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

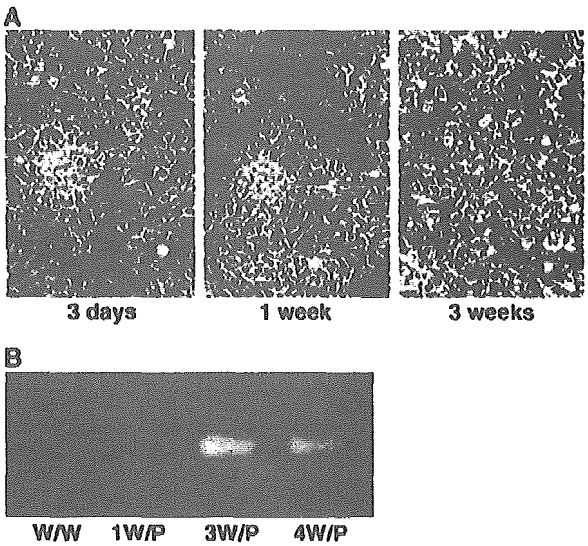

Fig. 1. Expression of fibroblast growth factor-1 (FGF-1) mRNA in rat fetal brain cells. A: Microscopic appearance of rat fetal brain cells in primary culture for 3 days, 1 week, and 3 weeks. B: FGF-1 mRNA. Total cellular RNA was prepared with Isogen (Wako Life Science) from brain cells in primary culture 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

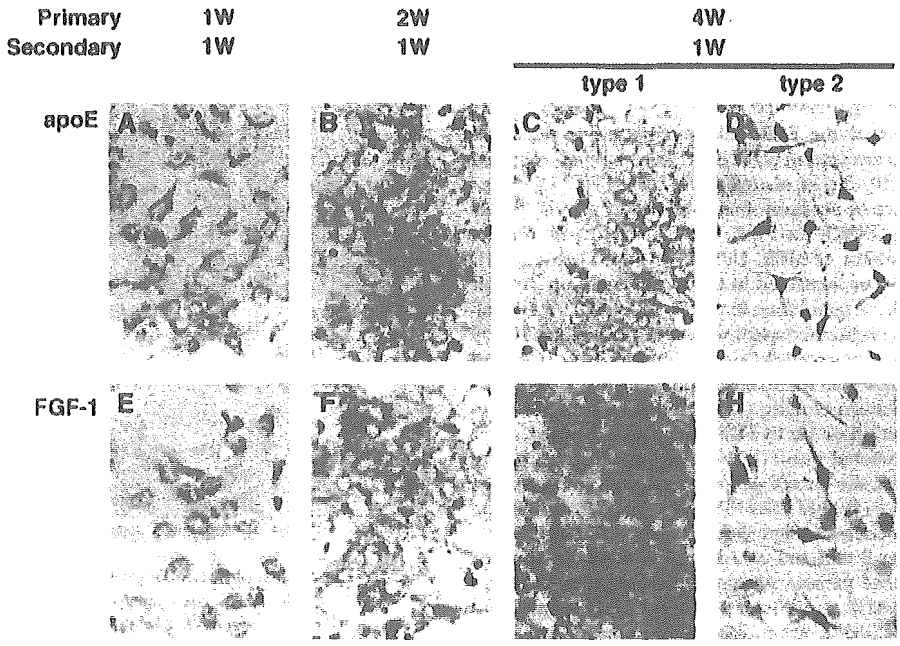

Fig. 2. Immunostaining of FGF-1 and apolipoprotein E (apoE) in astrocytes. The cells were prepared by 1 week secondary culture after primary culture for 1 week (A, E), 2 weeks (B, F), or 4 weeks (C, D and G, H) of rat fetal brain cells. In the preparations of 4 week primary and 1 week secondary culture, 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.

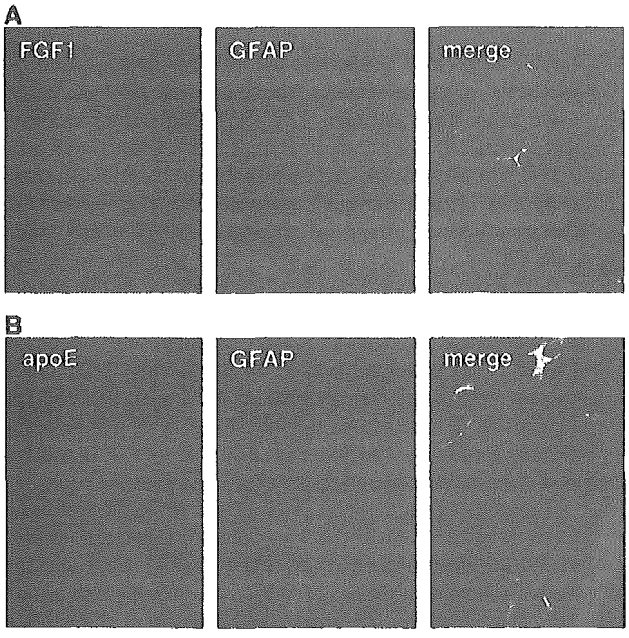

Fig. 3. Immunostaining of M/W cells. A: The cells were immunostained using goat anti-FGF-1 and mouse anti-glial fibrillary acidic protein (GFAP) antibodies and visualized with rhodamine-conjugated donkey anti-goat IgG antibody (Chemicon International) and fluorescein-conjugated horse anti-mouse IgG antibody (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.

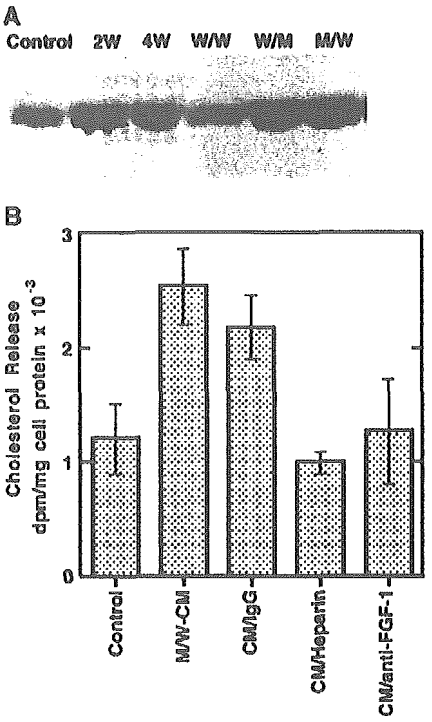

Fig. 4. FGF-1-like activity in conditioned media. A: The effect of the conditioned media of the various brain cells and astrocytes on the secretion of apoE by W/W cells. The conditioned media were prepared from the primary culture for 2 weeks (2W; 356 μg cell protein/ml/well) and 4 weeks (4W; 644 μg/ml/well) 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.

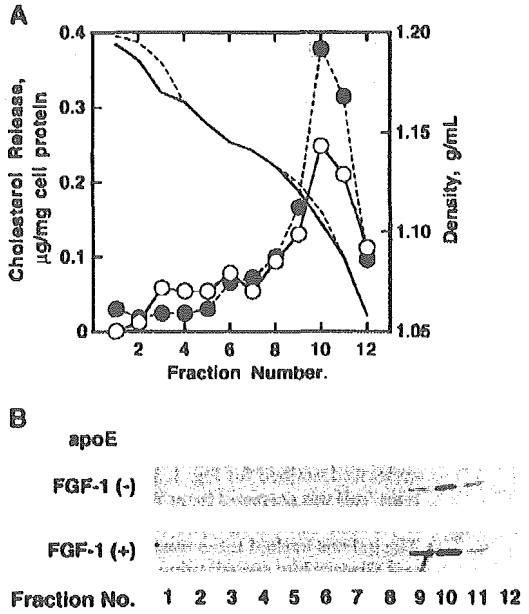

Fig. 5. Increase of apoE-HDL production by rat astrocytes stimulated by FGF-1. Rat astrocytes were incubated with or without FGF-1 (50 ng/ml) for 24 h and washed, and the conditioned medium was analyzed by density gradient ultracentrifugation after removal of cell debris as described in the text. A: 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

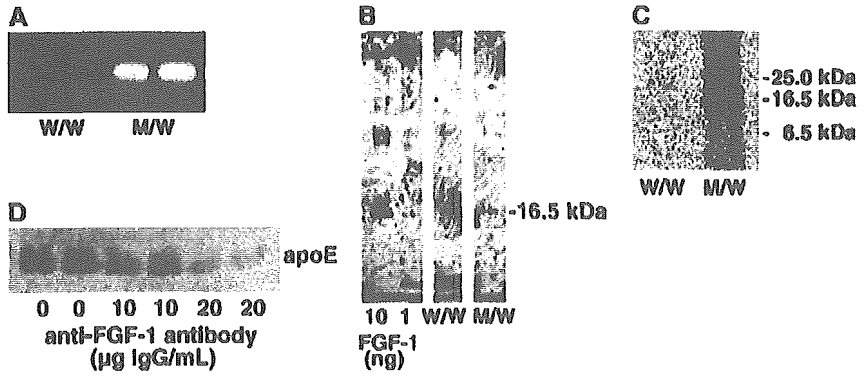

Fig. 6. Production and secretion of FGF-1 by M/W astrocytes. A: Expression of FGF-1 message in W/W cells and M/W cells. The cells were washed with DPBS three times and cultured in 0.1% BSA/F-10 for 24 h. Total cellular RNA was prepared from the cells, and RT-PCR was carried out using FGF-1 primer pairs 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 pretreatment 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.

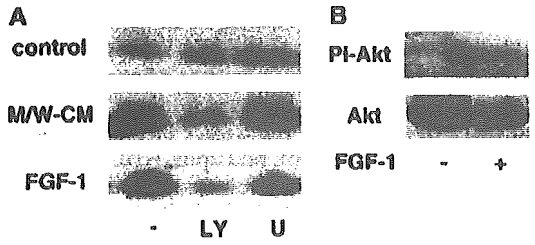

Fig. 7. Analysis of the signaling pathways. A: The rat astrocytes (W/W cells) were treated with LY294002 (LY; 10 μM) or U0126 (U; 10 μM) in the presence of FGF-1 as described in the text. The conditioned medium was analyzed by Western blotting. M/W-CM, M/W cell conditioned medium. B: The cells were treated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

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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Fenofibric Acid, an Active Form of Fenofibrate, Increases Apolipoprotein A-I–Mediated High-Density Lipoprotein Biogenesis by Enhancing Transcription of ATP-Binding Cassette Transporter A1 Gene in a Liver X Receptor–Dependent Manner

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Objective—Fibrates are widely used drugs to reduce plasma triglyceride and increase high-density lipoprotein. Their active forms, fibric acids, are peroxisome proliferator-activated receptor-α activators, but no direct evidence has been demonstrated for their activation of ATP-binding cassette transporter A1 (ABCA1) in relation to clinically used fibrates. We investigated the reaction of fenofibric acid in this regard.

Methods and Results—Fenofibric acid was examined for the effect of increase of ABCA1 activity. It enhanced ABCA1 gene transcription and its protein level in macrophage cell line cells and fibroblasts and increased apolipoprotein A-I–mediated cellular lipid release, all in a dose-dependent manner. Enhancement of the gene transcription was examined by using a reporter assay system for liver X receptor responsive element (LXRE) and its inactive mutant. The results demonstrated that the effect of fenofibric acid is dependent on active LXRE.

Conclusions—Fenofibric acid increased transcription of ABCA1 gene in a liver X receptor–dependent manner. (*Arterioscler Thromb Vasc Biol.* 2005;25:1193-1197.)

Key Words: fenofibrate ■ fibrates ■ PPARα ■ ABCA1 ■ HDL ■ cholesterol ■ atherosclerosis

High-density lipoprotein (HDL) is a negative risk factor in coronary atherogenesis,[1] and raising HDL is expected to protect us against atherosclerosis. Such an effect was demonstrated in experimental animals by specific gene expression[2] or inhibition of cholesteryl ester transfer protein (CETP).[3] Although no specific drug is available in clinical use for this purpose, a bile acid–sequestering resin and statins were shown to raise HDL by an unknown mechanism, besides lowering low-density lipoprotein, and subanalysis of these results indicated its independent effect of reducing the atherosclerosis risk.[4–6] Fibric acids, active forms of fibrate drugs and activators of peroxisome proliferator-activated receptor-α (PPARα),[7,8] are also known for an HDL-raising effect. This group of drugs has been widely used for a long time for the treatment of hyperlipoproteinemia, especially types IIb, III, and VI. Fibric acids enhance fatty acid catabolism and accordingly reduce plasma lipid level, predominantly triglyceride (TG). Increase of TG-rich lipoprotein results in increase of TG transfer to HDL in exchange with its cholesteryl ester by CETP, and therefore leads to production of small cholesterol-poor HDL as TG is hydrolyzed.[9,10] Consequently, reduction of TG-rich lipoprotein by fibrates leads to the increase of HDL cholesterol by reversing this mechanism.[11,12] Fibric acids were also shown to enhance transcription of the gene of apolipoprotein A-I (apoA-I) in the liver.[13–15] A PPARα activator, Wy14643, was shown to upregulate the gene of ATP-binding cassette transporter A1 (ABCA1)[16] that mediates and rate-limits biogenesis of HDL by the interaction of helical apolipoprotein and cells.[17–20] ABCA1 expression is enhanced by loading cholesterol to cells via the liver X receptor (LXR),[21,22] presumably because of the increase of oxysterol. The effect of Wy14643 was interpreted by the activation of the LXR pathway as it increased LXR.[16] However, there has been no direct demonstration of the ABCA1 upregulation by fibric acids derived from fibrate drugs clinically used. In mouse atherosclerosis models, PPARα agonists did not appear to enhance ABCA1 expression in atherosclerotic lesion despite their effect of the regression.[23,24] Here we report in vitro observation that fenofibric acid increases the expression of ABCA1 and apoA-I–mediated HDL production. The effect on ABCA1 expression was through the enhancement of the transcription of the ABCA1 gene being dependent on LXR.

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Materials and Methods

Cell Culture

RAW264 cells were maintained in Dulbecco modified Eagle medium (DMEM)/F-12 (1:1) medium (IWAKI Glass) containing 2% TCM serum replacement (purchased from KN) at 37°C in 5% CO_2.[25] Cells in 6-well plates at the concentration of 1.5×10^6 cells per well were incubated 24 hours before the experiments.[25] THP-1 cells (4.0×10^6 cells per well) were differentiated with 3.2×10^{-7} M phorbol 12-myristate 13-acetate (PMA; Wako) in 10% FBS (PAA Laboratories)-RPMI 1640 medium (IWAKI Glass) for 72 hours.[26] BALB/3T3 clone A31[27] (obtained from RIKEN Cell Bank) was incubated in Eagle's minimum essential medium (MEM) with 10% FCS. All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2. PPARα activators, fenofibric acid, or Wy14643 (Calbiochem-Novabiochem) were dissolved in dimethyl sulfoxide and added to the culture medium containing 0.2% BSA (Sigma).

Cellular Lipid Release

RAW264 cells were washed with PBS and cultured an additional 48 hours in the presence of fenofibric acid or Wy14643 in DMEM/F-12 (1:1) medium containing 2% TCM and 0.2% BSA. During the last 24 hours of the drug treatment, 300 μmol/L of dibutyryl cAMP (dbcAMP; Wako) and apoA-I (10 μg/mL) were added to the medium.[25] THP-1 cells and BALB/3T3 cells were also treated with the PPARα activators and apoA-I in 0.2% BSA-RPMI 1640 medium and 0.1% BSA-MEM, respectively. Cholesterol and choline-phospholipid released into the medium by apoA-I were determined enzymatically.[25] Adherent cells were dissolved in 0.1 N NaOH for protein determination by bicinchoninic acid protein assay system (Pierce).

Reporter Gene Assay

The constructs of luciferase reporter genes were prepared as described previously.[28] The 5′-flanking region of mouse ABCA1 gene (−1238/+57) was inserted into pGL3 vector (Promega) to generate ABCA1 promotor-luciferase reporter construct (pABCA1-Luc). The reporter plasmid with mutated and inactivated LXR-responsive element (LXRE) (mutant LXRE) was generated by using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutations introduced were identical to those reported previously.[21] Cells cultured in 24-well plates (3.0×10^3 cells per well) were washed once with PBS or pABCA1-Luc vector or pABCA1-mutant LXRE-Luc vector were cotransfected with phRL-tk vector (Promega) by Superfect transfection reagent (Qiagen). Three hours after the transfection, cells were washed with PBS and cultured in the presence of fenofibric acid or Wy14643 for 24 hours. Cellular luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega). Results were standardized by the Renilla luciferase activity derived from phRL-tk vector.

Immunoblotting of ABCA1

Cells incubated with fenofibrate or Wy14643 for 48 hours were harvested in cold PBS and pelleted by centrifugation. The cell pellet was suspended in 5 mmol/L Tris-HCl, pH 8.5, containing 1% protease inhibitor cocktails (Sigma) and placed on ice for 30 minutes. The cell suspension was centrifuged at 650*g* for 5 minutes, and the supernatant was centrifuged at 105 000*g* for 30 minutes to prepare the membrane fraction as a pellet. Immunoblotting of ABCA1 was performed according to the previous method.[26]

Results

Expression of ABCA1

The effects of fenofibric acid and Wy14643 on expression of ABCA1 are shown in Figures 1 and 2. The message of ABCA1 increased by fenofibric acid and Wy14643 in all types of cells examined: RAW264 cells treated with dbcAMP, PMA-differentiated THP-1 cells, and BALB/3T3 cells. ABCA1 protein also increased by the PPARα agonists being demonstrated by its immunoblotting analysis in all these cells (Figures 1 and 2).

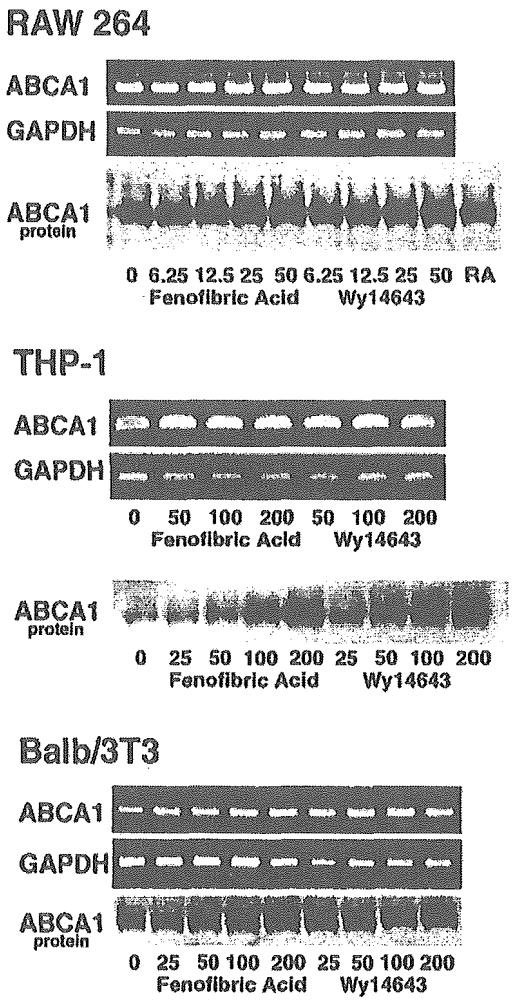

Figure 1. Effects of PPARα agonists fenofibric acid and Wy14643 on expression of ABCA1 in RAW264 cells pretreated with dbcAMP and THP-1 cells differentiated with PMA and BALB/3T3 fibroblasts. Messages of ABCA1 and GAPDH were detected by RT-PCR, and protein level of ABCA1 was determined by immunoblotting, as described in the text in each type of cell in the presence of the agonists (μmol/L) and 9-*cis*-retinoic acid (RA).

ApoA-I–Mediated Cellular Lipid Release

ApoA-I induced release of cellular cholesterol and phospholipids into the medium from the cells examined. PPARα agonists fenofibric acid and Wy14643 increased the apoA-I–mediated release of cholesterol and phospholipids in a dose-dependent manner (Figure 3). The increment of lipids released by the drugs was more prominent in cholesterol than phospholipid in RAW264 cells pretreated by dbcAMP. The maximum effect (102% increase in cholesterol release) was observed when the cells were treated with 25 μmol/L of fenofibric acid.

Reporter Gene Assay

Transcription of the ABCA1 gene was examined by using the reporter genes (pABCA1-Luc) in the dbcAMP-treated RAW264 cells (Figure 4). Fenofibric acid and Wy14642

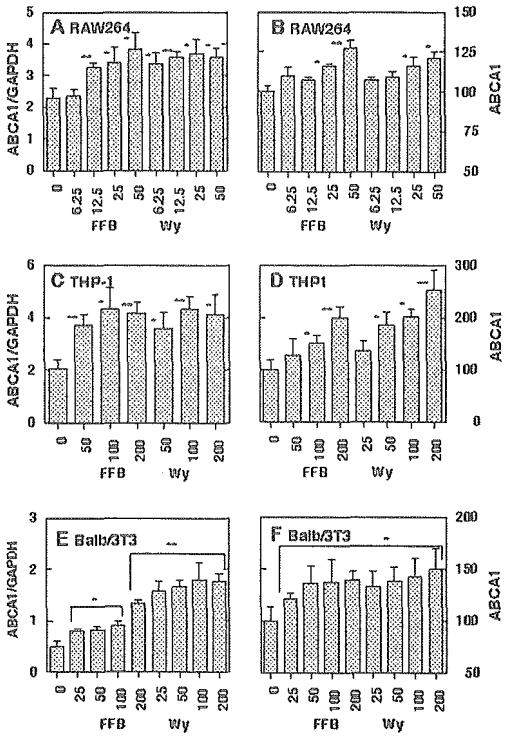

Figure 2. Effects of PPARα agonists fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L) on expression of ABCA1. The results of RT-PCR and Western blotting from the same experiments shown in Figure 1 were semiquantified by digital scanning in an Epson GT9500. Message of ABCA1 was standardized for that of GAPDH. Data points represent mean±SE of 3 independent experiments. Significance of the increase from the controls was examined by Student's *t* test and indicated as $^{*}P<0.05$ and $^{**}P<0.01$.

enhanced transcription of the ABCA1 reporter gene in a dose-dependent manner (Figure 4A and 4B). These effects were cancelled by substitute transfection of the mutant LXRE-containing reporter vector (pABCA1-mutant LXRE-

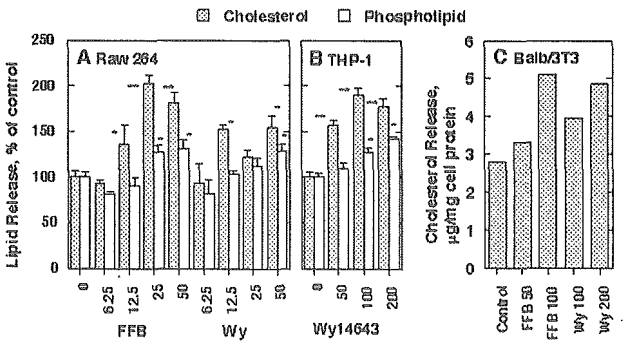

Figure 3. Lipid release by apoA-I from the cells examined in the presence of PPARα agonists fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L). Releases of cholesterol and phospholipid are expressed as percentage of the control for RAW264 cells (cholesterol 2.39 μg/mg cell protein and phospholipid 5.89 μg/mg cell protein), and for THP-1 cells (cholesterol 2.45 μg/mg cell protein and phospholipid 4.26 μg/mg cell protein). Data points in A and B represent mean±SE of triplicate measurement, and those in C represent the average of duplicate measurement. Significance of the increase from the controls was examined using Student's *t* test and indicated as $^{*}P<0.05$ and $^{**}P<0.01$.

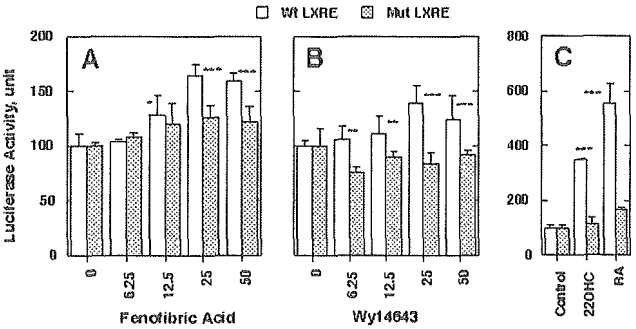

Figure 4. Luciferase reporter gene assay of ABCA1. The reporter genes for the ABCA1 promoter were constructed as described in the text. Wt-LXRE and Mut-LXRE indicate the genes without and with introduction of mutation in LXRE to inactivate the responsive element. The effects of fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L) were examined, as well as those of 9-*cis*-retionic acid (RA) and 22-oxysterol (220HC). Data points represent mean±SE of triplicate measurement. Significance of the increase was examined using Student's *t* test and indicated as $^{*}P<0.05$ from the blanks (no compound), $^{**}P<0.05$ from the controls (mutant LXRE), and $^{***}P<0.05$ from both.

Luc) to inactivate LXRE (Figure 4A and 4B), whereas 9-*cis*-retionic acid, a ligand for retinoid X receptor (RXR), and 22-oxysterol, a ligand for LXR, failed to increase the transcription of the mutant ABCA1 gene (Figure 4C).

Discussion

PPARs belong to the nuclear receptor superfamily group and act as ligand-activated transcription factors regulating the expression of certain target genes.[29] The PPAR family contains 3 different subtypes, designated PPARα, PPARβ/δ, and PPARγ. PPAR subtypes display distinct expression patterns, different ligand specificities, and distinct biological functions.[30–32] PPARs are activated by fatty acids and its metabolites and accordingly exert various effects in lipid homeostasis.[33] Several subtype-specific synthetic compounds have been developed for clinical use, including fibric acids (PPARα agonist) and glitazones (PPARγ agonist).[34] Fibrates are widely used drugs for hyperlipidemic patients because they significantly improve plasma lipid profiles by reducing TG-rich lipoprotein and raising HDL.[35,36] The primary effects of fibric acids, active forms of fibrates, on plasma lipids have been attributed to their PPARα-mediated expression of the genes of various enzymes that regulate lipid metabolism.[7,8] For HDL metabolism, the effects are partly explained by reduction of plasma TG itself and CETP reaction[9,10] and by increased expression of the apoA-I gene.[13–15] In addition, Wy14643, a nonclinical PPARα activator, was shown to enhance ABCA1 gene expression.[16] Because LXR was also activated in the condition used in that work,[16] and ABCA1 is known to be regulated by the LXR/RXR pathway, it was hypothesized that Wy14643 increases the transcription of ABCA1 gene via the LXR pathway.

We demonstrated the increase of ABCA1 by fenofibric acid, an active form of clinically used fibrate drug fenofibrate, in macrophage cell line cells and in mouse fibroblasts. These effects were also reproduced by a positive control Wy14643. To examine the mechanism, the reporter gene

assay was used via a promoter of the ABCA1 gene by introducing a mutation in LXR response element. Inactivation of this element was verified by abolishment of its response to 9-*cis* retinoic acid and 22-oxysterol, and fenofibrate failed to enhance transcription of the mutant reporter gene. Therefore, PPARα in fact activates the ABCA1 gene by the LXR-dependent pathway. The results were inconsistent with the finding that PPARs form a heterodimeric complex with the RXR (not LXR) and bind to specific PPAR-response elements in the promoter region of target gene.[37,38] However, a direct ligand of RXR, 9-*cis* retinoic acid, failed to activate the mutant gene, consistent with the established finding that dimerization of RXR with LXR is essential for enhancing ABCA1 gene transcription.[21]

Fenofibrate and Wy14643 reportedly have different affinity and distinct specificity to murine and human PPARs. However, both compounds showed equivalent capability in transactivation of the ABCA1 gene. Wy14643 activates not only PPARα but also PPARγ and PPARδ in cell-based transactivation assays[39] at the concentration >30 μmol/L. Activation of PPARδ was suggested to affect the ABCA1-mediated HDL biogenesis on the basis that an agonist of PPARδ induced HDL synthesis in culture cells and in monkeys.[40] Therefore, the effects of Wy14643 may include combined activation of various PPARs. In contrast, fenofibric acid is highly specific for activation of PPARα, at least up to 100 μmol/L.[39] Because C_{max} of fenofibrae is 30 μmol/L when it is orally administrated to human, it is most likely that the effect of this drug on the HDL biogenesis is based on the enhancement of ABCA1 expression by the mechanism shown in this article.

Fenofibrate has been shown to retard progression of coronary atherosclerosis,[41] consistent with the findings of reducing a risk of coronary heart disease by other fibrate drugs.[42,43] The clinical effects of these drugs are attributed to improvement of plasma lipoprotein profile by reducing TG and raising HDL. Although decrease of TG and increase of HDL are linked in human by the action of CETP,[10] the increase of ABCA1 activity may more directly contribute to raising HDL and prevention of lipid accumulation in vascular cells.

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Assembly of high density lipoprotein by the ABCA1/ apolipoprotein pathway

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Purpose of review
Mammalian somatic cells do not catabolize cholesterol and therefore need to export it for sterol homeostasis at the levels of cells and whole bodies. This mechanism may reduce intracellularly accumulated cholesterol in excess, and thereby would contribute to the prevention or cure of the initial stage of atherosclerotic vascular lesions.
Recent findings
HDL is thought to play a main role in this reaction on the basis of epidemiological evidence and in-vitro experimental data. Two independent mechanisms have been identified for this reaction. One is non-specific diffusion-mediated cholesterol 'efflux' from the cell surface, and cholesterol is trapped by various extracellular acceptors including lipoproteins. Extracellular cholesterol esterification on HDL provides a driving force for the net removal of cell cholesterol, and some cellular factors may enhance this reaction. The other mechanism is an apolipoprotein-mediated process to generate HDL by removing cellular phospholipid and cholesterol. This reaction is mediated by a membrane protein ABCA1, and lipid-free or lipid-poor helical apolipoproteins recruit cellular phospholipid and cholesterol to assemble HDL particles. The reaction is composed of two elements: the assembly of HDL particles with phospholipid by apolipoprotein, and cholesterol enrichment in HDL. ABCA1 is essential for the former step, and the latter step requires further intracellular events.
Summary
ABCA1 is a rate-limiting factor of HDL assembly and is regulated by transcriptional factors and posttranscriptional factors. Posttranscriptional regulation of ABCA1 involves the modulation of its calpain-mediated degradation.

Keywords
ABCA1, apolipoprotein, caveolin, cholesterol efflux, HDL, membrane

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Abbreviations

ABCA1	adenosine triphosphate-binding cassette transporter A1
ACAT	acyl coenzyme A : cholesterol acyltransferase
cAMP	cyclic adenosine monophosphate
LCAT	lecithin : cholesterol acyltransferase
LXR	liver X receptor
PKC	protein kinase C
RXR	retinoid X receptor

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Introduction

Cholesterol is a unique lipid molecule in animal cells. It acts as an essential constituent of cell membrane and is used as a precursor of steroid hormones and bile acids, but cannot be converted to energy. The biosynthesis of cholesterol is carried out in all the somatic cells, requiring a complicated 37 steps, although the liver is its major site in vertebrates. In contrast, the catabolism of cholesterol is very limited in most peripheral cells, such as limited and partial degradation by sterol 27-alpha-hydroxylase [1,2]. Most cholesterol molecules in the body of vertebrates are therefore transported to the major organ for its catabolism, the liver, except for a small but important part in steroidogenic cells. In the liver, cholesterol is converted to bile acids that are heavily reused in an enterohepatic circulation. This entire scheme of cholesterol metabolism indicates that our system is designed to recognize it as an important and valuable molecule that should not be wasted. In other words, we are well prepared for the crisis management of cholesterol shortage, but very poorly for its overload.

Among the various aspects of cholesterol homeostasis, the regulation of cholesterol biosynthesis and receptor-mediated lipoprotein uptake have been studied extensively for a long time. In addition to the discovery and extensive characterization of the LDL receptor and its reaction [3], recent developments in the study of the sterol regulatory binding protein system have established the regulatory mechanisms for cholesterol biosynthesis at the molecular level [4,5]. The release of cholesterol from somatic cells is also an important part of cholesterol homeostasis both at the cellular level and the whole body, especially in vertebrates. However, an understanding of cellular cholesterol release and intracellular cholesterol trafficking has lagged substantially. Knowledge has accumulated rapidly in this field of research in the past

several years, and significant progress has been made towards understanding the mechanism of cellular cholesterol release.

Extracellular cholesterol transport is largely borne by plasma lipoproteins. The delivery of cholesterol from the liver to the peripheral cells is mainly carried out by LDL and other apoB-containing lipoproteins by receptor-mediated endocytosis of the lipoproteins [3], and perhaps to a minor degree by HDL via the selective uptake of its cholesteryl ester in certain organs [6,7]. On the other hand, the release of cellular cholesterol and its transport to the liver are mediated by HDL. This pathway as a whole is under kinetic control and in a steady state by the assembly and clearance of plasma lipoproteins and extracellular cholesterol metabolism mediated by lecithin : cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein and other active molecules [8]. The most critical step seems to be the release of cholesterol from the cells, which is also a very important component of cellular cholesterol homeostasis. This pathway is often referred to as the concept of 'reverse cholesterol transport' for an anti-atherosclerosis mechanism by HDL, based on two lines of evidence that the plasma HDL level is negatively correlated with the increased risk of atherosclerotic vascular lesions [9], and incubation of the cells in culture with HDL results in a reduction in cellular cholesterol [10].

Two major mechanisms are proposed for the cellular cholesterol release step [11–13]. One is a non-specific cholesterol efflux from the cellular surface by physicochemical cholesterol exchange between the cell membrane and extracellular 'acceptors', perhaps mediated by its diffusion in an aqueous phase, in which the net release of cellular cholesterol is driven by extracellular cholesterol esterification by LCAT. HDL is a major cholesterol acceptor in this reaction with respect to its capacity for cholesterol accommodation, and provides a major and optimum site for the LCAT reaction. Cellular factors may also modulate this reaction. Scavenger receptor B1 seems to expedite the cholesterol exchange rate between the cell membrane and HDL, perhaps through a specific mode of binding to HDL [14,15]. ABCG4 alters the direction of intracellular cholesterol distribution to increase its release by this pathway [16•].

The other mechanism is an assembly of new HDL particles with cellular phospholipid and cholesterol upon the interaction of helical apolipoproteins with cells, in which various specific cellular functions are required, including a cellular interaction site for apolipoproteinand specific intracellular cholesterol traffickingfor this HDL assembly. Adenosine triphosphate-binding cassette transporter A1 (ABCA1) is a key cellular factor. This reaction seems to be a major source of plasma HDL. HDL thus plays dual roles in the removal of cell cholesterol in cholesterol homeostasis, as an efficient acceptor of cellular cholesterol in the diffusion-mediated pathway, and as a major player in the HDL assembly pathway.

Tangier disease and ABCA1: apolipoprotein-mediated HDL assembly is a major source of plasma HDL

The first findings of HDL assembly by cellular lipid and extracellular helical apolipoproteins was the observation that apolipoproteins of HDL such as apoA-I, A-II and E remove phospholipid and cholesterol from mouse peritoneal macrophages and generate new HDL particles [17]. The lipoprotein thus generated meets the criteria of prebeta-HDL with respect to physical and chemical properties [17], morphological appearance [18,19], and biochemical characteristics such as reactivity to LCAT [20,21]. Cholesterol in the cells decreased reciprocally especially in the compartment accumulated as cholesteryl ester [17]. The reaction can be carried out by various helical apolipoproteins having amphiphilic helices composed of some 20 to 22 amino acid residues, so that apoA-I, A-II, A-VI, E and insect apoIII all generate HDL [17,22,23], and so do synthetic amphiphilic peptides as far as they meet such criteria whether composed of D or L-amino acids [23–25]. It seems that certain numbers of the helical segments are required to carry out the reaction.

Although the rate of cholesterol release is much higher than phospholipid in the non-specific diffusion-mediated lipid exchange reaction, the apolipoprotein recruits primarily phospholipid rather than cholesterol to form stable HDL particles in this HDL assembly pathway [26]. The HDL generated by this reaction contains largely phospholipid and unesterified cholesterol, and the LCAT-mediated cholesterol esterification on the HDL generated perhaps helps the maturation of this HDL as it generates core cholesteryl ester [20,21]. However, unlike cholesterol release by a non-specific diffusion-mediated reaction, cholesterol esterification does not result in further enhancement of cellular cholesterol release when the HDL generated is already cholesterol rich [20].

The apolipoprotein-mediated cellular lipid release and HDL assembly clearly involve cellular factors. The apolipoprotein-mediated reaction requires a certain specific interaction site(s) on cells for helical apolipoprotein. This view is supported by the experimental results that apolipoproteins do not interact with certain types of cells, and generate no HDL, such as erythrocytes [27,28] and rat hepatoma cells Fu5AH [29,30]. In mouse macrophage

cells RAW264, the apolipoprotein-mediated generation of HDL is induced by the treatment of cells with cyclic adenosine monophosphate (cAMP) in parallel with the apolipoprotein binding to the cellular surface [31,32]. The requirement of intact Golgi function was also demonstrated for apolipoprotein–cell interaction [33]. As mentioned later, probucol suppressed apolipoprotein binding and HDL assembly [34,35]. The proteolytic treatment of cells also abolished the apolipoprotein-mediated reaction [28]. These results strongly indicated that a membrane protein component(s) is involved in the interaction of helical apolipoproteins with the cell surface.

Tangier disease is a genetic disorder known as one of the familial HDL defficiencies [36]. The cells from patients with this disease were shown to be impaired in the interaction with apolipoprotein and lack the HDL assembly [37,38]. Mutations were identified in the gene of the ABCA1 in patients with this disease and other types of familial HDL deficiencies [39–44], opening a new horizon for the study of HDL metabolism. Disruption of this gene resulted in HDL deficiency in mice, so that it is generally true that the product of this gene, ABCA1, is essential for production of plasma HDL [45,46].

Although apolipoproteins do not interact with Tangier cells and generate no HDL [37,38], the cells seem intact for non-specific diffusion-based cholesterol release [37]. This means that ABCA1 may act as a direct interaction site for apolipoproteins to generate HDL. To support this idea, induction of the HDL assembly reaction in RAW264 cells by cAMP is accompanied by the induction of apoA-I binding and the expression of ABCA1 [47,48]. Moreover, the direct interaction of helical apolipoproteins with ABCA1 was demonstrated by chemical cross-linking in the cells where the protein is transfected [49,50•]. ABCA1 thus essentially functions as a mediator for apolipoprotein cell binding, and direct interaction between these two molecules is indicated. Hot spots for the ABCA1 mutations are concentrated in the cytosolic adenosine triphosphate-binding cassette domains and the cell surface domains, according to the model proposed [51•], so that the interaction of ABCA1 with extracellular apolipoproteins seems to be an essential part of the ABCA1 function.

A lack of the ABCA1–apolipoprotein reaction results in a deficiency of plasma HDL. Therefore, the ABCA1-mediated reaction is a major source of the generation of HDL. It is, however, of interest that ABCA7 also mediates the HDL assembly in a very similar manner to ABCA1 [52•,53•]. This reaction may be important locally as a back-up system for ABCA1, but may not significantly contribute to the regulation of plasma HDL concentration.

Mechanism for HDL assembly reaction

HDL-like particles can be generated *in vitro* with helical apolipoproteins and phospholipid, with or without core lipid and cholesterol, without specific catalysts except for a requirement of energy for dispersion to homogeneity [54]. The reaction always ends up with particles of certain sizes composed of at least a few hundreds of phospholipid molecules. This means that HDL-like particles are a thermodynamically stable molecular assembly for helical apolipoproteins and phospholipid. On the other hand, the cholesterol–apolioprotein complex has never been generated in such a manner. When apolipoproteins interact with cells through ABCA1 to generate HDL, this should be the same based on the physicochemical nature of the apolipoprotein–phospholipid interaction. 'Lipidation' of apolipoprotein should take place primarily with phospholipid in a kind of snap-in manner rather than by 'gradual growth'. HDL particles are generated primarily with membrane phospholipid as discussed below.

The cholesterol content in HDL varies depending on the type and condition of cells. Table 1 summarizes the reactivity of various cells to apolipoprotein-mediated and non-specific diffusion-mediated cholesterol release. Three parameters are used to classify the cells: non-specific cholesterol release, the removal of phospholipid by apolipoprotein and that of cholesterol. All the cells are active in the non-specific reaction, although there may be some kinetic difference. For apolipoprotein-mediated HDL assembly, cells are categorized into three groups [62]. Group 1 cells interact with apolipoprotein and produce cholesterol-rich HDL. Group 2 cells produce cholesterol-poor HDL, suggesting that the cells are functional for the assembly of HDL with cellular phospholipid but cholesterol is poorly available for the reaction. Group 3 cells do not interact with apolipoprotein and produce no HDL, represented by Tangier cells. The expression of ABCA1 is a necessary factor for the generation of HDL but not for the incorporation of cholesterol into the HDL generated. Therefore, ABCA1 primarily functions to mediate the interaction of apolipoprotein and membrane phospholipid to generate HDL particles, and the enrichment of cholesterol in HDL may be an independent process.

Rat vascular smooth muscle cells generate cholesterol-poor HDL by an apolipoprotein–cell interaction [26], and treatment with cytokines and protein kinase C (PKC) activators increased the incorporation of cholesterol into HDL [58]. In contrast, the treatment of macrophages and the above-conditioned vascular smooth muscle cells with PKC inhibitors decreased the cholesterol content in HDL generated by apolipoprotein [55,58]. Cellular cholesterol levels may also contribute

Table 1. Reactivity of various cells to apolipoprotein-mediated HDL generation and diffusion-mediated cholesterol release

	HDL generation				
Cell	PL	Cholesterol	Diffusion	ABCA1	Ref.
MPM	+	+	+	(+)	[17]
MPM, PKC inhibitor-treated	+	±	+	(+)	[55]
RAW264	−	−	+	−	[31]
RAW264, cAMP-treated	+	+	+	+	[31,47]
Human fibroblasts	+	+	+	(+)	[20]
Human fibroblasts, probucol	ND	−	ND	+	[56*]
Rat fibroblasts	+	+	+	(+)	[57]
CHO cells	+	+	ND	(+)	[18]
Rat VSMC	+	±	+	ND	[58]
Rat VSMC, differentiated	+	+	+	ND	[58]
Monkey VSMC	ND	+	+	ND	[59]
Rabbit VSMC	ND	+	+	ND	[59]
5FuAH	−	−	+	ND	[29]
THP-1 undifferentiated	+	−	+	−	[60]
THP-1 differentiated	+	+	+	+	[60]
Tangier fibroblast	−	−	+	−	[37,38]
MPM, probucol-treated *in vitro*	−	−	+	ND	[34]
MPM, probucol-treated *in vivo*	−	−	+	ND	[35]
Rat astrocytes	+	±	+	(+)	[57]
Rat astrocytes, SPMase-treated	+	+	+	+	[61]
Erythrocytes	−	−	+	ND	[27,28]
MPM, trypsin treated	−	−	+	ND	[28]
WI38	+	+	+	+	[62]
MRC5	+	+	+	+	[62]
BalbC/3T3	+	+	+	+	[62]
L929	−	+	+	+	[62]
CHO-K1	−	+	+	+	[62]
COS7	−	−	+	−	[62]

cAMP, Cyclic adenosine monophosphate; CHO, Chinese hamster ovary; MPM, mouse peritoneal macrophage; ND, not determined; PKC, protein kinase C; PL, phospholipid; SPMase, sphingomyelinase; VSMC, vascular smooth muscle cells. + The presence of ABCA1 was determined in other papers.

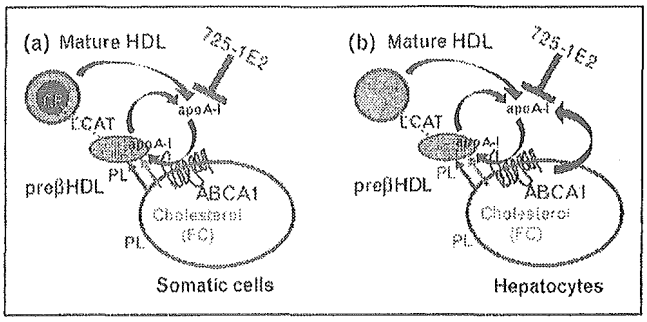
to the regulation of the cholesterol content in the HDL produced [63,64].

Cholesterol is mobilized from an intracellular compartment(s) to the pool used by the apolipoprotein-mediated HDL generation. When the cells were incubated with whole HDL particles, activation of PKC was observed [65]. The stimulation and inhibition of PKC modulated cholesterol content in HDL generated by apolipoprotein as mentioned above, and at the same time the intracellular cholesterol compartment available for the esterification by acyl coenzyme A : cholesterol acyltransferase (ACAT) was changed reciprocally to the HDL cholesterol content [55,58]. The involvement of PKC was thus implicated in the process of cholesterol mobilization by apolipoprotein. Cholesterol release by the apolipoprotein-mediated HDL assembly is almost linear up to 24 h in the time course, whereas the decrease in the ACAT-available cholesterol compartment rapidly reaches its maximum in a few hours [55]. The decrease in this compartment was shown as the most extreme case by the stimulation of cells by peroxidase-treated HDL [66,67], in which apoA-I/A-II heterodimer presented as a complex with lipids is responsible for this stimulation [68]. In contrast, no such rapid and dramatic change takes place when cell cholesterol is removed by the diffusion-mediated pathway [58]. Therefore, a specific intracellular signal rather than a mere general decrease in membrane cholesterol is involved in triggering the mobilization of cholesterol from a certain intracellular compartment. Similar findings were also demonstrated with the 'HDL-mediated' cell cholesterol removal. Fab fragments of anti-apoA-I antibody inhibited the removal of cholesterol from the intracellular pool but not from the plasma membrane [69]. Fibroblasts of Tangier disease patients showed an impairment of intracellular cholesterol removal by HDL [37,70].

A rapid signal seems to be generated by apoA-I to activate PKC in relation to the mobilization of the intracellular cholesterol compartment utilized for esterification by ACAT. Interesting findings related to such cholesterol mobilization have been made with caveolin and astrocytes. In the brain, plasma lipoproteins do not cross the blood–brain barrier and extracellular transport is carried out by the independent intrabrain HDL system. HDL seems to be generated by astroglia and microglia mainly by apoE synthesized by these cells [71,72]. Rat astrocytes secrete cholesterol-rich HDL by endogenous apoE and cholesterol-poor HDL by exogenous apolipoproteins including apoA-I and apoE [57]. The interaction of cholesterol with sphingomyelin in the plasma membrane was shown to be a factor to regulate the incorporation of cholesterol into the HDL generated, so that a sphingomyelin-rich membrane domain is a candidate compartment selectively used by the HDL assembly reaction by apolipoprotein [61,73].

Caveolin-1 is a molecule involved in intracellular cholesterol trafficking to the cellular surface, perhaps specific to a domain such as the caveola, often defined as detergent-resistant membrane rich in cholesterol and sphingomyelin. Caveolin-1 is found associated with detergent-resistant membrane [74], which is regulated by the membrane cholesterol content [75], and its expression is related to cellular cholesterol levels [76,77]. Caveolin-1 enhances cholesterol transport from the intracellular compartment to the plasma membrane [78] by forming a complex with a chaperone protein(s) in the cytosol [79]. The involvement of caveolin in cholesterol export to HDL was demonstrated [76], and more specifically in cholesterol trafficking to apolipoprotein-mediated HDL generation [60]. ApoA-I induces the rapid translocation of caveolin-1 [80] and cholesterol to cytosolic lipid-protein particles, as

Figure 1. Assembly of HDL by apoA-I and ABCA1



(a) In somatic cells that do not produce helical apolipoprotein [88°]. Extracellular apoA-I interacts with cellular phospholipid (PL) in the presence of ABCA1 to assemble preβ or nascent HDL particles. Their free cholesterol (FC) content is regulated by cellular factors, as described in the text. As lecithin : cholesterol acyltransferase (LCAT) reacts on HDL to produce cholesteryl ester (CE) as a core lipid, the lipoprotein particles get more spherical. ApoA-I dissociates from the HDL at any step of maturation and interacts with the cell surface to produce more HDL. (b) In hepatocytes [87°]. In addition to extracellular sources, apoA-I is also supplied from an authentic source and produces HDL by the same mechanism in an autocrine manner. A monoclonal antibody selectively against lipid-free apoA-I, 725-1E2, blocked the production of HDL in both cases [87°,88°].

well as phospholipase Cγ and PKCα [81°]. Diacyl glycerol is also generated on this particle.

It is still unclear whether cholesterol is incorporated after apolipoprotein-phospholipid particles are assembled or the lipid source for HDL assembly is enriched in cholesterol by the cholesterol mobilization system discussed above.

Apolipoprotein–cell interactions mediated by ABCA1 generate HDL and are a major source of plasma HDL, at least in mice and humans. However, many questions about the site and mechanism for the assembly of HDL particles still remain unanswered. Major sites for synthesis of helical apolipoproteins, especially for the main apolipoprotein of HDL apoA-I, are believed to be the liver and intestine. In contrast to apoB-containing lipoproteins, no HDL particles, not even premature HDL, has been reported to be found in the secretary pathways such as the endoplasmic reticulum and the Golgi apparatus with the cells of these organs. Nevertheless, HDL particles are found in culture media of the hepatocytes [82,83] or in the perfusate of the liver [84,85], mostly as a so-called nascent HDL that is composed mostly of surface lipids, phospholipid and cholesterol, not containing much core lipid, and consequently in a disc-like shape. The question then becomes how and where these particles are formed. If the apolipoprotein–cell interaction is a major mechanism for the production of HDL, it is possible that HDL is assembled by an autocrine mechanism, such that apoA-I or E are first secreted by the cells and then interact with the cell surface to generate HDL [31,86]. This hypothesis has been more directly supported by using an antibody specific to lipid-free apoA-I to inhibit ABCA1-dependent HDL assembly by hepatocytes [87°]. The same antibody also selectively inhibited an ABCA1-dependent part of cholesterol release to HDL from the cells that do not produce apolipoprotein [88°]. Therefore, lipid-free apolipoprotein is to be released whether from cells or from HDL particles to interact with cellular ABCA1 for the assembly of HDL particles from cellular lipid (Fig. 1). Alternatively, apolipoproteins may interact partly with the membrane sometime before secretion through the same mechanism as extracellular apolipoprotein reacts [89,90°]. This view may be consistent with the finding of an abnormal Golgi structure in the hepatocytes of ABCA1 knockout mice [45], and the differential generation of HDL with endogenous apoE and exogenous apoA-I by rat astrocytes [57].

How does HDL remove cell cholesterol?

HDL has been studied for its capacity as a whole particle to remove cellular cholesterol in most of the experiments published. When the cells are incubated with HDL particles, cellular cholesterol decreased substantially [10]. This net removal appears with the characteristics of both the diffusion-mediated and the apolipoprotein-mediated pathways; LCAT induces the net release of cell cholesterol by whole HDL particles, even from cells without having the capacity for interaction with apolipoprotein [27,91,92]. Even without LCAT activity, HDL is also capable of reducing cell cholesterol [34], being accompanied with various cellular events and reactions, most of which are reproduced by apolipoproteins, such as the removal of intracellular cholesterol [93], the rapid reduction of the ACAT-available cholesterol pool [66], and PKC activation [65]. HDL as a whole lipoprotein particle thus mediates cellular cholesterol removal by both of these two major pathways; diffusion-mediated cellular cholesterol release, and apolipoprotein-mediated removal of cellular lipid that leads to the assembly of new HDL particles with cellular phospholipid and cholesterol.

The concept of a diffusion-mediated reaction was first proposed by Glomset [94]. Various factors may be involved in its regulatory system, such as cholesterol compartments in the plasma membrane and HDL structure, but the major driving force for the net removal of cellular cholesterol is LCAT. On the other hand, it is not exactly known how the HDL apolipoproteins interact with the cell. Apolipoproteins do not interact with cells to generate HDL when they are bound to the LDL-size lipid microemulsion surface [95]. Therefore, it is likely that apolipoprotein interacts with the cells in its 'free' form.

Helical apolipoproteins are in equilibrium between a lipid-bound form and a dissociated form from the lipid surface

presumably free in solution. Although the dissociation constant of apolipoproteins is not known directly for the HDL surface, the constant measured for the LDL-size lipid particles are all in the order of 10^{-7} M, which may not be irrelevant for the HDL surface [96,97]. Assuming that the dissociation constant of apoA-I is in this range and the binding capacity of HDL is just enough to accommodate the total plasma apoA-I, a few per cent of plasma apoA-I can be lipid-free in the aqueous phase in equilibrium. It should be noted that the K_m for the HDL assembly reaction is less than 1% of plasma apoA-I concentration, so that this protein in a free form can carry out the reaction at the V_{max}. Also, there are a few reactions that reportedly release helical apolipoproteins from the HDL surface in plasma such as cholesteryl ester transfer protein in the presence of free fatty acids [98–100]. Phospholipid transfer protein [101] by itself also releases apolipoprotein from HDL, and the transfer of cellular phospholipid and cholesterol to HDL was indeed enhanced by phospholipid transfer protein [102]. Apolipoproteins can be transferred from HDL to the cell surface simply because of the higher affinity of free apolipoproteins for cells than for the lipid surface [59].

Finally, it was shown that a monoclonal antibody specific for lipid-free apoA-I selectively inhibited an ABCA1-dependent part of the cell cholesterol release to HDL, and that ABCA1-dependent binding to cells only took place with HDL apoprotein but not HDL lipid [88°]. In that paper, kinetic analysis of the data indicated that apoA-I has an affinity for HDL as high as that for the cellular surface, and apoA-I could still be transferred from HDL to the cell surface (Fig. 1). It is thus not irrelevant to speculate that apolipoproteins dissociate from HDL and interact with cells in their lipid-free form to generate new HDL particles. However, this must be proved by more solid evidence.

The question also to be asked is how lipid-free or lipid-poor stages of apolipoproteins are required for the assembly of HDL. This question is related to the hypothesis that lipid-poor HDL is an efficient 'acceptor' for cellular cholesterol. Cellular cholesterol appears in the so-called 'prebeta-HDL' fractions when the cells are incubated with plasma [103,104]. This phenomenon, however, can also be interpreted as these HDL being newly generated by apolipoprotein–cell interactions. This idea is supported by the finding that proteolytic treatment of the cell surface abolishes this first appearance of cell cholesterol in prebeta-HDL [105].

Regulation of ABCA1 activity and HDL assembly

ABCA1 is a rate-limiting step for HDL assembly. Therefore, regulation of its function is important for attenuation of the plasma HDL level [51°]. Expression of the *ABCA1* gene is regulated primarily by the liver X receptor (LXR)/retinoid X receptor (RXR) system [106–108,109°]. The physiological ligand for LXR is oxisterol, and it is of physiological relevance as the level of this ligand may increase in proportion to the cellular cholesterol level. ABCA1 is indeed upregulated by loading cells with cholesterol and downregulated by depleting cholesterol [110]. Agonists of both receptors have also been shown to upregulate the *ABCA1* gene, increase the cellular ABCA1 level and enhance the release of cellular lipid [111]. It is thus rational to conceive that ABCA1 functions for cholesterol homeostasis by reducing cellular cholesterol when it is overloaded.

Upregulation of the *ABCA1* gene is also reported by other factors. Peroxisome proliferator-activated receptor alpha agonists, Wy14643 and fenofibric acid, increased its transcription, seemingly in an LXR-dependent manner [112,113]. cAMP strongly induces the *ABCA1* gene transcription to increase ABCA1 expression and apoA-I-mediated cell lipid release in certain types of cells, especially in macrophage cell line cells such as Law264, THP-1 and J774 cells [31,47,48,114], seemingly in an LXR/RXR-independent manner. Some calcium antagonists also induced *ABCA1* gene transcription independently of the LXR/RXR system, resulting in an increase in ABCA1 and apoA-I-mediated lipid release [115°]. Regulation of *ABCA1* gene transcription thus directly associates with the level of ABCA1 protein and the release of cellular lipid by apolipoprotein. The in-vivo administration of an LXR/RXR ligand indeed results in an increase in plasma HDL [116°].

On the other hand, ABCA1 is rapidly degraded by calpain-mediated proteolysis [117,118°]. Helical apolipoproteins protect ABCA1 from this calpain-mediated degradation, and thereby increase the ABCA1 level as its degradation is slowed down [117]. Phosphorylation of ABCA1 is induced by helical apolipoproteins that are capable of generating HDL, and this seems to be related to its protection from proteolysis [119°]. The same results have been demonstrated with various apolipoproteins and synthetic peptides that interact with ABCA1 and generate HDL with cellular lipids [120°].

As to the signal to trigger ABCA1 phosphorylation, the replenishment of sphingomyelin is suggested to be involved [119°,121]. When HDL is assembled with cellular phospholipid, sphingomyelin is removed together with glycerophospholipid (mostly phosphatidylcholine). To replenish sphingomyelin, phosphorylcholin is generated from phosphatidylcholine by phospholipase C and transferred to ceramide. Diacyl glycerol is released in these reactions and PKC is activated by this process (Fig. 2). This is a relatively slow reaction, and distinguished from the rapid production of diacyl glycerol,

activation of PKC and mobilization of intracellular cholesterol [81°,122°].

These findings are somewhat inconsistent with other observations that the PEST sequence in mouse ABCA1 is responsible for its phosphorylation, and apoA-I stimulation of the cells dephosphorylate this site in relation to its stabilization against calpain [123°]. The reason is unknown for the discrepancy between the two streams of the evidence.

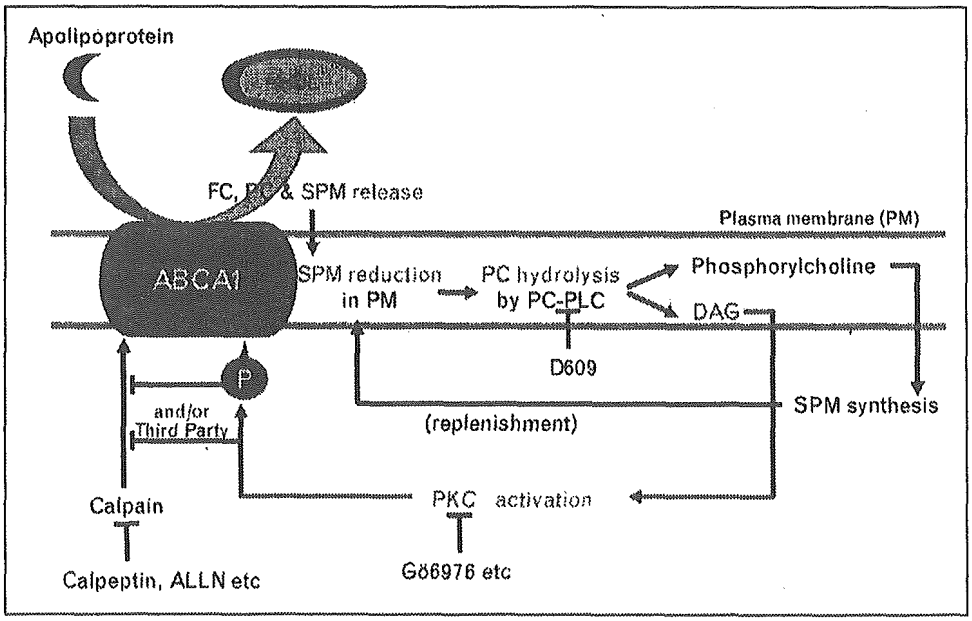
The lipid-lowering drug, probucol, has been known for its strong anti-lipid-oxidative nature that provided the background to interpret its clinical effect of reducing cutaneous xanthomas [124] and the prevention of atherosclerosis in animal experiments [125,126]. However, probucol also has the effect of lowering HDL [127], and this apparent conflict made this drug very controversial [128]. Interestingly, probucol was shown to inhibit the apolipoprotein–cell interaction and the generation of HDL with cellular lipids in a very similar manner to that of cells from the patients with Tangier disease. In probucol-treated cells, the lack of apolipoprotein binding has been demonstrated as a cause of the impairment of HDL assembly, both *in vitro* [34,85°] and *in vivo* [35]. The kinetics of HDL metabolism in the probucol-fed mice is also similar to that observed with Tangier patients [35].

Probucol does not alter the intracellular distribution of ABCA1 or the traffic of cholesterol. It inactivates ABCA1 in the plasma membrane, not only for its functions that mediate apolipoprotein binding to the cells and the assembly of HDL, but also for its susceptibility to calpain-mediated proteolytic degradation [56°]. Accordingly, the cellular ABCA1 level is increased by probucol although it is totally unfunctional [56°].

A different observation was reported that probucol prevents ABCA1 from reaching the plasma membrane in different cells in different conditions [129°]. The reason for the difference is unkown. Interestingly, cyclosporine A inactivates ABCA1 in a very similar manner to that of probucol; the inactivation of ABCA1 for its biological functions and susceptibility to calpain in the plasma membrane [130°].

The finding of the mutation in ABCA1 opened a new gate for studying cellular cholesterol homeostasis with respect to its releasing mechanism. This protein undoubtedly plays an essential role in the apolipoprotein-mediated assembly of HDL. It is, however, still unclear how ABCA1 functions to mediate the interaction of helical apolipoprotein with phospholipid in the cell membrane. In order to maintain cholesterol homeostasis, diffusion-mediated physicochemical cholesterol release functions as much as the apolipoprotein-mediated pathway, both at the cellular level and for the whole body. Therefore, Tangier patients may not develop general and massive cholesterol accumulation because the diffusion-mediated system is preserved [36]. This is same in LCAT-deficient patients who lack a driving force for the net cholesterol release by the diffusion-mediated system but not the apolipoprotein-mediated reaction [131]. The two systems thus back each other up to maintain cellular and body cholesterol homeostasis [132].

Figure 2. Stabilization of ABCA1 through the HDL assembly reaction by helical apolipoprotein



As described in the text, the removal of sphingomyelin (SPM) by the HDL assembly reaction causes its replenishment by activating phosphatidylcholine phospholipase C (PC-PLC), and phosphorylcholine is transferred to ceramide to regenerate sphingomyelin [121]. The other product of phospholipase C, diacylglyceride (DAG), may activate protein kinase C (PKC) α, which associates with the phosphorylation of ABCA1 and its stabilization against calpain-mediated degradation [119°]. D609 (a phospholipase C inhibitor), Gö6976, (a protein kinase C inhibitor), and ALLN and calpeptin (inhibitors of thiol proteases and calpain, respectively) all blocked ABCA1 phosphorylation and stabilization [119°]. FC, Free cholesterol. This figure is taken from Yamauchi *et al.* [119°].

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