An acidic fibroblast growth factor-like factor secreted into the brain cell culture medium upregulates apoE synthesis, HDL secretion and cholesterol metabolism in rat astrocytes

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Abstract

Production and release of apolipoprotein (apo) E and cholesterol were highly upregulated in the astrocytes prepared by 1-week secondary culture after 1-month primary culture of rat fetal brain cells (M/W cells) in comparison to the cells prepared by a conventional method of 1-week primary and 1-week secondary culture (W/W cells). Both cell preparations were mostly composed of astrocytes with small population of other glial cells, except that type-2 astrocyte-like cells accounted for 5–15\% of M/W cells indicating more activated and/or matured status. The conditioned medium of the 1-month primary culture stimulated W/W cells to increase the release of apoE and cholesterol into the medium. The treatment of W/W cells by acidic fibroblast growth factor (aFGF) similarly upregulated biosyntheses and release of apoE and cholesterol. The effect of the conditioned medium was completely inhibited by pretreatment with an anti-aFGF antibody. The increase of the aFGF message was demonstrated in the brain cells after 1-month primary culture. The findings suggested that an aFGF-like trophic factor upregulates biosynthesis and secretion of apoE-high density lipoprotein (HDL) in astrocytes probably by autocrine stimulation in this culture system. Since this cytokine is highly expressed in the development or post-injury period of the brain, it putatively activates intercellular cholesterol transport to support construction or recovery of the brain. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

More than 25\% of total body cholesterol in human is found in the central nervous system (CNS) and it accounts for 10\% of the brain dry mass. While extracellular cholesterol transport is generally carried by the plasma lipoprotein system as an important part of cholesterol homeostasis in mammalian bodies, the cells in the CNS are segregated from the lipoproteins in the systemic circulation by the blood brain barrier and use their own lipoprotein system distinct from plasma lipoproteins. Lipoproteins found in cerebrospinal fluid are all high density lipoproteins (HDL) that contain apolipoprotein(apo) E, A-I, A-IV or J [1], and are believed to compose a specific system for regulation of cholesterol homeostasis in the CNS.

Apo E, a glycosylated 34-kDa apolipoprotein, is a major apolipoprotein in cerebrospinal fluid. It is synthesized and secreted by astrocytes and microglia being complexed with phospholipid and cholesterol as HDL particle [2–6]. Synthesis of apoE by astrocytes largely depends on the stage of cellular differentiation and many astrocytoma cells do not synthesize apoE [7,8]. However, the mechanism is unknown for assembly and secretion of HDL particles by astrocytes with endogenously synthesized apoE, and an exact function of such apoE-HDL is unclear in cholesterol homeostasis among glia and neurons in the CNS. We have reported that the astrocytes secrete apoE along with cellular cholesterol and phospholipid to generate cholesterol-rich HDL while
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 3H-cholesterol ester and labeling of astrocytes

LDL isolated by ultracentrifugation from fresh plasma of a healthy volunteer was incubated with lipid microemulsion containing [1,2-3H]cholesterol 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 [3H]acetate (20 µCi/ml, New England Nuclear) in a fresh 0.02% BSA/F-10. The cholesterol synthesis was measured also by using [3H] mevalonolactone (5 µCi/100 nmol/ml) for the downstream 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 [3H]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-
vested by scraping with a rubber policeman and centrifuged at 1000 × 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 × 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 7-[3-14C]-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 [14C]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 3H-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 [3H]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 free cholesterol from total cholesterol. **: P<0.05 and ***: P<0.01 from W/W cells in panel (e).

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

The data indicate percentage to the total cells of the cells positively identified as astrocytes, oligodendroglias 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 overlaid 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 [3H]-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 (estersified 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'-GGCAGCCTCTCCATCTCCTC 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.

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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 wereseeded at 3.4 × 10⁴ 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, oligodendroglia and microglia, 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 [³H]-acetate, or (c) 5 μCi/ml of [³H]-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-[³-¹⁴C] 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 manner (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 washed, 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 [3H]-acetate for another 12 h in the presence of each growth factor. 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 [3H]-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 [3H]-acetate for 3 h in the fresh medium. The newly synthesized cholesterol (c) and mevalonate 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.]
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 to aFGF. The treatment resulted in the complete loss of the activity of M-CM to stimulate W/W cells to increase the release of cholesterol and apoE.

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. 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 [3H]-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.

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'-AAAGCCCGTCGTTCCATGG and 5'-GTGCGACACTGGAGTTGAGGGAC) [74] with 30 cycles according to the methods as described in Fig. 2.
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<sup>ras</sup>/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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