture of the brain cells, is responsible for stimulation of apoE synthesis, HDL generation and subsequent changes in cholesterol homeostasis in the astrocytes.

Several cytokines have been examined for the effect on astrocytes in terms of the secretion of apoE [44]. Epidermal growth factor reportedly increased apoE secretion by human astrocytes of high passage, whereas interleukin 1α and 1β, interferon γ, and basic FGF did not. Acidic FGF, a heparin-binding growth factor 1, is known as a potent mitogen for normal and transformed glial cells, and induces the morphological differentiation of these astroglial cells [45–49]. It has been thought that aFGF is primarily produced in neurons in vivo [50–55], but astrocytes are also identified as its potential source [56–59]. We have not yet determined which cell produces aFGF-like trophic factor in the long-term primary culture of fetal rat brain cells. However, neurons almost disappear during the initial 2 weeks of the primary culture and were hardly identified both in W/W and in M/W cells (Table 1). Therefore, it is likely that aFGF produced and released by astrocytes acts in an autocrine manner to stimulate apoE synthesis and HDL production.

Function of apoE and apoE-HDL in the CNS has not been fully understood. Many reports indicated the importance of this system in recovery from the injury of the nerve system by showing the increase of the production and secretion of apoE during and after the nerve degeneration in the CNS or in chronic degenerative disease of the brain [3,11,13–18]. Such a condition may cause astrocytes to produce and release aFGF to result in autocrine stimulation of apoE biosynthesis. The lipoprotein may be used as a cholesterol carrier to support neurite outgrowth stimulated by nerve growth factor [60]. The findings that aFGF enhanced production and secretion of nerve growth factor by astrocytes is consistent with this hypothesis [61–63]. Acidic FGF stimulates p21^{ras}/Erk signaling pathway in rat astrocytes [64], so that it at least acts through the membrane receptors, and less likely through FGF receptor 1 ,which is reportedly present predominantly in nuclei [65].

Injury of neurites induced expression of aFGF also in neurites and Schwann cells in vitro [66]. Acidic FGF appears to be highly expressed in neurons surviving in Alzheimer's disease [67], and is immunologically detected in the neurons of rat brain after experimental cerebral infarction [68]. Therefore, aFGF may also act as a paracrine cytokine released also from neurons to stimulate astrocytes.

Interestingly, aFGF does not have a signal sequence so that it is unlikely to be secreted by a regular secretory pathway [69,70]. Some specific mechanisms may therefore be required for the release of this cytokine such as an

increase of the membrane permeability or simple disruption of the membrane [71,72]. Although the astrocytes are most likely to release aFGF in the current experimental system, it is still important to identify which cells produce aFGF during the long-term primary culture of fetal rat brain cells and how it is released into the medium, in order to understand the mechanism for cholesterol homeostasis in the CNS. Physiological relevance of the current findings remains to be confirmed by demonstrating the parallel observations in vivo, such as the increase of aFGF in astrocytes in the brain after certain types of stress or injury. Our preliminary results indicate the post-injury increase of aFGF in astrocytes in the region of the mouse brain. Investigations are currently ongoing to answer these questions.

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Apolipoprotein-mediated Cellular Lipid Release Requires Replenishment of Sphingomyelin in a Phosphatidylcholine-specific Phospholipase C-dependent Manner*

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When sphingomyelin is digested by sphingomyelinase in the plasma membrane of rat astrocytes, productions of sphingomyelin, diacylglycerol, and phosphatidylcholine are stimulated. D609, an inhibitor of phosphatidylcholine-specific phospholipase C, suppressed these effects. Similarly, when apolipoprotein A-I removed cellular cholesterol, phosphatidylcholine, and sphingomyelin to generate high density lipoprotein, cholesterol synthesis from acetate subsequently increased, and sphingomyelin synthesis from acetate and serine also increased. D609 inhibited these effects again. D609 also inhibited the cholesterol removal by apoA-I not only from the astrocytes but also from BALB/3T3 and RAW264 cells. D609 decreased cholesterol synthesis, although D609 did not directly inhibit hydroxymethylglutaryl-CoA reductase. ApoA-I-stimulated translocation of newly synthesized cholesterol to cytosol was also decreased by D609. A diacylglycerol analog increased the apoA-I-mediated cholesterol release, whereas ceramide did not influence it. We concluded that removal of cellular sphingomyelin by apolipoproteins is replenished by transfer of phosphorylcholine from phosphatidylcholine to ceramide, and this reaction may limit the removal of cholesterol by apoA-I. This reaction also produces diacylglycerol that potentially triggers subsequent cellular signal cascades and regulates intracellular cholesterol trafficking.

Plasma high density lipoprotein (HDL)[1] is mainly generated by the interaction of helical apolipoproteins with cells to remove their phospholipid and cholesterol (1). This reaction requires a key membrane protein, ATP-binding cassette transporter A1 (ABCA1) (2–5). The mechanism for this HDL assembly with cellular lipid and apolipoproteins is not fully understood, and a role of ABCA1 in this reaction is also unclear at the current stage. Most of somatic cells are capable of generating HDL by this pathway, and this is one of the main mechanisms for exporting cholesterol of peripheral cells to the liver, its major catabolic site to bile acids. However, the principal organ of the production of HDL by this mechanism also seems to be the liver (6).

On the other hand, astrocytes generate HDL *in vitro* with apolipoprotein (apo) E endogenously synthesized and with other exogenously given helical apolipoproteins such as apoA-I (7). The HDL particles thus produced are thought to function as a main extracellular lipid carrier in the brain. HDL production with endogenous apoE is up-regulated by acidic fibroblast growth factor released presumably by the astrocytes themselves potentially by an autocrine mechanism upon the requirement such as the case of brain injury (8).

Whereas HDL assembled with endogenous apoE is rich in cholesterol, exogenous apolipoproteins generate HDL relatively cholesterol-poor (7). Digestion of sphingomyelin in the cell surface results in enrichment with cholesterol of the HDL assembled with exogenous apoA-I, and the replenishment of sphingomyelin restored the original profile of the production of cholesterol-poor HDL (9). Therefore, the restriction of cholesterol molecules by sphingomyelin in the membrane can be one of the factors to regulate the release of cell cholesterol by an apolipoprotein-mediated pathway.

Sphingomyelin interacts with cholesterol in the membrane and forms a cluster domain rich in cholesterol and sphingolipids (10–12). This is a basic driving force to create a domain structure called rafts or caveolae in the plasma membrane, which are thought to participate in various specific cellular functions such as signal transduction and cholesterol trafficking (13, 14). The results above indicated that the cholesterol molecules assembled to HDL with exogenous apolipoproteins are also provided from these domains. The apolipoprotein-cell interaction removes phosphatidylcholine and sphingomyelin as major phospholipids to generate HDL. Removal of sphingomyelin was parallel with the cholesterol removal under certain conditions (15, 16).

The other important factor of this reaction is involvement of intracellular signal transduction to mobilize cell cholesterol for incorporation into the HDL. Protein kinase C is indicated as one of the signal mediators for this pathway, but the details of this aspect remain unclear (17, 18).

In this paper, we have investigated a role of sphingomyelin and its metabolism in the release of cholesterol by the apolipoprotein-mediated HDL assembly pathway. The experimental data implicated that the compensatory synthesis of sphingomyelin for its removal is required for continuous release of cholesterol by apolipoprotein, and this reaction produces diacylglyceride from phosphatidylcholine, which can be a potential trigger of intracellular signal transduction.

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[1] The abbreviations used are: HDL, high density lipoprotein; apo, apolipoprotein; ABCA1, ATP-binding cassette transporter A1; FCS, fetal calf serum; LDL, low density lipoprotein; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; HMG-CoA, hydroxymethylglutaryl-CoA; SPMase, sphingomyelinase.

FIG. 1. **Increase of production of sphingomyelin, phosphatidylcholine, and diacylglyceride by the treatment with sphingomyelinase (SPMase) of rat astrocytes.** The cells were washed with DPBS/G and incubated in F-10 medium containing 0.02% bovine serum albumin (0.02% BSA/F-10) in the presence (*closed circles*) or absence (*open circles*) of 100 milliunits of sphingomyelinase for 1 h. The cells were washed with DPBS/G three times and incubated with 20 μCi/20 μM/ml [^{3}H]acetate (*A* and *B*) or 10 μCi/ml of [^{3}H]glycerol (*C*) for 1, 3, 5, and 7 h. Lipid was extracted from the whole cells with organic solvent as described in the text. After separating the lipid by TLC, radioactivity in sphingomyelin (*A*), phosphatidylcholine (*B*), and diacylglyceride (*C*) was determined. The data represent the average ± S.E. of triplicate assays. * and ** indicate $p < 0.05$ and 0.01 from the cells without the SPMase treatment.

FIG. 2. **Effect of D609 on the sphingomyelin (SPM) replenishment reaction.** Rat astrocytes were incubated in 0.02% BSA/F-10 medium containing [^{14}C]serine (2 μCi/ml) for 16 h. The cells were washed and treated with (*dark bars*) or without (*light bars*) 100 milliunits of sphingomyelinase for 1 h (*A* and *B*), followed by washing and replacement with a 0.02% BSA/F-10. The cells were then incubated with or without D609 (50 μg/ml) for 3 h. Cellular lipid was extracted and analyzed by TLC for radioactivity of sphingomyelin (*A*) and ceramide (*B*). Alternatively, the cells were incubated in a fresh 0.02% BSA/F-10 containing [^{3}H]acetate (40 μCi/ml) for 3 h, washed, and treated with (*closed circles*) or without (*open circles*) 100 milliunits of sphingomyelinase for 2 h. After washing, the cells were incubated in a fresh 0.02% BSA/F-10 with D609 at the indicated concentration for 2 h. Lipid was extracted from the cells, separated by TLC, and radioactivity of diacylglyceride (*DG*) (*C*) and sphingomyelin (*D*) was determined. Finally, the cells were incubated with (*dark bars* and *closed circles*) or without (*light bars* and *open circles*) 100 milliunits/ml of sphingomyelinase for 1 h. After washing, the cells were treated with D609 (0, 20 and 50 μg/ml) for 3 h in 0.02% BSA/F-10 again and then incubated with [^{3}H]acetate (20 μCi/ml) for 1 h. The cellular lipid was extracted, and the count in sphingomyelin was determined after separating by TLC (*E*). The same data were displayed as a percent decrease (*F*). The data represent the mean ± S.E. of triplicate assay.

EXPERIMENTAL PROCEDURES

Cell Culture and Conditioning—Rat fetal astrocytes were prepared as described elsewhere (19). The cerebra without surface blood vessels and meningeal layers were obtained from 17-day fetal Wistar rats. They were dissected into about 2-mm cubes and were treated with 0.1% trypsin solution in Dulbecco's phosphate-buffered saline containing 0.15% glucose (DPBS/G) at room temperature for 5 min. The cells were seeded in 10% fetal calf serum (FCS) in F-10 medium and cultured for 1 week (primary culture). After the treatment with 0.1% trypsin in DPBS/G again, the cells were cultured in 10% FCS/F-10 in a 6-well multiple tray (3-cm culture plates, Costar 3516) for 1 week. BALB/3T3 and RAW264 cells were obtained from the ATCC and maintained as described previously (16). The cells were treated with sphingomyelinase (Sigma), 100 milliunits, for 1 h, with human apoA-I isolated from human plasma HDL, 10 μg/ml, for appropriate period, or with tricyclodecan-9-yl-xanthogenate (D609)(Calbiochem), a competitive inhibitor of phosphatidylcholine-specific phospholipase C, 50 μg/ml (20–23).

Preparation of LDL Containing Radiolabeled Cholesteryl Ester—Lipoproteins of the plasma from a healthy volunteer were labeled with [1,2-^{3}H]cholesteryl oleate (Amersham Biosciences), and the LDL fraction was isolated by ultracentrifugation and dialyzed against 5 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, according to the method described previously (24).

Cellular Lipids Metabolism—Rat astrocytes at the confluent stage in 3-cm culture plates were washed three times with DPBS/G and then cultured in 1 ml of F-10 medium containing 0.1% bovine serum albumin (BSA) for 24 h. After replacement with 1 ml of the fresh medium, the cellular lipids were labeled by incubating for 1–24 h with 20 or 40 μCi/ml of [^{3}H]acetate (PerkinElmer Life Sciences) for cholesterol, phosphatidylcholine, sphingomyelin, and diacylglyceride, and 5 or 10 μCi/ml of [1(3)-^{3}H]glycerol (Amersham Biosciences) for diacylglyceride, 1 μCi/ml of L-[U-^{14}C]serine (Amersham Biosciences) for ceramide and sphingomyelin, or 25 μg of protein/ml of the labeled LDL for cholesterol. Lipid was extracted with hexane/isopropyl alcohol (3:2, v/v) or chloroform/methanol (2:1, v/v) from the cells or the conditioned medium, respectively. Radioactivity of cholesterol, phosphatidylcholine, sphingomyelin, diacylglyceride, and ceramide was measured by a liquid scintillation counter after separation by TLC with the solvents of diethyl ether, benzene, ethanol, and acetic acid (40:50:2:0.2) for separation of cholesterol and diacylglyceride; chloroform, methanol, acetic acid, and water (250:150:40:10) for phosphatidylcholine and sphingomyelin; 2-butanol, acetic acid, and water (3:1:1) for ceramide. Cellular protein was determined by the method of Lowry *et al.* (25). The experiments with other cell line cells were carried out in the same manner.

Assay of HMG-CoA Reductase Activity—HMG-CoA reductase assay was carried out according to the method described elsewhere (26). Briefly, the cell pellets were harvested by scraping with a rubber policeman and centrifugation at 1,000 × *g* for 10 min. The cell extracts were prepared by the treatment of the cell pellet with the buffer containing 50 mM imidazole, 5 mM EDTA, 200 mM KCl, and 0.25% Brij 97, pH 7.4, at 37 °C for 10 min and centrifugation at 12,000 × *g* for 15 min. The aliquot of the detergent-solubilized extract was incubated with 0.2 M potassium phosphate buffer containing 40 mM glucose 6-phosphate, 5 mM NADP, 8 mM dithiothreitol, 20 units/ml glucose-6-phosphate dehydrogenase, and 2 μCi/ml DL-3-[3-^{14}C]hydroxyl-3-methylglutaryl-CoA (American Radiolabeled Chemicals, Inc.) at 37 °C for 2 h. The reaction was terminated with 0.2 N HCl, and the mixture was incubated at 37 °C for 15 min to allow lactonization of [^{14}C]mevalonic acid. The labeled mevalonolactone was identified by TLC by using acetone/benzene (1:1, v/v).

Cytosol Preparation—The cytosol of rat astrocytes was prepared according to the method of Thom *et al.* (27) and Ito *et al.* (28). Briefly, the cells were harvested with a rubber policeman after washing four times with DPBS/G. The cell pellet prepared by centrifugation at 1,000 rpm for 10 min was treated with cold extract solution composed of 0.02 M boric acid, 0.3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine, pH 10.0, for 15 min with 25× strong agitation per 10 s every 5 min. The suspension was neutralized with 0.5 N HCl and then centrifuged at 3,000 rpm for 10 min. After removal of nuclei and cell debris, the suspension was centrifuged at 300,000 × *g* for 30 min at 4 °C to obtain supernatant as a cytosol fraction.

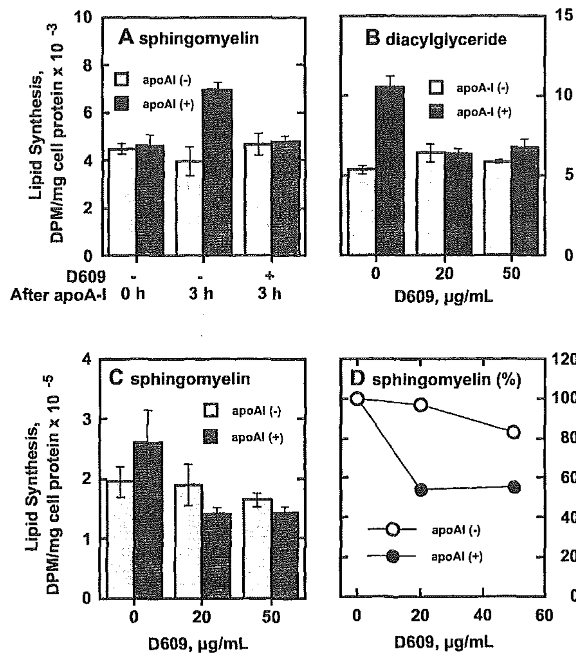

FIG. 3. **Effect of D609 on production of diacylglyceride and sphingomyelin in rat astrocytes when cellular lipid is removed by apoA-I.** *A*, the cells were labeled by incubating in 0.1% BSA/F-10 with [^{14}C]serine (2 μCi/ml) for 16 h. The cells were washed and then incubated with (*dark bars*) or without (*light bars*) 5 μg/ml apoA-I for 2 h in a fresh 0.02% BSA/F-10, followed by washing and the incubation with D609 (50 μg/ml) for 3 h. Lipid was extracted, and radioactivity was determined for sphingomyelin fraction. *B*, the cells were labeled in 0.1% BSA/F-10 with [^{3}H]acetate (20 μCi/ml) for 3 h, washed, and incubated in a fresh 0.02% BSA/F-10 with (*dark bars*) or without (*light bars*) apoA-I (5 μg/ml) for 2 h in the presence of D609 (0, 20, and 50 μg/ml). Lipid was extracted, and radioactivity in diacylglyceride was determined. *C*, the cells were incubated with (*dark bars* and *closed circles*) and without (*light bars* and *open circles*) apoA-I (5 μg/ml) in the presence of D609 (0, 20, and 50 μg/ml) for 2 h and then washed. The cells were then labeled with [^{3}H]acetate (20 μCi/ml) for 2 h in fresh 0.02% BSA/F-10 in the presence of D609 at the same concentration as the pretreatment. Radioactivity in cellular sphingomyelin was determined. *D*, the data in *C* are displayed as a percentage of the control. The data represent the mean ± S.E. of triplicate assay.

RESULTS

Rat astrocytes were treated with sphingomyelinase for 1 h, and incorporation of [^{3}H]acetate into sphingomyelin and phosphatidylcholine and incorporation of [^{3}H]glycerol into diacylglyceride were monitored in a time-dependent manner. Syntheses of all of these lipids were increased by enzyme treatment (Fig. 1). Increase was apparent with sphingomyelin (Fig. 1*A*) and diacylglyceride (Fig. 1*C*) at 3 h after the treatment, but it took over 5 h before the increase of phosphatidylcholine synthesis became detectable (Fig. 1*B*). After the cell was labeled with [^{3}H]serine, rat astrocytes were treated with sphingomyelinase and then incubated with D609 (Fig. 2, *A* and *B*). Radioactivity in sphingomyelin slightly decreased after 3 h perhaps reflecting the metabolic turnover. When sphingomyelin was digested, the count somewhat recovered after 3 h, and D609

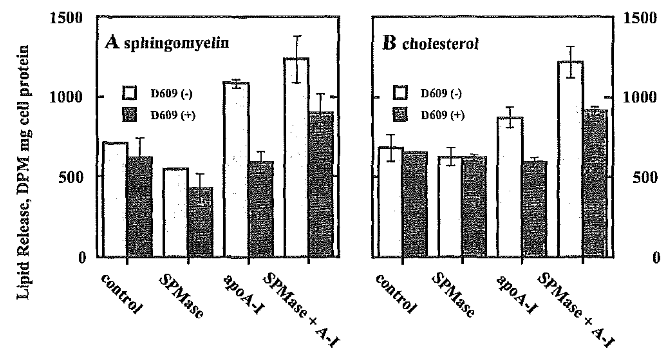

FIG. 4. **Effect of D609 on apoA-I-mediated cholesterol release of rat astrocytes treated with SPMase.** Rat astrocytes were incubated in a fresh 0.1% BSA/F-10 for 24 h. The cells were labeled with [^{3}H]acetate (40 μCi/ml) for 3 h in 0.02% BSA/F-10, washed, and treated with SPMase (0 or 100 milliunits) in the presence (*dark bars*) or absence (*light bars*) of 50 μg/ml of D609 for 1 h in a fresh 0.02% BSA/F-10. The cells were further incubated with apoA-I (5 μg/ml) in the presence or absence of 50 μg/ml of D609 for 4 h after washing. Lipid was extracted from the conditioned medium, and radioactivity in sphingomyelin (*A*) and cholesterol (*B*) was determined. The data represent the mean ± S.E. of triplicate assays.

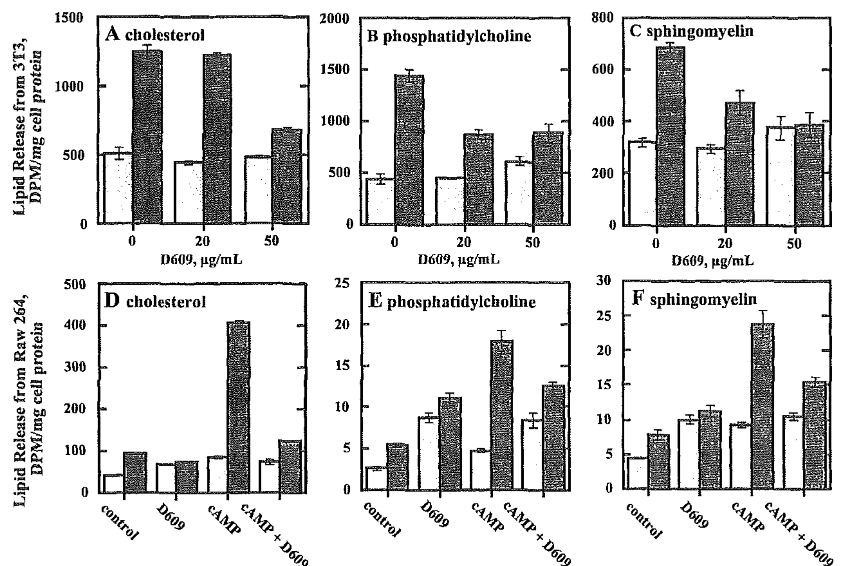

FIG. 5. **Effect of D609 on the apoA-I-mediated cholesterol release from BALB/3T3 and RAW264 cells.** BALB/3T3 cells were incubated with [^{3}H]acetate (20 μCi/ml) for 16 h in 0.1% BSA/F-10 then further incubated with (*dark bars*) or without (*light bars*) 5 μg/ml of apoA-I for 4 h in the presence of D609 (0, 20, or 50 μg/ml) in a fresh 0.02% BSA/F-10. Lipid was extracted from the conditioned medium, and radioactivity was determined in cholesterol (*A*), phosphatidylcholine (*B*), and sphingomyelin (*C*). RAW264 cells were incubated with dibutyryl cAMP (0 or 300 μM) and [^{3}H]acetate (20 μCi/ml) for 16 h in a fresh 0.1% BSA/F-10, washed, and further incubated with (*dark bars*) or without (*light bars*) apoA-I (5 μg/ml) in the presence or absence of 50 μg/ml D609 for 4 h in 0.02% BSA/F-10. Lipid was extracted from the conditioned medium, and radioactivity was determined in cholesterol (*D*), phosphatidylcholine (*E*), and sphingomyelin (*F*). The data represent the mean ± S.E. of triplicate assays.

inhibited this recovery (Fig. 2*A*). Radioactivity in ceramide reciprocally increased by D609 indicating that recovery reaction uses ceramide (Fig. 2*B*). D609 suppressed the incorporation of acetate into diacylglyceride, more greatly in the sphingomyelinase-treated cells than the untreated cells (Fig. 2*C*). Inhibition of sphingomyelin synthesis by D609 was also greater in the treated cells than the non-treated cells (Fig. 2, *D–F*). The results indicated that acute decrease of sphingomyelin by its digestion was rescued by the pathway including the transfer reaction of phosphorylcholine from phosphatidylcholine to ceramide. The decrease in diacylglyceride production by D609 was consistent with the notion that it inhibits phosphatidylcholine-specific phospholipase C, and the release of diacylglyceride was reduced.

When the cells were incubated with apoA-I to induce the removal of cellular lipid, incorporation of serine into sphingomyelin (Fig. 3, A, *C*, and *D*) and production of diacylglyceride (Fig. 3*B*) were all increased, and these increases were suppressed by D609. The responses were very similar to those after the sphingomyelinase treatment, indicating that the reduction of sphingomyelin after the removal by apoA-I is compensated by the same phosphorylcholine transfer pathway from phosphatidylcholine. Sphingomyelin synthesis was inhibited by D609 only to the extent to cancel the increase by apoA-I (Fig. 3, *A* and *D*), indicating that acute replenishment of sphingomyelin is dependent on phosphatidylcholine-specific phospholipase C, but the base-line synthesis is rather by other pathways. As shown in Fig. 4, the releases of cholesterol and sphingomyelin by apoA-I were both suppressed by D609 regardless of the pretreatment of the cells with sphingomyelinase. Inhibition of the apoA-I-mediated lipid release by D609 was observed in a similar manner with other cell lines such as BALB/3T3 and dibutyryl cAMP-treated RAW264 cell (Fig. 5). Thus, replenishment of sphingomyelin is required for continuation of cholesterol removal by apoA-I.

Inhibition of the cholesterol release by D609 resulted in suppression of cholesterol biosynthesis, not only at the step of mevalonate synthesis but also further downstream of the pathway (Fig. 6, *A–C*), although D609 does not show a direct inhibitory effect on HMG-CoA reductase (Fig. 6*D*). Thus, D609 indirectly influenced the cholesterol biosynthesis pathway through modulation of the apoA-I-mediated change of intracellular cholesterol homeostasis. Translocation of newly synthesized cholesterol to the cytosol was markedly inhibited by D609 along with significant suppression of the apoA-I-mediated translocation of sphingomyelin and phosphatidylcholine to the cytosol (Fig. 7).

It has been indicated that apolipoprotein-mediated cellular lipid release involves intracellular signal transduction (17, 18, 29–32). When sphingomyelin is removed by apoA-I, the replenishment reaction would modulate the levels of the lipid signal mediators by producing diacylglycerol and using ceramide. The direct effect of these compounds was therefore estimated. As shown in Fig. 8, C_6-ceramide failed to decrease the apoA-I-mediated cholesterol release. On the other hand, the effect of phorbol 12-myristate 13-acetate (PMA) that mimics diacylglyceride was positive on the apoA-I-mediated cholesterol release (Fig. 8*C*). These findings suggested that the increase of diacylglyceride by D609 rather than consumption of ceramide may also be a factor that influences the apoA-I-mediated cholesterol release.

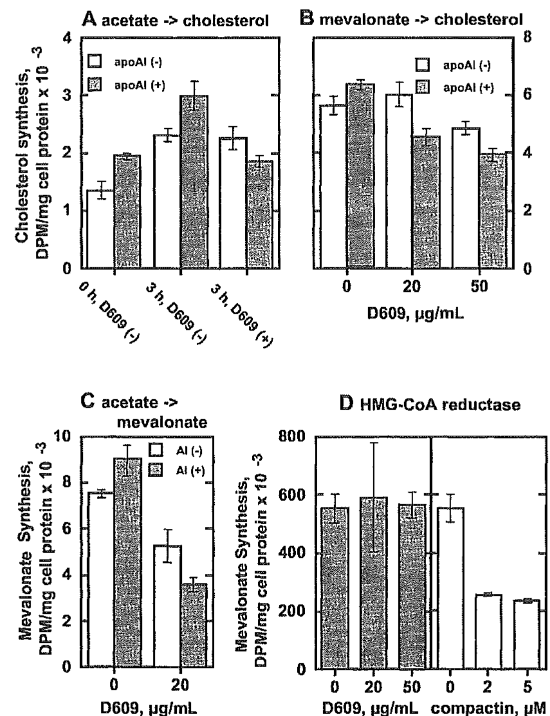

FIG. 6. **Effect of D609 on cholesterol biosynthesis when apoA-I removes cellular lipid.** *A*, rat astrocytes were incubated with (*dark bars*) or without (*light bars*) apoA-I (5 µg/ml) for 3 h, followed by the incubation with [^{3}H]acetate (20 µCi/ml) for 30 min and washing. The cells were then incubated with or without D609 (50 µg/ml) for 3 h in a fresh 0.02% BSA/F-10. Lipid was extracted from the cells, and radioactivity in cholesterol was determined. *B*, rat astrocytes were incubated with (*dark bars*) or without (*light bars*) 5 µg/ml of apoA-I for 3 h in a fresh 0.02% BSA/F-10, washed, and labeled with [^{3}H]mevalonate (8 µCi/ml) for 1 h in the presence of D609 (0, 20, or 50 µg/ml) in 0.02% BSA/F-10. Lipid was extracted from the cells, and radioactivity in cholesterol was determined. *C*, rat astrocytes were treated with (*dark bars*) or without (*light bars*) apoA-I (5 µg/ml) in 0.02% BSA/F-10 for 3 h in the presence or absence of D609 (20 µg/ml) and then washed. The cells were incubated with [^{3}H]acetate (20 µCi/ml) for 2 h, followed by washing and incubation with 0.2 M HCl for 15 min. After sonication of the cell suspension, an aliquot of the supernatant of the centrifugation at 15,000 rpm for 30 min was analyzed by TLC to determine radioactivity in mevalonate. *D*, the cell pellet of rat astrocytes was prepared by scraping with a rubber policeman and centrifugation at 1,000 × *g* for 10 min and was treated with a buffer containing 50 mM imidazole, 5 mM EDTA, 200 mM KCl, and 0.25% Brij 97, pH 7.4, at 37 °C for 10 min followed by centrifugation at 12,000 × *g* for 15 min. The aliquot of the detergent-solubilized extract was incubated with the buffer containing 0.2 M potassium phosphate, 40 of glucose 6-phosphate, 5 mM NADP, 8 mM dithiothreitol, 20 units/ml glucose-6-phosphate dehydrogenase, and 2 µCi/ml DL-3-[3-^{14}C]hydroxy-3-methylglutaryl-CoA with or without D609 (0, 20 or 50 µg/ml) or compactin (0, 2, or 5 µM) at 37 °C for 2 h. The reaction was terminated with 0.2 N HCl, followed by incubation at 37 °C for 15 min. The labeled mevalonolactone was identified by TLC by using acetone/benzene (1:1, v/v). The data represent the mean ± S.E. of triplicate assays.

DISCUSSION

The results are summarized as follows. Digestion of sphingomyelin in plasma membrane stimulates the biosynthesis of sphingomyelin by the pathway of phosphorylcholine transfer from phosphatidylcholine to ceramide. The same cellular reaction is observed when apoA-I removes cellular sphingomyelin to produce HDL. This compensatory synthesis of sphingomyelin subsequently produces diacylglyceride. D609 has been first reported as an antiviral compound (20) and is widely accepted as a selective and competitive inhibitor of phosphatidylcholine-specific phospholipase C (21–23). This compound inhibited not only this pathway but also the apoA-I-mediated change in cellular cholesterol metabolism, its release, the increase of the biosynthesis, and its translocation to the cytosol.

We reported previously that sphingomyelin-cholesterol interaction in plasma membrane is one of the regulatory factors for the apoA-I-mediated release of cholesterol, especially in the astrocyte, by restricting cholesterol molecules (9). Massive

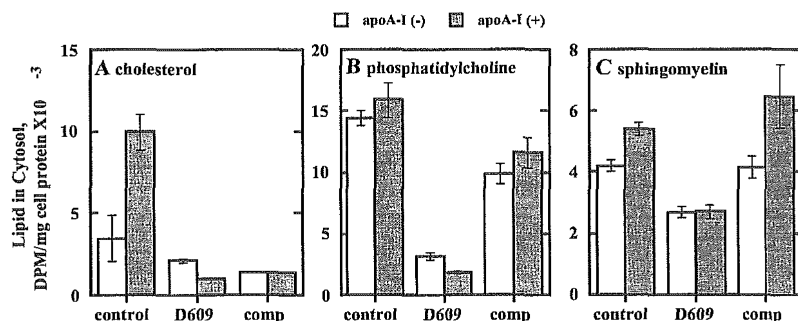

FIG. 7. **Cholesterol translocation to the cytosol by apoA-I.** Rat astrocytes were pulse-labeled with 20 μCi/ml [^{3}H]acetate in 0.02% BSA/F-10 in the presence and absence of D609 (50 μg/ml) or compactin (3 μM) for 3 h. After washing 3 times with DPBS/G, the cells were incubated with (*dark bars*) or without (*light bars*) 5 μg/ml apoA-I in 0.02% BSA/F-10 containing 1 mM sodium acetate for 90 min. Lipid was extracted with the chloroform/methanol (2:1) solution from the cytosol fraction prepared as described in the text, and radioactivity of sphingomyelin, phosphatidylcholine, or cholesterol was determined after separation by TLC. The data represent the mean ± S.E. of the triplicate samples.

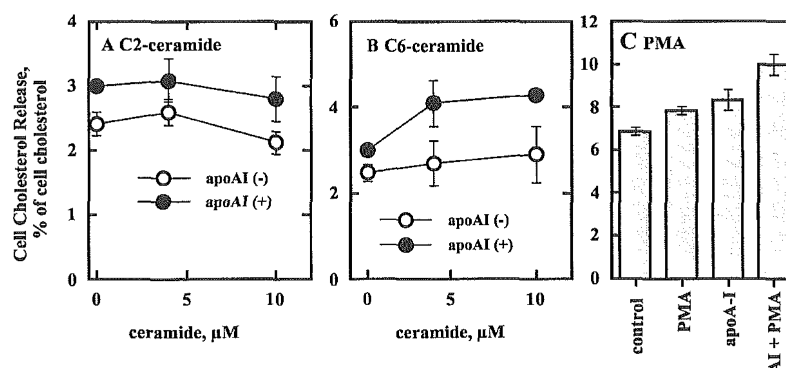

FIG. 8. **Effects of ceramide and PMA on the apoA-I-mediated cholesterol release.** Rat astrocytes were labeled with [^{3}H]acetate (20 μCi/ml) for 18 h in a fresh 0.1% BSA/F-10, washed, and stimulated by C_2- or C_6-ceramide at the indicated concentration for 1 h in fresh 0.02% BSA/F-10. The cells were then incubated with (*closed circles*) or without (*open circles*) apoA-I (5 μg/ml) for 7 h in fresh 0.02% BSA/F-10. Lipid was extracted from the conditioned medium and analyzed by TLC. Alternatively, the cells were incubated with LDL containing [^{3}H]cholesteryl ester (25 μg of protein/ml) in a fresh 0.1% BSA/F-10 for 24 h. After washing and incubation for 24 h in 0.1% BSA/F-10, the cells were treated with or without 50 nM PMA in a fresh 0.02% BSA/F-10 for 1 h. The cells were washed and then incubated with (*closed circles*) or without (*open circles*) 5 μg/ml apoA-I in 0.02% BSA/F-10 for 8 h. Lipid in the conditioned medium was extracted and analyzed by TLC for determination of radioactivity in cholesterol. The data were presented as the percentage of radioactivity of cellular cholesterol and represent the mean ± S.E. for triplicate assay.

digestion of sphingomyelin increased the incorporation of cholesterol into the HDL generated by exogenous apoA-I. Sphingomyelin is rapidly recovered in the cells, and the effect of the digestion on the apoA-I-mediated cholesterol release gradually diminished as the recovery goes. Exogenous supply of sphingomyelin also reversed the effect of digestion.

The data presented in this work indicated that the replenishment of sphingomyelin is carried out mainly by the transfer reaction of phosphorylcholine from phosphatidylcholine to ceramide. Interestingly, the apoA-I-mediated generation of HDL, which accompanies substantial removal of cellular sphingomyelin, also stimulates this sphingomyelin replenishment pathway. In apparent contrast to the reactions after massive digestion of sphingomyelin, continuous cholesterol release by apoA-I seems to require this replenishment of sphingomyelin. Therefore, the effect of sphingomyelin digestion seems mainly due to the relief of the cholesterol molecule and induction of its intracellular redistribution. On the other hand, cholesterol and sphingomyelin may be removed together from the membrane by the apoA-I-mediated reaction, and the endogenous supply of sphingomyelin seems to be required for continuation of this reaction.

The apoA-I-mediated cellular lipid release and assembly of HDL are accompanied by the related intracellular events of cholesterol metabolism, such as translocation of cholesterol from the compartment used by acyl-CoA:cholesterol acyltransferase (32, 33), and to the cytosol to form lipid-protein complex particles and the increase of biosynthesis of cholesterol (28). These related reactions are also suppressed by inhibiting the replenishment synthesis of sphingomyelin. The findings strongly implicate an essential role of sphingomyelin in the incorporation of cholesterol into the HDL generated by apolipoprotein-cell interaction. The intracellular cholesterol transport for the HDL assembly depends on the intracellular supply of sphingomyelin, so that formation of a cholesterol-sphingomyelin domain in the membrane may be important for the HDL assembly reaction.

It is indicated by a few authors including ourselves (17, 18, 29–32) that intracellular signaling is involved in activation of cholesterol trafficking for its incorporation into HDL. From this viewpoint, it is noteworthy that sphingomyelin synthesis reactions for the replenishment apparently involve lipid signal mediators. Phosphorylcholine is released from phosphatidylcholine by leaving diacylglycerol and is transferred to ceramide to produce sphingomyelin. The inhibition of apoA-I-mediated events by D609 can therefore be interpreted by a decrease in diacylglyceride or by an increase of ceramide. C_6-ceramide, a ceramide analog, did not influence the apoA-I-mediated cholesterol release, implying that ceramide is not involved in direct regulation of these reactions. On the other hand, a functional analog of diacylglyceride, PMA, stimulated the apoA-I-mediated cholesterol release, suggesting that increase of diacylglycerol may be related to the enhancement of intracellular cholesterol trafficking. This is also consistent with the previous reports that protein kinase C activation can be involved in mobilization of cellular cholesterol molecules for assembly of HDL.

Inhibition of sphingomyelin synthesis by D609 markedly inhibited cholesterol synthesis in rat astrocytes treated with apoA-I. It is generally accepted that syntheses of cholesterol and sphingomyelin are regulated independently by such evidence that suicide inhibitors of serine palmitoyltransferase, β-chloroalanine, and cycloserine do not alter cholesterol biosynthesis (34). However, some linkage between them can be considered as cholesterol synthesis was significantly increased when fibroblast membranes were enriched with sphingomyelin (35).

In the present work, suppression of cholesterol biosynthesis by inhibition of sphingomyelin synthesis did not accompany the decrease of HMG-CoA reductase. On the other hand, HMG-CoA reductase inhibitors did not influence phospholipid metabolism (data not shown). However, it is too premature to conclude about the mechanism for direct relationship between sphingomyelin metabolism and regulation of cholesterol homeostasis.

Luberto and Hannun (36) reported that D609 inhibited sphingomyelin synthase activity of SV40-transformed human lung fibroblasts but not phosphatidylcholine-specific phospholipase C activity, suggesting that the release of phosphorylcholine from phosphatidylcholine may be partially due to sphingomyelin synthase. Nevertheless, the present data suggested that the effect of D609 was achieved by inhibition of the release of phosphorylcholine from phosphatidylcholine no matter what the mechanism is.

For further confirmation of an important role of sphingomyelin replenishment in the apoA-mediated cholesterol release, more direct inhibition of sphingomyelin synthesis should be attempted.

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Reviews

The Very Low-density Lipoprotein (VLDL) Receptor: Characterization and Functions as a Peripheral Lipoprotein Receptor

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The very low-density lipoprotein (VLDL) receptor is a member of the low-density lipoprotein (LDL) receptor family. *In vitro* and *in vivo* studies have shown that VLDL receptor binds triglyceride (TG)-rich lipoproteins but not LDL, and functions as a peripheral remnant lipoprotein receptor. VLDL receptor is expressed abundantly in fatty acid-active tissues (heart, skeletal muscle and fat), the brain and macrophages. It is likely that VLDL receptor functions in concert with lipoprotein lipase (LPL), which hydrolyses TG in VLDL and chylomicron. In contrast to the LDL receptor, VLDL receptor binds apolipoprotein (apo) E2/2 VLDL particles as well as apoE3/3 VLDL, and the expression is not down-regulated by intracellular lipoproteins. Recently, various functions of the VLDL receptor have been reported in lipoprotein metabolism, metabolic syndrome/ atherosclerosis, cardiac fatty acid metabolism, neuronal migration and angiogenesis/ tumor growth. Gene therapy of VLDL receptor into the liver showed a benefit effect for lipoprotein metabolism in both LDL receptor knockout and apoE mutant mice. Beyond its function as a peripheral lipoprotein receptor, possibilities of its physiological function have been extended to include signal transduction, angiogenesis and tumor growth. *J Atheroscler Thromb, 2004; 11: 200–208.*

Key words: VLDL receptor, LDL receptor family, Lipoprotein metabolism, Signal transduction

Introduction

The low-density lipoprotein (LDL) receptor family is a growing receptor family composed of more than ten receptors. LDL receptor, a genetic defect of which induces familial hypercholesterolemia (FH), has been discovered and elucidated as an LDL receptor pathway (1, 2). Plasma LDL particles are recognized, internalized and degraded by the coated pit-located hepatic LDL receptors. FH heterozygotes (about 1 per 500 people) express half of the normal number of functional LDL receptors, and rare FH homozygotes (about 1 per million people) express few to no functional LDL receptors on their cell surface. Their plasma cholesterol levels rise from 300 to 500 mg/dl, and 600 to 1200 mg/dl, respectively. Even though the physiological functions of LDL receptor have been established, the functions of other LDL receptor family members are under investigation. In the LDL receptor family, the very

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low-density lipoprotein (VLDL) receptor and apolipoprotein (apo) E receptor 2 (apoER2) are most similar to the LDL receptor structurally (3). In this review, we describe a special feature of the VLDL receptor as a peripheral lipoprotein receptor.

Characterization of the VLDL Receptor

Cloning of the VLDL receptor

In 1998, Herz *et al.* cloned LDL receptor-related protein-1 (LRP-1), which is the second protein of the LDL receptor family from the human liver cDNA library (4). Its protein is a large cell surface protein containing 4,544 amino acids (to 600 kDa) and consists of five common structural domains resembling those of LDL receptor: (i) ligand-binding type cysteine-rich repeats (ii) epidermal growth factor (EGF) receptor-like cysteine-rich repeats (iii) YWTD domains (iv) a single membrane-spanning segment, and (v) a cytoplasmic domain. LRP-1 binds apoE-containing lipoproteins but not LDL, and functions as a remnant lipoprotein receptor in the liver in cooperation with LDL receptor. Recently, novel functions on amyloid β-protein and tissue-type plasminogen activator (tPA) in the brain also have been elucidated (5, 6).

At that time, we considered that LRP-1 was too large of a protein and speculated that another lipoprotein receptor would have a sequence and structure more similar to those of LDL receptor. To exclude the rabbit LDL receptor, the entire pooled cDNA library was digested with Sal I and recircularized with T4 DNA ligase. The presence of a unique Sal I site in the rabbit LDL receptor cDNA and the Okayama-Berg vector resulted in the loss of any LDL receptor cDNAs after recircularization and retransformation. The resulting LDL receptor-subtracted cDNA library was screened with the 1.9-kb Sma I-Sal I fragment from the rabbit LDL receptor cDNA under low-stringency hybridization conditions (7, 8). Surprisingly, a cloned new cDNA encoded a protein with striking homology to the LDL receptor (Fig. 1). We considered that the new gene was a brother of the LDL receptor but LRP-1 was a distant relative. The mature protein consisted of five domains spanning 846 amino acids: 328 N-terminal amino acids including an 8-fold repeat of 40 amino acids homologous to the ligand binding repeat of the LDL receptor; 396 amino acid residues homologous to the epidermal growth factor precursor including three cysteine-rich repeats; a region immediately outside of the plasma membrane, rich in serine and threonines; 22 amino acids transversing the plasma membrane; and 54 amino acids including the NPVY sequence required for clustering of the LDL receptor in coated pits and that projects into the cytoplasm. Following the rabbit VLDL receptor cDNA cloning, we also cloned the human VLDL receptor cDNA from the THP-1 monocytic leukemia cell cDNA library (9). The human VLDL receptor gene contains 19 exons spanning approximately 40 kb. The exon-intron organization of the gene is almost the same as that of the LDL receptor gene, except for an extra exon that encodes an additional repeat in the ligand-binding domain (LDL receptor contains a 7-fold repeat and VLDL receptor has an 8-fold repeat). The VLDL receptor mRNAs produce two kinds of VLDL receptor proteins by alternative splicing type 1 VLDL receptor and type 2 VLDL receptor, which lacks the O-linked sugar domain encoded by exon 16. Although the structure and organization of the VLDL receptor gene is highly similar to those of the LDL receptor gene, the two genes are located on different chromosomes: the LDL receptor gene is on chromosome 19 and the VLDL receptor gene on chromosome 9.

Tissue distribution

The VLDL receptor mRNAs are highly abundant in the heart, muscle, adipose tissue, and brain, and are barely detectable in the liver, in which the LDL receptors are expressed abundantly (7). We also confirmed the VLDL receptor expression in THP-1 cells, PMA-induced (THP-1) macrophages, HL-60 cells, human monocyte-derived macrophages, rabbit alveolar macrophages and rat cultured cardiomyocytes (9–13). Immunoreactive VLDL receptor protein was detected in the endothelium of the capillaries and small arterioles but not in the veins or venules of bovine skeletal muscle, heart, ovary and brain. The VLDL receptor was also detected at high levels on the endothelial surface of bovine coronary arteries but not in the aortic endothelium (14). Interestingly, the type 1 VLDL receptor containing the O-linked sugar domain was preferentially expressed in the heart, brain, and skeletal muscle, whereas bovine aortic endothelial cells expressed only type 2 VLDL receptor (15). In human and

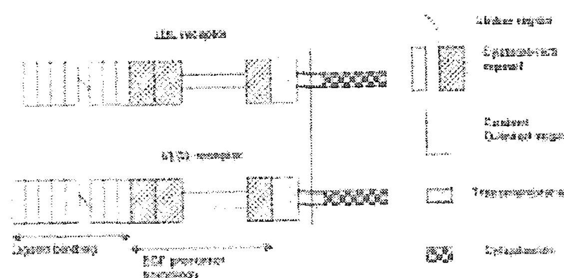

Fig. 1. Structure of LDL receptor and VLDL receptor.
The number of cysteine-rich repeats in the ligand-binding domain is different between the two receptors. LDL receptor contains a 7-fold repeat and VLDL receptor has an 8-fold repeat. The two receptors have eight and ten residues in the linker region, in the LDL receptor between repeats 4 and 5, and in the VLDL receptor between repeats 5 and 6.

rabbit atherosclerotic lesions, the VLDL receptor was expressed in macrophages and smooth muscle cells, and its expression was highly induced in atherosclerotic lesions (16–19). The VLDL receptor is most abundantly expressed in the heart. An in situ hybridization study revealed that VLDL receptor mRNA was detected within the human myocardium, and the hybridization pattern corresponded to that of myofibrils. In the human liver, the presence of VLDL receptor mRNA was only detected within vascular structures and sinusoidal lining cells (Kuppfer cells), with no signal detected in hepatocytes (16). In the human brain, the VLDL receptor was present on resting and activated microglia associated with senile plaques and cortical neurons. A novel splicing variant lacking exon 4 in the cysteine-rich repeats region was discovered by RT-PCR more often than type 1 and type 2 VLDL receptors, only in the brain (20).

Ligand-binding specificity of the VLDL receptor as a lipoprotein receptor

To confirm the ligand-binding specificity of the new cloned gene that was similar to the LDL receptor structurally, we transfected the cloned cDNA into ldlA-7 cells (LDL receptor-deficient CHO cells) and examined the ligand-binding specificity compared to that of LDL receptor transfectants. The new gene produced proteins that bound apoE-containing lipoproteins including VLDL, intermediate density lipoprotein (IDL) from Watanabe heritable hyperlipidemic (WHHL) rabbits and β-migrating VLDL (β-VLDL) from cholesterol-fed rabbits, but did not bind LDL from WHHL rabbits, whereas CHO cells transfected with the human LDL receptor cDNA bound both apoB- and apoE-containing lipoproteins including VLDL, IDL, LDL from WHHL rabbits and β-VLDL from cholesterol-fed rabbits. ^{125}I-labeled β-VLDL binding to the transfected cells was inhibited by unlabeled apoE-liposomes, indicating that the receptor recognizes apoE (7, 8). These binding properties were confirmed by a more detailed examination which indicated that the VLDL receptor never bound LDL. The VLDL receptor was more slowly processed than the LDL receptor, and did not show the increase in affinity and decrease in binding of β-VLDL on cooling to 4 degrees that was exhibited by the LDL receptor (21). On the other hand, the VLDL from fasted normal human subjects bound with lower affinity than the VLDL prepared from WHHL rabbits or the β-VLDL from cholesterol-fed rabbits. A ligand blot study clearly showed that human VLDL receptor bound β-VLDL from fasted cholesterol-fed rabbits with higher affinity than human fasted VLDL particles (Fig. 2A), and cold LDL completed ^{125}I-β-VLDL binding to the LDL receptor protein but not to the VLDL receptor protein (Fig. 2B). The low affinity binding of fasted human VLDL to the VLDL receptor could be overcome by enriching VLDL with either apoE or lipoprotein lipase (LPL) (22). There are three mechanisms between LPL and the VLDL receptor: (i) directly binding to the VLDL receptor through LPL, (ii) mediating the TG-rich lipoprotein particles through heparan sulfate proteoglycans, and (iii) its lipolytic activity, converting VLDL particles to smaller remnants (apoE-rich particles) before they can become endocytosed by VLDL receptor through apoE. Fluorescence microscopic examination indicated that Pseudomonas LPL that was dissimilar to human LPL structurally, but had a lipolytic activity, directly enhanced the binding of the fasted human VLDL to the VLDL receptor (Fig. 3). We consider that the third mechanism is the most important factor in VLDL receptor binding specificity. Niemeier A *et al.* showed the same mechanism for chylomicron particles. The VLDL receptor mediated the uptake of chylomicron remnant (CR), and this uptake was further increased by the addition of apoE and inactivated LPL (23). Taking into account that the VLDL receptor and LPL are expressed in

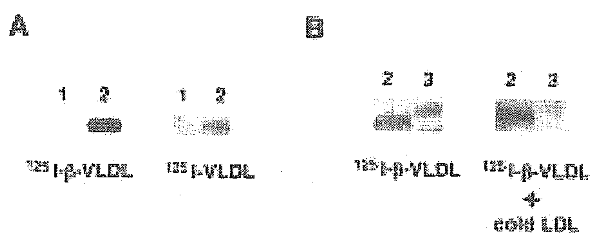

Fig. 2. Ligand blots study.
A: Human VLDL receptor over-expressing CHO cells recognized ^{125}I-rabbit β-VLDL with a higher affinity than ^{125}I-human fasted VLDL. (1: mock transfectants, 2: VLDL receptor transfectants)
B: Both VLDL receptor and LDL receptor recognized ^{125}I-rabbit β-VLDL. Only LDL receptor band disappeared by the addition of a 15-fold concentration of cold LDL, indicating that LDL was not a ligand of the VLDL receptor. (2: VLDL receptor transfectants, 3: LDL receptor transfectants)

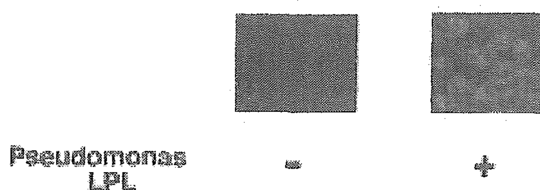

Fig. 3. Fluorescence microscopic examination in VLDL receptor transfectants.
Fluorescence (DiI)-labeled fasted human VLDL (1.5 μg/ml) was not recognized by human VLDL receptor, but treatment of Pseudomonas LPL (5.0 μg/ml) on VLDL accelerated the binding of human VLDL to VLDL receptor.