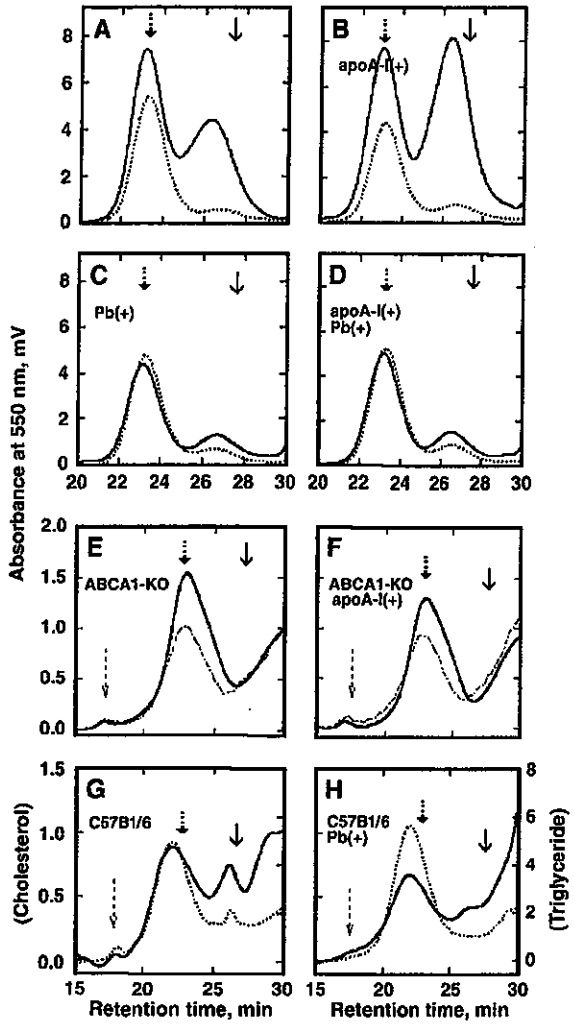


Fig. 4. The effect of ABCA1 inactivation on the generation of HDL by hepatocytes as analyzed by HPLC. A–D: HepG2 cells. A: The conditioned medium of HepG2 cells after 16 h. B: The conditioned medium incubated with apoA-I (10 μg/ml) for 16 h. C: The conditioned medium of probucol-loaded HepG2 cells after 16 h. D: The conditioned medium of probucol-loaded HepG2 cells with apoA-I (10 μg/ml) for 16 h. E–F: Primary cultured hepatocytes. E: The conditioned medium of the hepatocytes isolated from an ABCA1-deficient mouse after 16 h of incubation. F: The conditioned medium of the hepatocytes of an ABCA1-deficient mouse after incubation with apoA-I (10 μg/ml) for 16 h. G: The conditioned medium of the hepatocytes obtained from C57Bl/6 mice fed with control chow after 16 h of incubation. H: The conditioned medium of the hepatocytes obtained from C57Bl/6 mice fed with 0.5% probucol-containing chow. The medium was analyzed by HPLC using two tandem gel-permeation columns (Lipopropak XL; 7.8 mm × 300 mm; Tosoh) to yield the lipoprotein profiles as described in the text. Solid lines represent cholesterol, dotted lines represent triglyceride, and dot-and-broken lines (in E and F) represent choline-phospholipid. Arrows with a solid line, a thick broken line, and a thin broken line indicate the eluting positions of plasma HDL, LDL, and VLDL, respectively. Pseudopeaks generated by the color of the medium caused baseline shifts in the analysis of the primary culture medium as the sensitivity of the monitoring lipid was increased. KO, knockout.

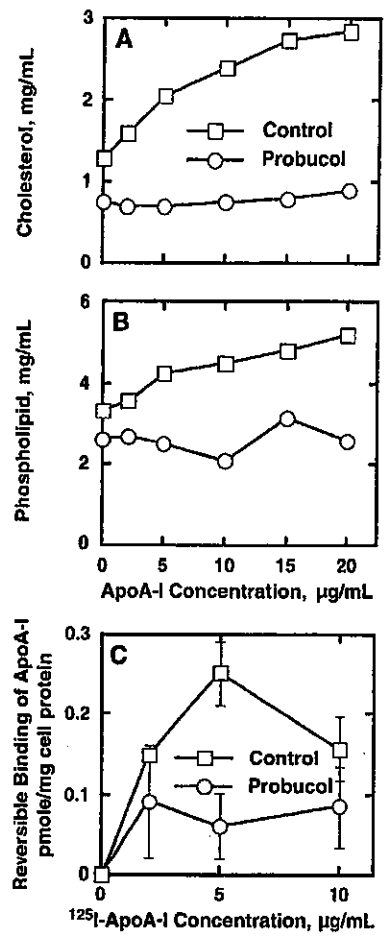


Fig. 5. Effect of probucol loading on apoA-I-mediated cellular lipid release from HepG2 cells and exchangeable binding apoA-I to HepG2 cells. A and B: Lipid release. HepG2 cells were incubated with either control LDL or probucol-containing LDL for 24 h. Cellular cholesterol and phospholipid release were measured after incubation for 16 h with various amounts of apoA-I. HDL-cholesterol (A) and HDL-phospholipid (B) were determined by an HPLC system. Open squares, control LDL-loaded cells; open circles, probucol-LDL loaded cells. C: ApoA-I binding. The indicated amounts of ^{125}I-apoA-I-incubated HepG2 cells were preloaded with control LDL or probucol-containing LDL for 2 h at 4°C. The cells were washed two times with ice-cold PBS, and the bound ^{125}I-apoA-I was displaced two times by 50 μg/ml nonlabeled apoA-I for 9 h. The difference in the total radioactivity after the first washing and after the second displacement was calculated as displaceable or exchangeable binding of apoA-I to the cells. Squares, binding to the cells loaded with control LDL; circles, binding to the cells loaded with probucol-containing LDL. Error bars indicate ± SEM.

of acetone and dissolved in 10 μl of sample buffer for SDS-polyacrylamide electrophoresis, incubated at 100°C for 5 min, and 5 μl of 9 M urea was added. The electrophoresis was performed on a polyacrylamide gel with a gradient of 4–20%. The conditioned media and fractionated media by ultracentrifugation were directly analyzed by immunoblotting against apoA-I. Anti-human albumin IgG fraction of rabbit antiserum was purchased from Sigma-Aldrich Inc. and used as a secondary antibody. Blot-

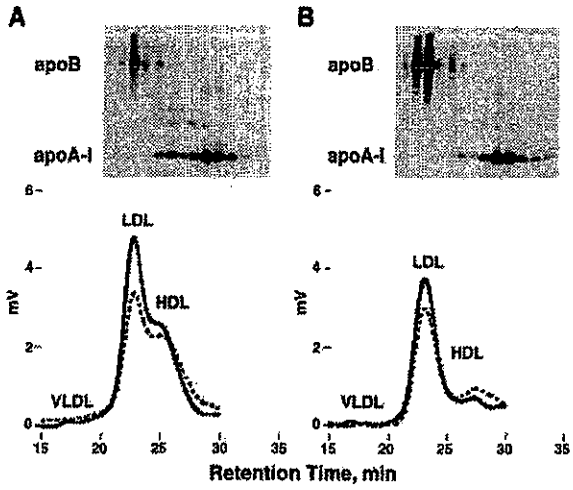


Fig. 6. Analysis of apolipoproteins in the HPLC fractions of the HepG2 conditioned medium. The conditioned medium of HepG2 cells (A) and probucol-loaded HepG2 cells (B) was tested after 16 h of incubation. The medium was analyzed by HPLC and fractionated as described in the text. The upper panels show immunoblot analysis of the fractions for apoB and apoA-I, and the lower panels represent HPLC elution profiles of lipids (solid lines, cholesterol; dotted lines, choline-phospholipid). Positions of lanes in the immunoblot analysis are adjusted for the HPLC elution times. VLDL, LDL, and HDL indicate the eluting positions for plasma lipoproteins.

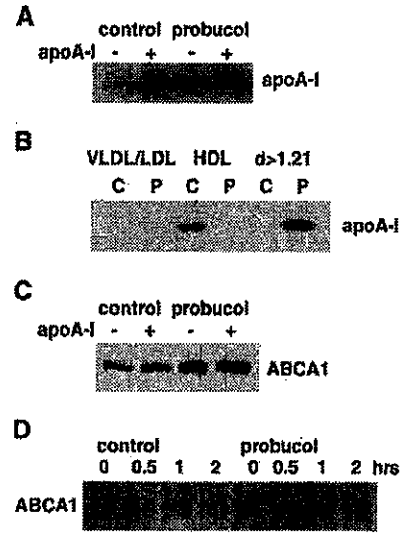


Fig. 7. Immunoblot analysis of apoA-I and ABCA1. A: ApoA-I in the conditioned medium of HepG2 cells was analyzed after preincubation with control LDL and probucol-containing LDL and subsequent incubation in the presence and absence of 10 μg/ml apoA-I for 16 h. B: Density distribution of apoA-I in the medium. ApoA-I was analyzed in density subfractions of the conditioned medium of HepG2 cells preincubated with 50 μg/ml control LDL (C) or probucol-containing LDL (P) for 24 h and then incubated for 16 h. The medium was fractionated by sequential ultracentrifugation for the VLDL/LDL fraction ($d < 1.063$ g/ml), the HDL fraction ($1.063 < d < 1.21$ g/ml), and the bottom fraction ($d > 1.21$ g/ml). Each fraction, corresponding to 10 μl of the original medium, was dialyzed and analyzed for apoA-I. C: ABCA1 in the plasma membrane of HepG2 cells prepared as described for A. D: Clearance of ABCA1 in HepG2 cells. The cells were preloaded with control or probucol-containing LDL, and ABCA1 was examined after incubation with 71 μM cycloheximide.

ted bands were visualized by the chemiluminescence method (ECL Western Blotting Detection System; Amersham Pharmacia Biotech).

Clearance rate of ABCA1

The clearance rate of ABCA1 was analyzed by immunoblotting of the protein in the bulk cellular membrane fraction (21). HepG2 cells were loaded with control and probucol-containing LDL for 2 h and incubated in the presence of 71 μM cycloheximide to inhibit the synthesis of new protein. The cells were harvested and applied for ABCA1 immunoblotting using a specific antibody raised against a C-terminal peptide of human ABCA1 (21).

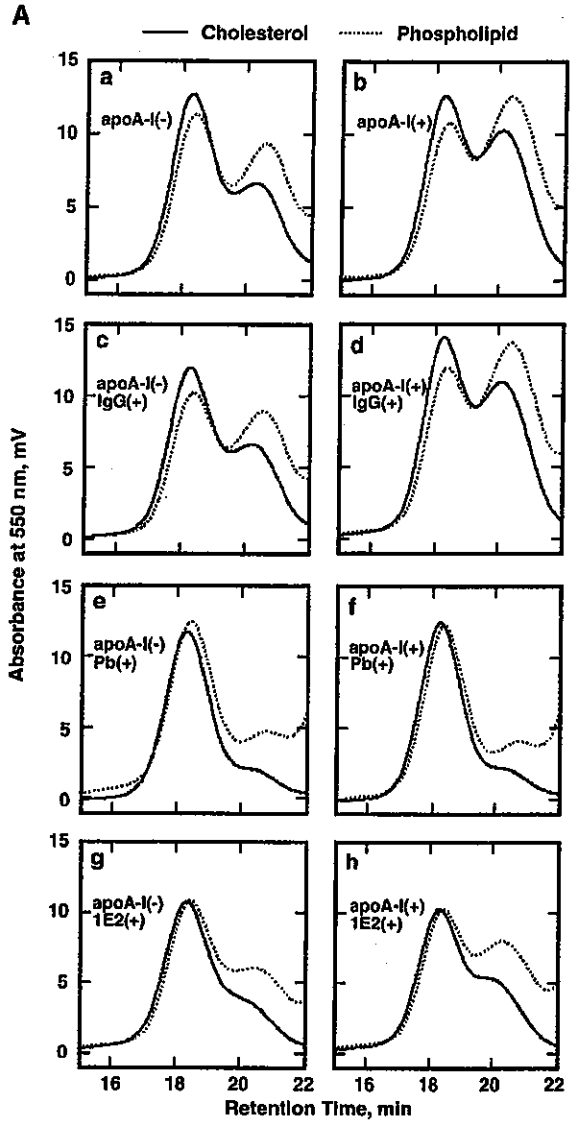
Other methods

Cellular lipid was analyzed as described previously (20). The sample was also used for probucol analysis using an HPLC system (22). Protein was measured with the bicinchoninic acid method using BSA as a standard.

RESULTS

Apolipoprotein-induced cellular lipid release from HepG2 cells is shown in **Fig. 1**. There was a significant spontaneous release of cholesterol and choline-phospholipid by HepG2 cells (indicated at zero concentration of apolipoproteins). Both apoA-I and apoA-II, when exogenously added to the culture medium, induced further release of the lipids in a dose-dependent manner. The medium was analyzed for lipoprotein particles using gel-permeation HPLC (**Fig. 2**). Two major peaks were identified corresponding to LDL and HDL sizes by human plasma reference, whereas no significant peak was detected at the position of VLDL (Fig. 2A). The peaks were indeed verified to correspond to density classification of lipoprotein as fractions $d < 1.063$ and $d > 1.063$ (Fig. 2C, E). The conditioned medium incubated with 10 μg/ml human apoA-I showed an increase of the HDL peak (Fig. 2B, D, F). Thus, increase of the release of cholesterol and phospholipid by apolipoprotein was shown to be attributable to the additional production of the HDL-size particles. Generation of lipoprotein was also examined for primary cultured mouse hepatocytes (**Fig. 3**). The cells were incubated with and without exogenous 10 μg/ml human apoA-I for 24 h, and the conditioned medium was fractionated by ultracentrifugation. Cholesterol content in the HDL fraction ($d > 1.063$) was increased by 2-fold, and the VLDL-LDL fraction ($d < 1.063$) was insignificantly decreased. These results indicated that the system is functional both in HepG2 cells and in mouse hepatocytes for generation of the HDL-size lipoprotein by helical apolipoprotein.

To examine the contribution of ABCA1 to this system, the effect of an inactivator of ABCA1, probucol, was analyzed in those cells. Probucol was loaded to HepG2 cells, and the culture medium was analyzed by HPLC after 16 h of incubation with and without 10 μg/ml apoA-I. The



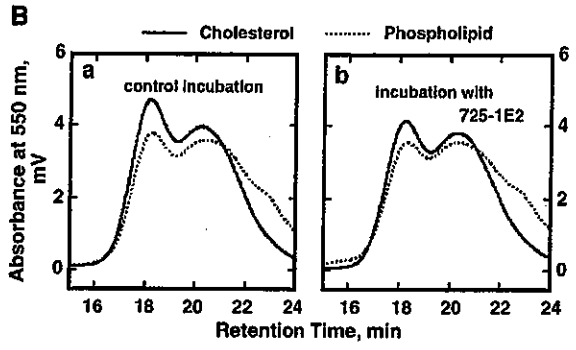


Fig. 8. Inhibition of HDL production by HepG2 cells by a monoclonal antibody against lipid-free apoA-I. A: HepG2 cells were incubated with 0.02% BSA-containing MEM-α medium in the presence of 1 mg/ml monoclonal antibody 725-1E2 specific to lipid-free apoA-I and 10 μg/ml apoA-I for 16 h, and the medium was analyzed for its lipoprotein profile using HPLC with one gel-permeation column (Lipopropak XL; 7.8 mm × 300 mm; Tosoh). A-a: The medium of the cells by plain incubation. A-b: The medium of the cells incubated in the presence of apoA-I. A-c: The medium after incubation in the presence of control mouse IgG. A-d: The medium incubated in the presence of apoA-I and control IgG. A-e: The medium of the probucol (Pb)-preloaded cells. A-f: The medium of the cell preloaded with probucol after incubation in the presence of apoA-I. A-g: The medium of the cells incubated in the presence of the free apoA-I-specific antibody 725-1E2. A-h: The medium of the cells incubated with the antibody 725-1E2 in the presence of apoA-I. B: The conditioned medium of HepG2 cells was incubated for 16 h in the absence (B-a) and presence (B-b) of the antibody, and the lipoprotein profile was analyzed by HPLC. Solid lines, cholesterol; dotted lines, choline-phospholipid.

data for cellular lipid and probucol are listed in **Table 1**. There was no difference in lipid composition between the control and probucol-loaded cells. The latter cells contained probucol at ~1 μg/mg cell proteins, which was equivalent to our previous data with mouse peritoneal macrophages to which probucol was delivered by acetylated LDL (9). LDL- and HDL-size particles were identified in the medium, and the HDL fraction was increased by apoA-I (**Fig. 4A, B**). Probucol markedly decreased the HDL peak with both spontaneous production or exogenously added apoA-I (Fig. 4C, D). This finding was confirmed by electrophoretic analysis of the medium on an agarose gel (data not shown). The medium of mouse primary hepatocytes was also examined. The hepatocytes isolated from ABCA1-deficient mice produced no HDL, and apoA-I did not induce the production of HDL either (Fig. 4E, F). The effect of probucol was examined in hepatocytes prepared from C57Bl/6 mice by feeding control chow or 0.5% probucol-containing chow. The HDL peak decreased by probucol feeding (Fig. 4G, H). **Figure 5** shows the effect of probucol on the apoA-I-induced lipid release from HepG2 cells. The results indicated again that probucol inhibits the lipid release for both the baseline and an additional part induced by apoA-I. The cell-specific (reversible) binding of apoA-I was examined using ^{125}I-labeled apoA-I for HepG2 cells. Probucol inhibited the binding of apoA-I (Fig. 5C).

ApoB and apoA-I were analyzed in the HPLC fractions of the HepG2-conditioned medium (**Fig. 6**). ApoB was detected in the LDL fractions, and the probucol treatment did not alter this distribution. ApoA-I in the HDL fractions was markedly decreased by the probucol treatment. ApoA-I was also present in the free protein fractions, and this is

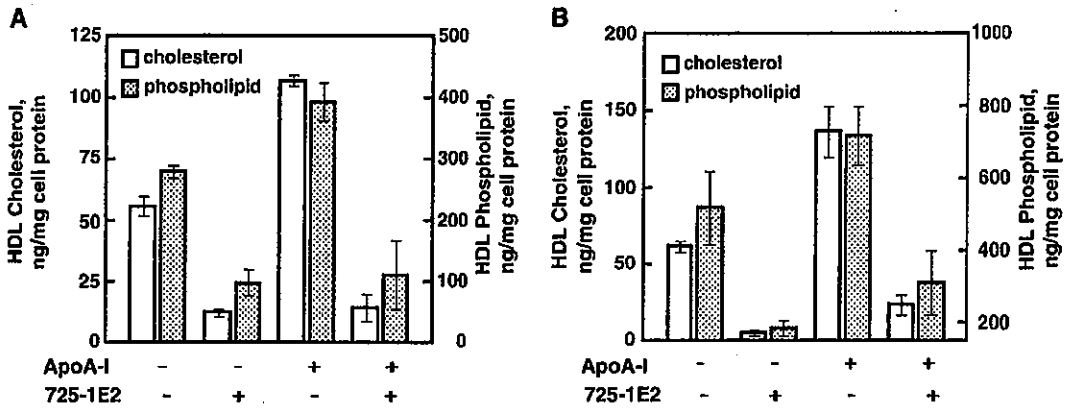


Fig. 9. Effect of free apoA-I-specific monoclonal antibody on HDL production by HepG2 cells. A: Cholesterol and phospholipid in HDL in the medium of HepG2 cells incubated in the presence of the antibody 725-1E2 and apoA-I, based on HPLC analysis. The data represent means ± SEM of triplicate assays. B: Cholesterol and phospholipid in HDL in the medium of HepG2 cells incubated in the presence of the antibody 725-1E2 and apoA-I, based on ultracentrifugal analysis. Data represent means ± SEM of triplicate assays.

consistent with the findings from the analysis of human plasma by HPLC in the diluted condition equivalent to the culture medium (×250) (data not shown). Although this HPLC method was thoroughly validated for lipoprotein lipid elution profile by its excellent correlation with the other gel-permeation method with a Superose column, apoA-I may dissociate from HDL particles during the analysis, presumably as a result of the use of 0.005% Brij-35 and extreme dilution of lipoprotein (17, 19).

Because of this limitation of the HPLC method, the medium was analyzed by ultracentrifugation to identify the status of apoA-I in the medium. When ABCA1 was inactivated by probucol in HepG2 cells, overall secretion of apoA-I was not influenced, as demonstrated in **Fig. 7A**. However, although apoA-I was localized in the HDL fraction ($1.063 < d < 1.21$ g/ml) in the control conditioned medium, it was found exclusively in the $d > 1.21$ g/ml fraction in the medium of probucol-loaded cells (Fig. 7B). Thus, apoA-I is secreted by the cells mostly in its free form when ABCA1 is inactivated.

Stabilization and increase of ABCA1 by additional apoA-I was not observed in this cell line, presumably because it is already affected by endogenously secreted apolipoproteins (Fig. 7C). However, ABCA1 was resistant against degradation in the probucol-loaded HepG2 cells (Fig. 7C, D) in the same manner as we demonstrated in fibroblasts (11).

Finally, we attempted to inactivate free apoA-I in the medium by trapping it using a monoclonal antibody to lipid-free apoA-I, 725-1E2 (15). As shown in **Fig. 8**, production of the HDL-size particles by both endogenous and exogenous apoA-I was effectively suppressed when this antibody was present in the medium during the incubation of HepG2 cells (Fig. 8Ag, Ah), whereas control IgG did not influence the profile (Fig. 8Ac, Ad). On the other hand, this antibody did not change lipoprotein profile when incubated with the HepG2-conditioned medium after removal of the cells (Fig. 8Ba, Bb). These results are quantitatively illustrated in **Fig. 9A**. Alternatively, the medium was analyzed by ultracentrifugation after the same experiments, and these results are shown in Fig. 9B. Similar to the results of the HPLC analysis, HDL production was selectively suppressed by the lipid-free apoA-I monoclonal antibody 725-1E2.

DISCUSSION

The results of this work are summarized as follows. *1*) HepG2 cells and mouse primary cultured hepatocytes produce HDL with endogenously synthesized apolipoprotein as well as with exogenously added apolipoprotein. *2*) Production of HDL by both pathways is inhibited by the inactivation of ABCA1, but secretion of endogenous apoA-I was not decreased as much. ApoA-I is secreted as a free form when ABCA1 is inactivated. *3*) A monoclonal antibody against lipid-free apoA-I, 725-1E2, suppresses the production of HDL by HepG2 cells but has no effect on lipoprotein when directly incubated with the HepG2-conditioned medium. Based on these observations, we conclude that the majority of HDL produced by hepatocytes are generated by the interaction of apoA-I that is secreted in a free or lipid-poor form with ABCA1 of the hepatocytes in an autocrine-like manner.

Many reports have characterized the HDL-like particles secreted in the culture medium by HepG2 cells and other liver cell lines (23–25). We essentially confirmed these findings with HepG2 cells and, in addition, demonstrated the increase of production of such particles by exogenously added apoA-I and apoA-II. Thus, the HepG2 cells have the same pathway to generate HDL as many other cells upon interaction with helical apolipoproteins, presumably dependent on ABCA1 (26).

Probucol has been shown to inhibit the apolipoprotein-cell interaction and accordingly to suppress the generation of HDL (9, 10). We recently demonstrated that this compound inactivates ABCA1 in the plasma membrane with respect to its activity as well as its calpain-mediated degradation (11). Probucol inhibited the production of

HDL, whether by endogenous or exogenous apolipoprotein, in hepatocytes. These findings suggested that endogenous and exogenous apolipoprotein, mainly apoA-I, generate HDL by a common mechanism, most likely by the ABCA1 pathway. Under this condition, the amount of the secreted apoA-I did not change, and it was secreted mostly in its free form (Fig. 7A, B). Therefore, the interaction of apoA-I with ABCA1 does not influence the rate of its secretion, but most apoA-I remains in a free form in the medium when it is unable to assemble HDL.

A monoclonal antibody raised against apoA-I, 725-1E2, has been characterized to be selective for lipid-free apoA-I (15). By using this antibody, we attempted to alter the production of HDL by HepG2 cells. In the presence of this antibody in the culture medium of HepG2 cells, the production of HDL markedly decreased. This finding strongly suggested that most apoA-I is secreted in its free form and trapped by the antibody, resulting in a decrease of HDL production. This view was supported by the finding that the incubation of the antibody with the HepG2-conditioned medium did not cause a change of the lipoprotein profile. Therefore, the effect of the antibody was not likely caused by the reaction with HDL after its generation by the cells.

It was reported that a certain portion of apoA-I (20%) was intracellularly lipidated in HepG2 cells and in primary cultured hepatocytes (8, 27), and some of the lipidation may not be mediated by ABCA1 (8). However, the present results indicated that the majority of HDL particles are produced by a common mechanism for endogenous and exogenous apolipoproteins, both of which can be inhibited by probucol. Also, most of the HDL production was inhibited by lipid-free apoA-I-specific antibody, indicating an autocrine mechanism: apoA-I is secreted as a free form and then interacts with hepatocytes to generate HDL. These findings by no means exclude the possibility of intracellular lipidation of apoA-I and the presence of the ABCA1-independent pathway as a minor source of HDL production.

ApoA-I is secreted as pro-apoA-I and converted to a mature form by a metalloprotease in blood plasma (28). Both pro-apoA-I and mature apoA-I were identically capable of generating HDL by reacting with ABCA1 (15), so perhaps there is no need to consider apoA-I maturation for this autocrine mechanism.

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Astrocytes produce and secrete FGF-1, which promotes the production of apoE-HDL in a manner of autocrine action

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Abstract **The astrocytes prepared by 1 week secondary culture after 1 month primary culture of rat brain cells (M/W cells) synthesized and secreted apolipoprotein E (apoE) and cholesterol more than the astrocytes prepared by conventional 1 week primary and 1 week secondary culture (W/W cells) (Ueno, S., J. Ito, Y. Nagayasu, T. Furukawa, and S. Yokoyama. 2002. 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. *Biochim. Biophys. Acta.* 1589: 261–272). M/W cells also highly expressed fibroblast growth factor-1 (FGF-1) mRNA. FGF-1 was identified in the cell lysate of both cell types, but M/W cells released more of it into the medium. Immunostaining of FGF-1 and apoE revealed that both localized in the cells that produce glial fibrillary acidic protein. The conditioned media of M/W cells and FGF-1 stimulated W/W cells to release apoE and cholesterol to generate more HDL. Pretreatment with a goat anti-FGF-1 antibody or heparin depleted the stimulatory activity of M/W cell-conditioned medium. The presence of the anti-FGF-1 antibody in the medium suppressed apoE secretion by M/W cells. Differential inhibition of signaling pathways suggested that FGF-1 stimulates apoE synthesis via the phosphoinositide 3-OH kinase for PI3K/Akt pathway. Thus, astrocytes release FGF-1, which promotes apoE-HDL production by an autocrine mechanism. These results are consistent with our in vivo observation that astrocytes produce FGF-1 before the increase of apoE in the postinjury lesion of the mouse brain (Tada, T., J. Ito, M. Asai, and S. Yokoyama. 2004. Fibroblast growth factor 1 is produced prior to apolipoprotein E in the astrocytes after cryo-injury of mouse brain. *Neurochem. Int.* 45: 23–30).—Ito, J., Y. Nagayasu, R. Lu, A. K. M. Hayashi, and S. Yokoyama. Astrocytes produce and secrete FGF-1, which promotes the production of apoE-HDL in a manner of autocrine action. *J. Lipid Res.* 2005. 46: 679–686.**

Supplementary key words apolipoprotein E • fibroblast growth factor-1 • high density lipoprotein • brain damage • cholesterol

The brain cells are segregated from lipoproteins in the systemic circulation by the blood-brain barrier, so that cholesterol homeostasis in the brain is dependent on its specific extracellular lipid transport system by apolipoproteins and lipoproteins (1–5). Helical apolipoproteins such as apolipoprotein E (apoE), apoA-I, apoD, apoA-IV, and apoJ have been identified in the cerebrospinal fluid as components of HDL, but the main apolipoproteins are apoE and apoA-I (6–10). The phenotype of human brain apoE does not change after liver transplantation, so that brain apoE is mostly produced in the brain (11).

It is known that apoE is produced mainly by astrocytes and partly by microglia in the brain, suggesting that astrocytes play an important role in cholesterol homeostasis in the central nervous system (5, 10, 12–16). Astrocytes produce cholesterol-rich HDL with cellular lipid by autologously synthesized apoE (8, 17). They also react with exogenous apoA-I to generate cholesterol-poor HDL through a unique system for intracellular cholesterol transport (18–20). Like plasma HDL, ATP binding cassette transporter A1 supports such production of brain HDL, although other pathways may also function as backup systems (21, 22). These lipoproteins are thought to play important roles in intercellular lipid transport in the brain. It has been noted that apoE synthesis is upregulated in the brain during development and after injury (13, 23–31), and this reaction is likely to be involved in the healing process of the injury (32–35).

Four types of apoE binding receptor are identified in the brain: very low density lipoprotein receptor, low density lipoprotein receptor, low density lipoprotein receptor-related protein, and apoE receptor-2; thus, apoE is thought to function as a recognition site of lipoproteins for lipid

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Abbreviations: apoE, apolipoprotein E; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; FGF-1, fibroblast growth factor-1; GFAP, glial fibrillary acidic protein; PI3K, phosphoinositide 3-OH kinase; TBS, salined 0.02 Tris-Hcl buffer.

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delivery among brain cells (36, 37). However, some of them are also likely to mediate signals for the migration of brain cells during the developmental integration of the brain (38).

We reported previously that astrocytes prepared by 1 month primary culture of rat fetal brain cells and subsequent 1 week secondary culture (M/W cells) synthesized and secreted apoE and cholesterol more actively than astrocytes prepared according to the conventional method of 1 week primary and 1 week secondary culture (W/W cells) (39). A fibroblast growth factor-1 (FGF-1)-like factor is secreted by the long-cultured rat fetal brain cells, and their conditioned media stimulated W/W astrocytes for the secretion of apoE. We also found that FGF-1 is produced by astrocytes adjacent to the cryoinjury lesions of mouse brain before the increase of apoE synthesis in vivo (40). We identified a promoter polymorphism of FGF-1 related to risk for Alzheimer's disease (41). Thus, we hypothesize that FGF-1 is a trigger stimulant of apoE synthesis and generation of HDL in the postinjury brain, presumably by an autocrine mechanism. In the present work, we attempted to identify the cells that secrete FGF-1 in the culture system and demonstrate an autocrine mechanism for this factor to stimulate apoE-HDL production. This is an important process to identify the triggering mechanism for the production of apoE and its HDL in the postinjury brain for recovery from damage.

MATERIALS AND METHODS

Preparation of fetal rat astrocytes

Astrocytes were prepared from the 17 day fetal brain of Wistar rats according to the method previously described (42). After removal of the meninges, the brain was cut into small pieces and treated with 0.1% trypsin solution in Dulbecco's phosphate-buffered saline (DPBS) containing 0.15% glucose (0.1% trypsin/DPBS/G) for 3 min at room temperature. The cell pellet by centrifugation at 1,000 rpm for 3 min was cultured in F-10 medium containing 10% fetal calf serum (10% FCS/F-10) at 37°C for 4 weeks as a primary culture. After treatment with 0.1% trypsin/DPBS/G containing 1 mM ethylenediaminetetraacetic acid, the cells were cultured in 10% FCS/F-10 for 1 week as a secondary culture (M/W cells). Alternatively, astrocytes were prepared by a conventional method of 1 week primary and subsequent 1 week secondary culture (W/W cells). Both preparations contained 95% astrocytes [glial fibrillary acidic protein (GFAP)-positive], 0.3% oligodendroglias (anti-myelin basic protein-positive), and 3% microglias (ED-1-positive) (39).

Synthesis and release of cellular cholesterol

Rat astrocytes at a confluent stage were washed with DPBS four times and incubated in 0.1% BSA/F-10 for 24 h. The cells were incubated with [^{3}H]acetate (New England Nuclear) in fresh 0.02% BSA/F-10 for certain periods of time. For the lipid-release experiments, the cells were washed three times with cold DPBS and further incubated in fresh 0.02% BSA/F-10 in the presence of 1 mM acetate. Cholesterol was extracted from the cells and the conditioned medium with hexane-isopropanol (3:2, v/v) and chloroform-methanol (2:1, v/v), respectively, and separated by TLC on Silica Gel-60 plates (E. Merck, Darmstadt, Germany). Radioactivity in the cholesterol fraction was counted (43). The medium was also analyzed by density gradient ultracentrifugation as described previously (17). After removing cell debris by centrifugation, the medium (8 ml) was overlaid on the sucrose solution (d = 1.175; 17 ml) and centrifuged at 1×10^5 *g* for 48 h. Samples were fractionated and analyzed for cholesterol mass by the enzymatic colorimetric method (44) and for apoE by Western blotting (see below).

Analysis of cell and medium protein by Western blotting

The cells were harvested with a rubber policeman after washing four times with DPBS. The cell pellet by centrifugation at 1,000 rpm for 10 min was treated with cold and salined 0.02 M Tris-HCl buffer, pH 7.5 (TBS), containing the protease inhibitor cocktail (Sigma) for 10 min with 25 agitations for 10 s every 5 min. The suspension was centrifuged at 3,000 rpm for 10 min for removal of nuclei and cell debris. The supernatant was sonicated and centrifuged at 370,000 *g* for 30 min to obtain supernatant as a cell protein extract fraction. Cell debris was removed from the conditioned medium by centrifugation at 15,000 rpm for 30 min. Protein in the cell extract or in the conditioned medium was precipitated by 10% trichloroacetate and centrifugation at 15,000 rpm for 20 min, separated by SDS-PAGE, and transferred to a Sequi-Blot™ polyvinylidene difluoride membrane (Bio-Rad). The membrane was immunostained with a goat anti-FGF-1 antibody (Santa Cruz Biotechnology) and a rabbit antibody against rat apoE, a generous gift from Dr. Jean Vance (University of Alberta).

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from rat astrocytes with Isogen (Wako Life Science) and reverse-transcribed to generate cDNA in a SuperScript Preamplification System (Gibco BRL). The cDNA was subjected to PCR using the DNA probes for rat apoE mRNA and FGF-1 mRNA as described in the previous paper (39). After electrophoresis of the products, an agarose gel was stained with freshly prepared SYBR Gold nucleic acid gel stain solution. The band was detected by an ultraviolet transilluminator (UVP NLM-20E) at 302 nm. The apoE primer pairs were 5′-GCGCACCTCCTGCATCTCCTC-3′ (sense) and 5′-AGGATCTATGCAACCGACTCG-3′ (antisense). The FGF-1 primers were 5′-AAGCCCGTCGGTGTCCATGG-3′ and 5′-GATGGCACAGTGGATGGGAC-3′.

Immunocytostaining of astrocytes

Astrocytes on a tissue culture chamber/slide (Mikes Scientific) were washed with DPBS and fixed with 100% methanol at −20°C for 30 min. The cells were treated with 1% Triton X-100 in 0.02 M phosphate buffered saline at room temperature for 2 min after washing with DPBS. The cells were washed with DPBS again, treated with goat anti-FGF-1 antibody, or rabbit anti-rat apoE antibody, at room temperature for 60 min and washed. After incubation with biotin-conjugated anti-goat IgG, or anti-rabbit IgG antibody (Histofine) for 30 min at room temperature, the cells were washed, treated with peroxidase-conjugated streptavidin (Histofine) for 15 min, and then washed. The cells were stained by reaction with 0.01% 3,3′-diaminobenzidine tetrahydrochloride (Dojindo)/0.03% H_2O_2/0.05 M Tris buffer, pH 7.5, for 5 min at room temperature.

Alternatively, M/W astrocytes were fluorescence immunostained after being fixed in organic solution composed of methanol, chloroform, and acetic acid (6:3:1) at −20°C for 3 h. After washing with cold TBS, the cells were reacted with either goat anti-FGF-1 or goat anti-rat apoE antibody (Santa Cruz Biotechnology) and mouse anti-GFAP antibody (BD Transduction Laboratories) in TBS containing 3% donkey serum and 3% horse serum at room temperature for 1 h. The cells were reacted with rhodamine-conjugated donkey anti-goat IgG antibody (Chemicon International) or fluorescein-conjugated horse anti-mouse IgG antibody (Vector Laboratories) in the presence of 3% donkey or

3% horse serum, respectively, at room temperature for 1 h after washing three times with TBS. The cells were observed by laser scanning confocal microscopy (LSM5; Zeiss, Jena, Germany).

Analysis of signaling pathways

For the analysis of the FGF-1-initiated signals to stimulate apoE synthesis, rat astrocytes (W/W cells) were washed, replaced with 0.1% BSA/F-10, and incubated for 24 h with FGF-1 (50 ng/ml) in the presence or absence of an inhibitor of phosphoinositide 3-OH kinase (PI3K), for PI3K, LY294002 (10 μM; Calbiochem), or an inhibitor of MEK, U0126 (10 μM; Calbiochem). The cells were further incubated in the same condition in fresh 0.02% BSA/F-10 for 8 h and then in 0.02% BSA/F-10 for 16 h after washing. The conditioned medium was centrifuged at 15,000 rpm for 60 min to remove cell debris, treated with 10% TCA, and centrifuged at 15,000 rpm for 20 min. The pellet was analyzed by SDS-PAGE and Western blotting using rabbit anti-rat apoE antibody. Phosphorylation of Akt by FGF-1 was also examined. After the cells were incubated with FGF-1 (50 ng/ml) in fresh 0.02% BSA/F-10 for 5 min, the cytosol was prepared as a supernatant of the cell treatment in 0.02 M Tris-HCl buffer, pH 7.5, containing protease inhibitor cocktail (Sigma) for 10 min with 10 s of agitation 25 times every 5 min and centrifugation at 90,000 rpm for 30 min. The cytosol protein precipitated with 10% TCA was analyzed by SDS-PAGE and Western blotting using mouse anti-protein kinase B (PKB) α/Akt antibody (BD Transduction Laboratories) and rabbit anti-phospho-Akt (Thr-308) antibody (Cell Signaling Technology).

RESULTS

During long-term culture of the rat brain cells, a large number of neurites were identified at 1 week, and astrocytes became predominant after 2–3 weeks, when neurons were hardly identified (**Fig. 1A**). Expression of the FGF-1 message was not apparent at 1 week primary culture and was markedly increased after 3 weeks (Fig. 1B). These findings indicated that FGF-1 was produced by astrocytes rather than neurons during the long-term primary culture of brain cells.

To identify the cells that produce FGF-1 and apoE more specifically, immunostaining was performed for FGF-1 and apoE in various astrocyte preparations by 1 week secondary culture after primary culture of the brain cells for 1, 2, and 4 weeks (**Fig. 2**). The increase of FGF-1 and apoE was observed in cells prepared after primary culture for both 2 and 4 weeks (Fig. 2A–C, 2E–G). In the astrocyte preparation of 4 week primary and 1 week secondary culture (M/W cells), a group of cells were found with an appearance of "type 2" astrocyte-like cells. Both proteins were also identified in these cells (Fig. 2D, H).

M/W cells were further analyzed by fluorescence immunostaining to confirm that both FGF-1 and apoE were produced by astrocytes. **Figure 3** shows that FGF-1 and apoE were both immunochemically identified in the GFAP-positive cells.

M/W cells were examined for the production and secretion of FGF-1. The conditioned media of the brain cell primary culture and of the astrocyte preparations were examined for effects on the astrocytes prepared by a conventional method of 1 week primary and 1 week secondary

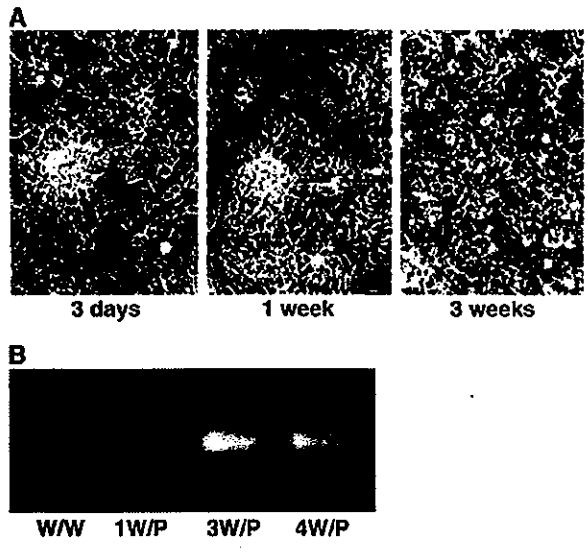

Fig. 1. Expression of fibroblast growth factor-1 (FGF-1) mRNA in rat fetal brain cells. A: Microscopic appearance of rat fetal brain cells in primary culture for 3 days, 1 week, and 3 weeks. B: FGF-1 mRNA. Total cellular RNA was prepared with Isogen (Wako Life Science) from brain cells in primary culture for 1 week (1W/P), 3 weeks (3W/P), and 4 weeks (4W/P) and from astrocytes prepared by conventional 1 week primary and 1 week secondary culture (W/W). Total mRNA (5 μg) was reverse-transcribed to cDNA using the SuperScript Preamplification System (Gibco BRL) for 10 min at 25°C, for 50 min at 50°C, and for 15 min at 70°C, and 0.5 μg of cDNA product was amplified using FGF-1 primers (5′-AAGCCCGTCGGTGTCCATGG-3′ and 5′-GATGGCACAGTGGATGGGAC-3′) for 30 cycles.

culture (W/W cells) (**Fig. 4**). The medium of the primary culture for 2, 3, and 4 weeks and that of M/W cells stimulated apoE secretion from W/W cells (Fig. 4A). The astrocytes after 1 week primary and 4 week secondary culture (W/M cells) also generated the conditioned medium to stimulate apoE secretion. Stimulation of cholesterol release from W/W cells by M/W cell-conditioned medium was neutralized by pretreatment of the medium with anti-FGF-1 antibody-Sepharose and with heparin-Sepharose (Fig. 4B). This finding is consistent with the results with the conditioned medium of long-cultured whole brain cells (39).

The effect of FGF-1 was further analyzed in the experiments shown in **Fig. 5**. Density gradient analysis of the medium revealed an increase of HDL production by astrocytes (W/W cells) when stimulated by FGF-1 (Fig. 5A). There was no shift of the density peak of HDL with this change. Figure 5B demonstrates the distribution of apoE in the density fractions. The increase of apoE by FGF-1 was observed coincidentally with the cholesterol peak.

The message and protein of FGF-1 in M/W astrocytes were examined. M/W cells expressed a greater level of FGF-1 mRNA than did W/W cells (**Fig. 6A**). On the other hand, a similar amount of FGF-1 was detected as a 16.5 kDa protein in the heparin-bound fraction of the extracts

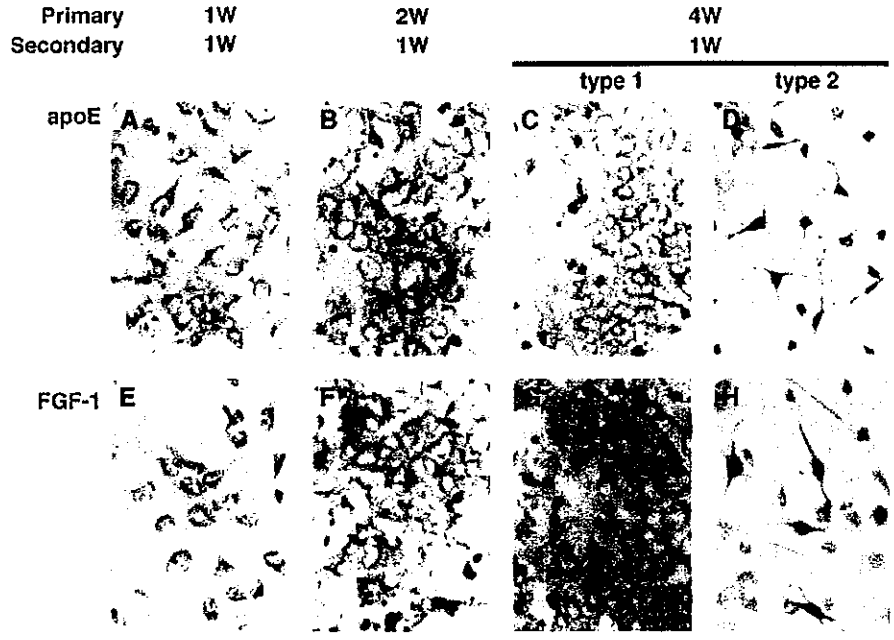

Fig. 2. Immunostaining of FGF-1 and apolipoprotein E (apoE) in astrocytes. The cells were prepared by 1 week secondary culture after primary culture for 1 week (A, E), 2 weeks (B, F), or 4 weeks (C, D and G, H) of rat fetal brain cells. In the preparations of 4 week primary and 1 week secondary culture, C and G represent the fields in which ordinary astrocytes (type 1) are predominant and D and H represent the fields in which cells with type 2-like appearance are predominant. The cells were immunostained with rabbit anti-rat apoE antibody (A–D) or goat anti-FGF-1 antibody (E–H) as described in Materials and Methods.

of both W/W and M/W cells by Western blotting analysis using an anti-FGF-1 antibody (Fig. 6B). However, a much greater amount of this protein was secreted into the culture medium by M/W cells than by W/W cells, as identified by immunoprecipitation with anti-FGF-1 antibody (Fig. 6C). Furthermore, secretion of apoE by M/W cell was markedly decreased when the cells were exposed to an anti-FGF-1 antibody during the detection period (Fig.

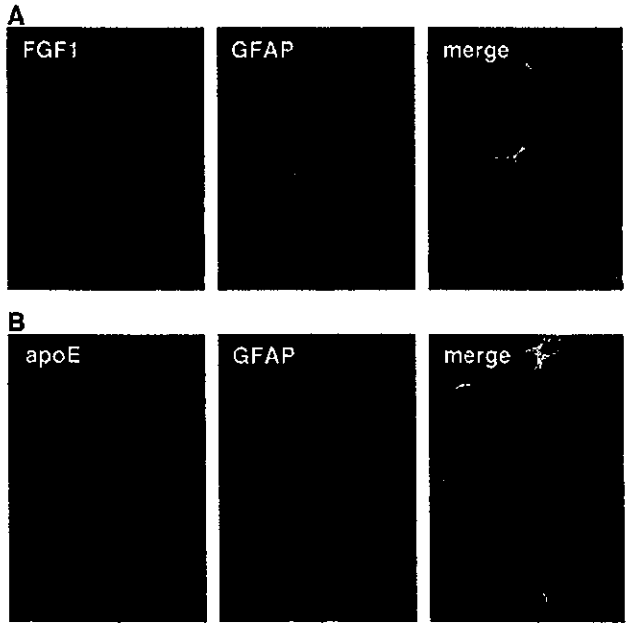

Fig. 3. Immunostaining of M/W cells. A: The cells were immunostained using goat anti-FGF-1 and mouse anti-glial fibrillary acidic protein (GFAP) antibodies and visualized with rhodamine-conjugated donkey anti-goat IgG antibody (Chemicon International) and fluorescein-conjugated horse anti-mouse IgG antibody (Vector Laboratories), respectively. The cells were observed by laser scanning confocal microscopy (LSM5; Zeiss, Jena, Germany). B: The cells were treated in the same manner except that goat anti-rat apoE antibody was used instead of anti-FGF-1 antibody.

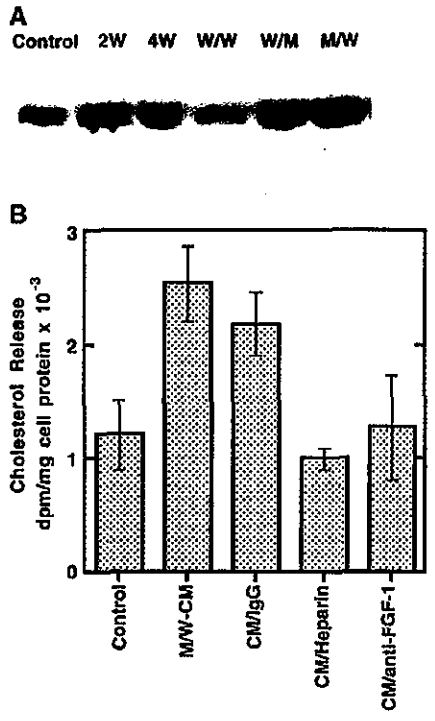

Fig. 4. FGF-1-like activity in conditioned media. A: The effect of the conditioned media of the various brain cells and astrocytes on the secretion of apoE by W/W cells. The conditioned media were prepared from the primary culture for 2 weeks (2W; 356 μg cell protein/ml/well) and 4 weeks (4W; 644 μg/ml/well) of W/W cells (W/W; 142 μg/ml/well) and from M/W cells (M/W; 409 μg/ml/well) and W/M cells prepared by 1 week primary and 4 week secondary culture (W/M; 376 μg/ml/well). W/W cells were incubated with each conditioned medium (500 μl in 1 ml of culture medium) for 24 h. After washing and replacement with fresh 0.02% BSA/F-10 medium, the 16 h cultured medium was analyzed by immunoblotting for apoE. B: Effect of treatment with anti-FGF-1 antibody or heparin of the conditioned medium of M/W cells on its stimulatory effects on W/W cells for the release of cholesterol. M/W-CM, M/W cell conditioned medium; CM/IgG, the medium pretreated with normal rabbit IgG-bound protein G-Sepharose; CM/Heparin, heparin-Sepharose CL-6B (Amersham Pharmacia); CM/anti-FGF-1, goat anti-FGF-1 antibody-bound protein G-Sepharose (Amersham Biosciences) at room temperature for 2 h. W/W cells were incubated with or without 500 μl of M/W-CM or pretreated M/W-CM for 24 h and washed, followed by incubation with 20 μCi/ml [^{3}H]acetate in 1 ml of 0.02% BSA/F-10 for 16 h. After washing three times with Dulbecco's phosphate-buffered saline (DPBS), the cells were incubated for 16 h in fresh 0.02% BSA/F-10. The lipid extracted from the conditioned medium was analyzed by TLC to determine radioactivity in cholesterol. Each column represents the average and standard error of triplicate samples.

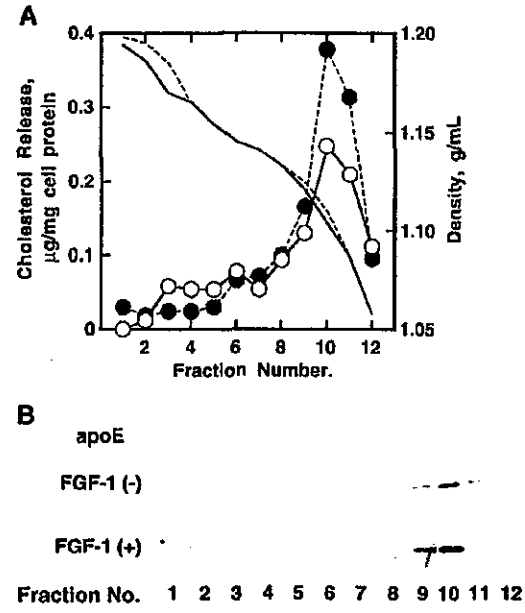

Fig. 5. Increase of apoE-HDL production by rat astrocytes stimulated by FGF-1. Rat astrocytes were incubated with or without FGF-1 (50 ng/ml) for 24 h and washed, and the conditioned medium was analyzed by density gradient ultracentrifugation after removal of cell debris as described in the text. A: Cholesterol mass was measured as described in the text. Open circles with solid lines indicate the conditioned medium without FGF-1, and closed circles with broken lines represent the conditioned medium with FGF-1. Lines without symbols represent the density of the fractions. B: The same sample fractions were analyzed for apoE by Western blotting as described in the text.

6D). Thus, we concluded that M/W astrocytes produce and secrete FGF-1 and that the secreted FGF-1 stimulates the cells to produce and secrete apoE by an autocrine mechanism.

Signaling pathways for the stimulation of apoE synthesis by FGF-1 were examined. The increase of apoE secretion by FGF-1 was suppressed by LY294002, a PI3K/Akt inhibitor, but not by U0126, a MEK inhibitor (Fig. 7A). Phosphorylation of Akt in cytosol was in fact induced by FGF-1 (Fig. 7B). Thus, FGF-1 stimulates apoE synthesis via the PI3K/Akt pathway rather than by the Ras/MEK/Erk pathway.

DISCUSSION

We previously reported that FGF-1-like factor is secreted by 4 week primary cultured rat brain cells into the culture medium and enhances the production and secretion of apoE and cholesterol/phospholipid in W/W cells (39). We also discovered in vivo that FGF-1 is produced before the production of apoE in astrocytes after cryoinjury of the mouse brain (40). From these findings, we hypothesized that FGF-1 is one of the trigger factors for the production and release of apoE and apoE-HDL in astrocytes after brain injury and that it is released by astrocytes themselves to carry this reaction in an autocrine manner. In the present study, we further investigated this reaction in vitro to demonstrate that astrocytes are in fact capable of releasing FGF-1 and autostimulating apoE-HDL production.

The results summarized below led us to conclude that M/W astrocytes release FGF-1 and stimulate the production of apoE-HDL by an autocrine reaction. *1*) The astrocytes prepared by 1 month primary and 1 week secondary

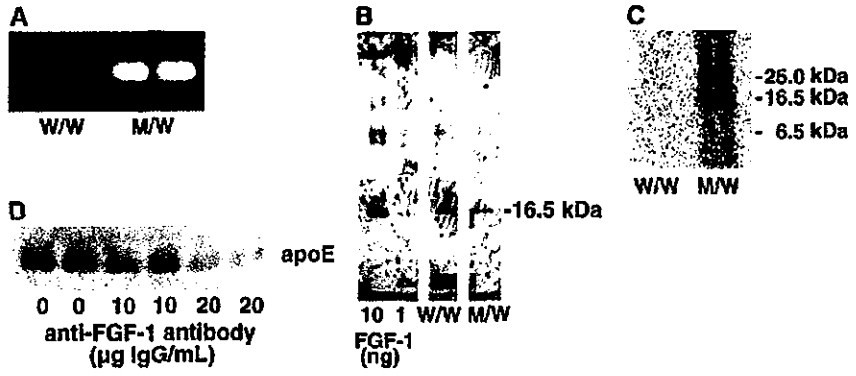

Fig. 6. Production and secretion of FGF-1 by M/W astrocytes. A: Expression of FGF-1 message in W/W cells and M/W cells. The cells were washed with DPBS three times and cultured in 0.1% BSA/F-10 for 24 h. Total cellular RNA was prepared from the cells, and RT-PCR was carried out using FGF-1 primer pairs with 28 cycles as described for Fig. 1. B: FGF-1 in astrocytes. Three milliliters of cell extract (250 μg of cytosol proteins/ml) was prepared from W/W and M/W astrocytes by sonication of the cells in TBS containing protease inhibitor cocktail (Sigma). The cell extract was mixed with heparin-Sepharose at room temperature for 2 h. The gel was washed three times with TBS containing protease inhibitor cocktail and applied for SDS-PAGE and Western blot analysis using goat anti-FGF-1 antibody. The left two lanes represent standard FGF-1. C: FGF-1 secreted into the conditioned medium of astrocytes. W/W and M/W cells (797 and 1,320 μg of total cell protein, respectively), each in two 10 cm Petri dishes, were metabolically labeled with 500 μCi/0.5 nM [^{35}S]methionine in 7 ml of 0.02% BSA/F-10 without methionine or cysteine for 10 h and washed four times. After the cells were incubated in fresh 0.02% BSA/F-10 for 16 h, the conditioned medium equivalent to 690 μg/cell protein was collected. FGF-1 was immunopurified using goat anti-FGF-1 antibody-bound protein G-Sepharose and analyzed by SDS-PAGE and autoradiography. D: Effect of FGF-1 antibody in the culture medium on apoE secretion by astrocytes. M/W cells were exposed to anti-FGF-1 antibody (Santa Cruz Biotechnology) at the indicated concentration in fresh 10% FCS/F-10 at 3, 5, and 7 days after subculture. The culture medium (equivalent to 45 μg of cell proteins) was collected at the end of the 7th day and analyzed by SDS-PAGE and Western blotting using an anti-rat apoE antibody.

culture of rat fetal brain cells (M/W cells) strongly expressed FGF-1 mRNA and synthesized and released FGF-1 into the conditioned medium, being identified as a heparin binding and anti-FGF-1 antibody-reactive protein of 16.5 kDa. 2) M/W cells themselves actively synthesized and secreted apoE with the cellular lipid to generate a greater amount of apoE-HDL, and this reaction was strongly suppressed by the presence of an anti-FGF-1 antibody in the medium during the culture. The conditioned medium of M/W cells stimulated apoE synthesis in the astrocytes (W/W cells), and this activity was abolished by pre-

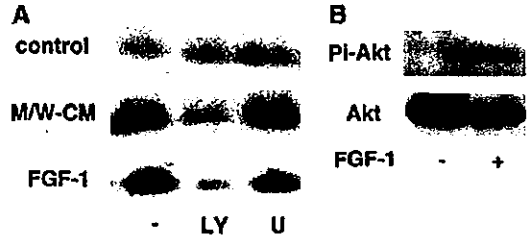

Fig. 7. Analysis of the signaling pathways. A: The rat astrocytes (W/W cells) were treated with LY294002 (LY; 10 μM) or U0126 (U; 10 μM) in the presence of FGF-1, as described in the text. The conditioned medium was analyzed by Western blotting. M/W-CM, M/W cell conditioned medium. B: The cells were treated with FGF-1 for 5 min, and the cytosol was analyzed for Akt and phosphorylated (Pi) Akt by Western blotting.

treatment of the medium with an anti-FGF-1 antibody. 3) The cells that produce FGF-1 and apoE were both GFAP-positive. 4) Results from the examination of signaling pathways suggested that FGF-1 stimulates apoE synthesis via PI3K/Akt activation.

Among the several cytokines examined for the stimulation of apoE synthesis and secretion in human astrocytes, epidermal growth factor stimulated apoE secretion, whereas interleukin 1α and 1β, interferon γ, and FGF-2 did not (45). In our previous work, we examined the effect of FGF-1, FGF-2, insulin, and interleukin 1β in rat astrocytes, and only FGF-1 stimulated the synthesis and secretion of apoE and lipid in rat astrocytes (39). FGF-1-like activity was found in the conditioned medium of brain cell culture to stimulate the astrocytes in the same manner. Our finding that FGF-1 is produced in astrocytes before the production of apoE in the brain injury lesion strongly supported the idea of its important role in the healing process. It has been thought that FGF-1 is primarily synthesized by neurons in vivo, but astrocytes are also identified as a potential source (46–52). The present results indicate that astrocytes are the main source of FGF-1 for the stimulation of apoE-HDL production by an autocrine mechanism.

Epidermal growth factor increases apoA-I expression in human hepatoma cells (HepG2) through the Ras-MAP kinase cascade and Sp1 (53). FGF-1 is known to induce signaling through P21ras/Erk (54, 55) and PI3K/Akt (56) in cells, including astrocytes. The present experiments using

inhibitors of these pathways preliminarily indicate that apoE synthesis is stimulated by FGF-1 via the PI3K/Akt pathway. It is still unknown how this signaling regulates apoE gene expression. On the other hand, the apoE gene is upregulated by liver X receptor/retinoid X receptor in macrophages/adipocytes (57), fibroblasts (58), and astrocytes (59). Thus, the present results do not exclude the possibility that FGF-1 may indirectly upregulate apoE gene expression via the enhancement of cholesterol metabolism. The involvement of cAMP- and protein kinase C (PKC)-related pathways was also suggested for apoE upregulation (60).

It is puzzling how FGF-1 is released by cells even without a signal peptide. FGF-9 produced in the brain and kidney is also secreted by cells in spite of the lack of a signal peptide (61). As FGF-9 is an *N*-glycosylated protein, it is thought to be processed and secreted via the Golgi apparatus. The FGF-1-transfected cells release FGF-1 into the culture medium only in heat-shock conditions (62). FGF-1 may thus be secreted by astrocytes under stress to stimulate the secretion of apoE-HDL, such as heat shock, oxidation, and long-term culture, as described in this work. These stress conditions may be related to the brain damage.

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