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Reactivity of Astrocytes to Fibroblast Growth Factor-1 for Biogenesis of Apolipoprotein E-High Density Lipoprotein is Down-regulated by Long-time Secondary Culture

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We previously showed that astrocytes produce and release fibroblast growth fator-1 (FGF-1) upon 1-month primary and 1-week secondary culture (M/W cells) and stimulate themselves by an autocrine manner to produce apoE-high-density lipoproteins (HDL), closely associated with their generation of apoE-HDL in brain injury. Astrocytes prepared by 1-week primary and 1-month secondary culture (W/M cells), however, expressed FGF-1 as much as M/W cells but produce apoE-HDL much less. The W/M cells conditioned medium in fact contained FGF-1 activity to stimulate astrocytes prepared by 1-week primary and 1-week-secondary culture (W/W cells). FGF-1 did not stimulate W/M cells for apoE-HDL biogenesis while it stimulated W/W cells. Phosphorylation of Akt, ERK and MEK were induced by FGF-1 in W/W cells but not in W/M cells. Finally, fibroblast growth factor receptor-1 in the membrane decreased in W/M cells in comparison to W/W cells. Interestingly, the reactivity of astrocytes to FGF-1 was recovered when W/M cells were transferred to the tertiary culture of 1 week. We concluded that astrocytes decrease their reactivity to FGF-1 for apoE-HDL biogenesis in certain conditions. The findings indicate astrocyte FGF-1 enhances biogenesis of apoE-HDL also by a paracrine mechanism.

Key words: astrocytes, apolipoprotein E, high-density lipoprotein, cholesterol, fibroblast growth factor-1.

Abbreviations: apoE, apolipoprotein E; HDL, high-density lipoprotein; FGF-1, fibroblast growth factor 1; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; TLC, thin layer chromatography; BSA, bovine serum albumin; FGFR-1, fibroblast growth factor receptor 1.

Astrocytes play many important roles in the brain for maintaining its function. One of those functions is to produce and secrete apolipoprotein E (apoE) and generate high-density lipoprotein (HDL) with the cellular lipid (*1*, *2*). ApoE production increases in the brain when it is injured, acutely and perhaps chronically as well (*3–12*). We found that healing of the experimental cryo-injury of the brain was substantially retarded in the apoE-deficient mice (*13*). Production of fibroblast growth factor-1 (FGF-1) was observed in astrocytes in the peri-injury regions 2 days after the injury both in the apoE-deficient and wild-type mice brain. ApoE production increased a few days later in the same regions of the wild-type mouse brain (*13*). *In vitro*, astrocytes produce and release FGF-1 into the medium when prepared as 1-month primary and 1-week secondary culture (M/W cells) (*14*). These cells themselves produce a large amount of apoE-HDL, and anti-FGF-1 antibody prevented this apoE-HDL production, so that increase of apoE-production in the astrocytes thus prepared seemed due to an autocrine reaction of FGF-1 (*15*). The conditioned medium of M/W cells and FGF-1 stimulated the astrocytes prepared in a conventional method as 1-week primary and 1-week secondary culture (W/W cells). Therefore, we hypothesize that FGF-1 is a trigger for astrocytes to stimulate generation of apoE-HDL for recovery of the brain injury by an autocrime mechanism (*15*).

We further investigated the mechanism for FGF-1 to stimulate apoE-HDL production with respect to intracellular signalling. We identified that FGF-1 initiates apoE gene transcription, biosynthesis of cholesterol and other lipid, and secretion of apoE-HDL, independently (*16*). The PI3K/Akt pathway up-regulates apoE-HDL secretion, the MEK/ERK pathway stimulates cholesterol biosynthesis and an unknown pathway enhances apoE transcription.

FGF-1 is produced and released by M/W cells that are kept for 1 month with other neural cells including neurons. Neurons are removed in the secondary culture by the trypsin treatment of the cells in primary culture, so that the astrocytes in the secondary are free from the influence of neurons. Experiment is thus designed to examine whether production of FGF-1 is induced by the influence of other neural cells such as neurons or by the long-time incubation itself. Astrocytes were therefore prepared after 1-week primary culture either by the conventional method of transferring the cells to the secondary culture of 1 week (W/W cells) or by its extension to 1 month (W/M cells).

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FGF-1 was produced and released by W/M cells as well showing that this is independent of co-culture with other neural cells and perhaps due to the long-time culture of astrocytes. In contrast to the findings with M/W cells, however, the reactivity of astrocytes to FGF-1 seemed to be altered in W/M cells. Although apoE-HDL secretion was found somewhat high in comparison to the conventionally prepared W/W cells, W/M cells showed lower apoE-HDL production than M/W cells. We investigated the underlying mechanism for this phenomenon and found that the cells in this condition produce and release FGF-1 so much as M/W cells but they are poorly reactive to FGF-1. Thus, astrocytes produce and release FGF-1 in certain conditions such as under stress but they may remain reactive to FGF-1 or reduce the reactivity to FGF-1 being dependent on their microenvironment. This finding suggests that FGF-1 acts on astrocytes to stimulate apoE-HDL secretion either (or both) by an autocrine or (and) paracrine reaction(s).

MATERIALS AND METHODS

Preparation of Fetal Rat Astrocytes—Astrocytes were prepared from the 17-day-old fetal brain of Wistar rat according to the method previously described (*14–17*). 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 r.p.m. for 3 min was cultured in F-10 medium containing 10% fetal calf serum (FCS) (10% FCS/F-10) at 37°C for 1 week as a primary culture. After treatment with 0.1% trypsin/DPBS/G containing 1 mM ethylenediaminetetra-acetic acid, the cells were cultured in 10% FCS/F-10 for 1 week (W/W cells) or for 4 weeks (W/M cells) as secondary culture (*14*). W/M cells were further transferred to the tertiary culture of 1 week (W/M/W cells). Population of W/M and W/M/W cells were predominant in astrocytes being consistent with that of W/W and M/W cells according to the criteria we previously reported (*14*).

Synthesis of Cellular Lipids—To measure *de novo* synthesis of lipid, astrocytes were incubated with [^{3}H]-acetate (20 μCi/ml) as indicated in each figure legend and washed three times with DPBS. Lipid was extracted from the cells with hexane/isopropanol (3:2, v/v), and radioactivity was counted in cholesterol, sphingomyelin and phosphatidylcholine after separation by thin layer chromatography (TLC) (*15*).

Cellular Lipid Release into the Medium—For standard measurement of cholesterol and other lipid released into medium (*15*), astrocytes were labelled by incubating with [^{3}H]-acetate (20–40 μCi/ml) in 0.1% bovine serum albumin (BSA)/F-10 as indicated in each figure legend. The cells were washed three times with DPBS and incubated in a fresh 0.02% BSA/F-10 for 5 h. The medium was collected and centrifuged at 15,000 r.p.m. for 30 min to remove the cell debris. Lipid was extracted from the medium with chloroform/methanol (2:1, v/v) and analysed by TLC with diethyl ether/benzene/ethanol/acetic acid (200:250:10:1) and chloroform/methanol/acetic acid/water (25:15:4:1) to determine radioactivity of cholesterol, sphingomyelin and phosphatidylcholine.

Analysis of Protein by Western Blotting—The method was described previously (*15*, *16*). The conditioned medium of astrocytes was treated with 10% trichloroacetic acid and centrifugation at 15,000 r.p.m. for 20 min after the cell debris was removed by centrifugation at 15,000 r.p.m. for 30 min. The cells ware treated with 0.02 M Tris–HCl, pH7.4 containing protease inhibitor cocktail for 15 min with 25 times strong agitations for 10 s every 5 min. After removing nuclei by centrifuging at 3,000 r.p.m. for 20 min, the supernatant was centrifuged at 90,000 r.p.m. for 30 min to obtain cytosol and membrane fractions as supernatant and precipitant. Each sample was analysed by 10% SDS–PAGE and immunostained with rabbit anti-rat apoE (a generous gift from Dr Jean Vance, The University of Alberta), a rabbit anti-phosphorylated Akt (Thr-308) antibody (Cell Signaling Technology), a mouse anti-protein kinase B (PKB) α/Akt antibody (BD Transduction Laboratories), a mouse anti-phosphorylated p44/p42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology), a rabbit anti-p44/42 MAP kinase antibody (Cell Signaling Technology), a rabbit anti-phosphorylated MEK 1/2 (Ser217/221) antibody (Cell Signaling Technology), a rabbit anti-MEK 1/2 antibody (Cell Signaling Technology), a goat anti-FGF-1 antibody (Santa Cluz Biotechnology) and a rabbit anti-Flg [FGF-receptor 1 (FGFR-1)] antibody (Santa Cluz Biotechnology).

Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR)—Total cellular RNA was extracted from rat astrocytes by RNAqueous^{TM} (Ambion), and aliquot of 0.5 μg was reverse-transcribed to cDNA using high-capacity cDNA Reverse Transcription Kits (Applied Biosystems). The cDNA was subjected to PCR by using the DNA probes for rat FGF-1-mRNA, apoE-mRNA, FGFR-1-mRNA [Gene Amp (Applied Biosystems)]. After an electrophoresis of the products, an agarose gel was stained with EtBr solution (Nippon Gene Co. Ltd., Tokyo). The band was detected by an ultraviolet transilluminator (UVP NLM-20 E) at 302 nm. The primer pairs were 5′-AAGCCCGTCGGTGTCCATGG-3′ (sense) and 5′-GATGGCACAGTGGATGGGAC-3′ (anti-sense) for FGF-1, 5′-CTGTTGGTCCCATTGCTGAC-3′ (sense) and 5′-TGTGTGACTTGGGAGCTCTG-3′ (anti-sense) for apoE and 5′-TTGTGGCCTTGACCTCCAAC-3′ (sense) and 5′-TCCCCTGAAGAGCAGGTAGA-3′ (anti-sense) for FGFR-1.

RESULTS

Expression of FGF-1 and apoE in Long-Time Cultured Astrocytes—The message of FGF-1 increased in rat astrocytes in both W/M and M/W cells (prepared by long-time culture, for secondary and primary, respectively), in comparison to the astrocytes by conventional preparation (W/W cells, 1 week for each primary and secondary culture) (Fig. 1A). The message and secretion of apoE also increased in the both cells but the increase was more prominent in M/W cells (Fig. 1B and C). The data with M/W cells are consistent with our previous results that M/W cells produce and release FGF-1 and stimulate

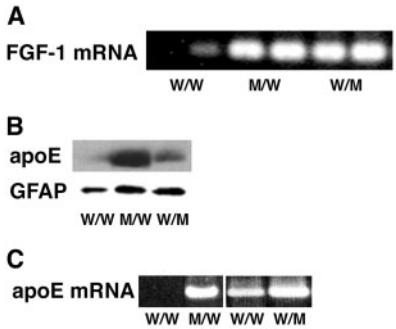

Fig. 1. **Expression of FGF-1 and apoE in astrocytes.** (A) Expression of FGF-1 mRNA in astrocytes prepared by 1-week primary and 1-week secondary culture (W/W), 1-month primary and 1-week secondary culture (M/W) and 1-week primary and 1-month secondary culture (W/M). Total cellular RNA was extracted and subjected for RT–PCR as described in the text. (B) ApoE secreted into the condition media from the W/W, M/W and W/M cells. After preparation, the cells were incubated for 24 h in a fresh 0.02% BSA/F10 medium. Each medium was analysed by western blotting as described in the text. Glial fibrillary acidic protein (GFAP) was also analysed for the cells to indicate equal activation or differentiation of the astrocytes in M/W and W/M cells. (C) Expression of apoE mRNA in astrocytes comparing W/W and M/W cells by RT–PCR. Total cellular RNA, 5 μg, was subjected to reverse transcription, and 0.5 μg of the produced cDNA was amplified by using apoE promer pairs as described in the text, by 26 cycles for W/W and M/W cells, and by 30 cycles for W/W and W/M cells.

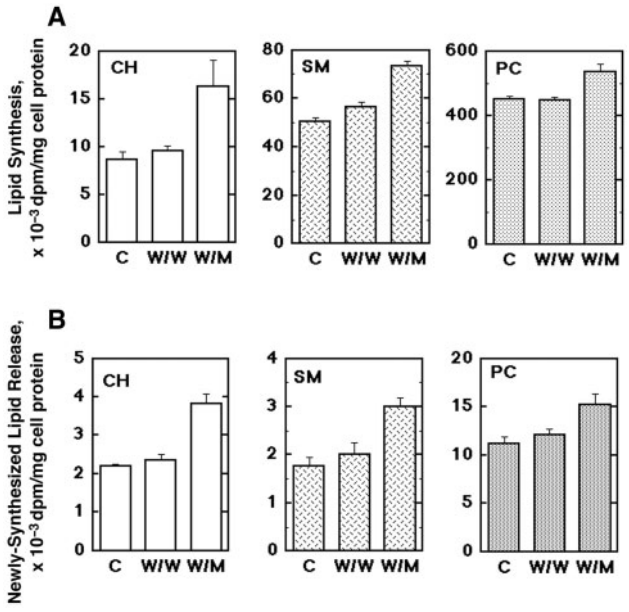

Fig. 2. **Effect of the conditioned medium of W/M cells on rat W/W astrocytes.** (A) Lipid biosynthesis was examined in W/W cells after stimulation by the conditioned medium of W/W and W/M cells. W/W cells were incubated in 500 μl of fresh 0.1% BSA/F-10 plus 500 μl of 0.1% BSA/F-10 or plus 500 μl of the conditioned medium of W/W or W/M cells for 24 h. After washing with DPBS, the cells were incubated with 20 μCi/ml of [^{3}H]-acetate in 1 ml of 0.02% BSA/F-10 for 3 h. The astrocytes were washed three times with DPBS and lipid was extracted from the cells and analysed by TLC to determine radioactivity in cholesterol (CH), sphingomyelin (SM) and phosphatidylcholine (PC) as described in the text. (B) Lipid release from W/W cells was measured. The cells incubated in 500 μl of fresh 0.1% BSA/F-10 plus 500 μl of 0.1% BSA/F-10 or plus 500 μl of the conditioned medium of W/W or W/M cells for 6 h, followed by incubation with 20 μCi/ml of [^{3}H]-acetate for 18 h without washing. After washing with DPBS three times, the cells were further incubated in 0.02% BSA/F-10 for 5 h. Lipid was extracted from the medium and analysed by TLC to determine radioactivity in cholesterol (CH), sphingomyelin (SM) and phosphatidylcholine (PC) as described in the text.

the cells for apoE-HDL biogenesis by an autocrine mechanism (*14–16*). ApoE secreted into the medium was all recovered with HDL fraction (d = 1.063–1.21) of the conditioned medium (data not shown), also being consistent with our previous results (*14–16*).

The Conditioned Medium of W/M Cells Stimulates W/W Cells—In order to examine whether FGF-1 is released into the medium in an active form in W/M cells, the conditioned medium was given to W/W cells and biosynthesis and release of cellular lipid was measured. Both biosynthesis and release of lipids were increased in W/W cells by the medium of W/M cells but not by the medium of W/W cells (Fig. 2). The results indicated that the W/M cell-conditioned medium contained FGF-1-like activity, similarly to M/W cells that were examined in our previous reports (*14*, *15*).

The Effects of FGF-1 on W/M Cells—The direct effect of FGF-1 was investigated on W/W cells and W/M cells. Figure 3A shows the effect of FGF-1 on cholesterol biosynthesis. FGF-1 induced increase of cholesterol biosynthesis in a dose-dependent manner in W/W cells and M/W cells, while there was no effect on W/M cells. Figure 3B demonstrates the dose-dependent effect of FGF-1 on W/W and M/W cells for increase of the release of cholesterol and phospholipids, and its no apparent effect on W/M cells for the same parameters. This was reflected in apoE secretion into the medium by astrocytes as it is increased by FGF-1 with W/W cells but not with W/M cells.

Signals Induced by FGF-1 in W/M Cells—FGF-1 was shown to induce signals of the PI3K/Akt pathway for apoE transport and secretion and the MEK/ERK pathway for lipid biosynthesis *via* the FGF receptor(s) (*16*). Therefore, protein phosphorylation was examined for Akt, ERK and MEK proteins upon stimulation of W/W and W/M asctrocytes by FGF-1. Figure 4A shows that each of these signal proteins was phosphorylated by FGF-1 in W/W cells whether present in the membrane or cytosol fractions, while those in W/M cells were not phosphorylated by FGF-1. Finally, the FGFR-1 was analysed for its message and protein. Expression of FGFR-1 mRNA was not different between W/W and W/M cells, and this was reflected by no apparent difference in cytosolic FGFR-1 (Fig. 4B). However, the FGFR-1 protein in membrane significantly decreased in W/M cells in comparison to W/W cells, demonstrated also in Fig. 4B. Molecular weight of the membrane FGFR-1 was similar to that of the larger molecule of the duplex bands of cytosolic FGFR-1, presumably indicating maturation of the receptor protein.

Recovery of the Reactivity of Astrocytes to FGF-1—Finally, the reactivity of astrocytes to FGF-1 was recovered by transferring the W/M cells to the tertiary

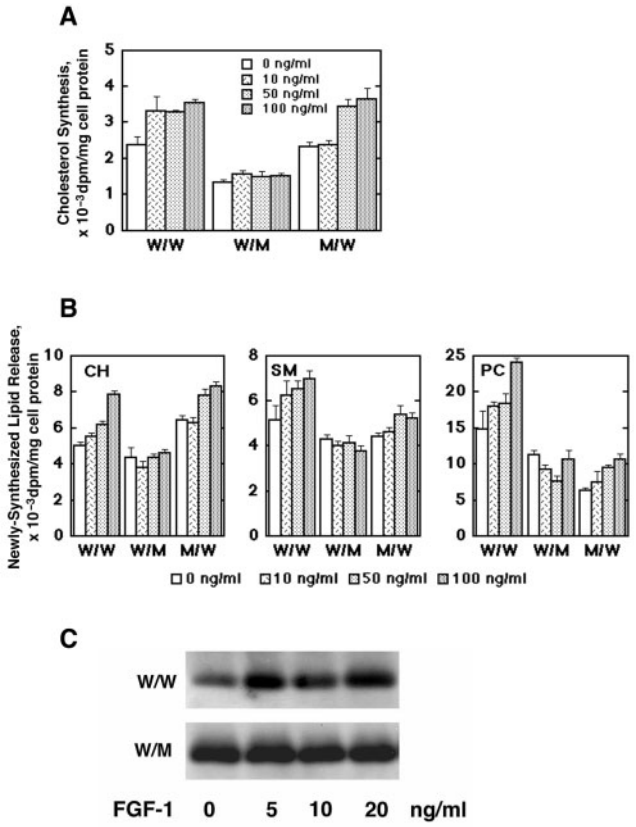

Fig. 3. **Effect of FGF-1 on astrocytes.** (A) W/W, W/M and M/W cells were incubated with FGF-1 (0, 10, 50 and 100 ng/ml) in 0.1% BSA/F-10 for 24 h. The cells were washed and labelled with 20 μCi/ml of [^{3}H]-acetate in a fresh 0.02% BSA/F-10 for 2 h. After washing, lipid was extracted from the cells and radioactivity in cholesterol was analysed by TLC as described in the text. (B) Cellular lipid release by the W/W, W/M and M/W astrocytes. The cells were incubated with the indicated amount of FGF-1 as above and [^{3}H]-acetate as described for 3 h. After washing with DPBS three times, the cells were further incubated in 0.02% BSA/F-10 for 5 h. Lipid was extracted from the conditioned medium after removal of cell debris to analyse by TLC for cholesterol (CH), sphingomyelin (SM) and phosphatidylcholine (PC). (C) Stimulation of apoE secretion by FGF-1. W/W cells and W/M cells were incubated with various amount of FGF-1 as described above for 24 h. After washing and incubation of the cells with 0.02% BSA/F-10 for 24 h, the conditioned medium was analysed for apoE secretion by western blotting.

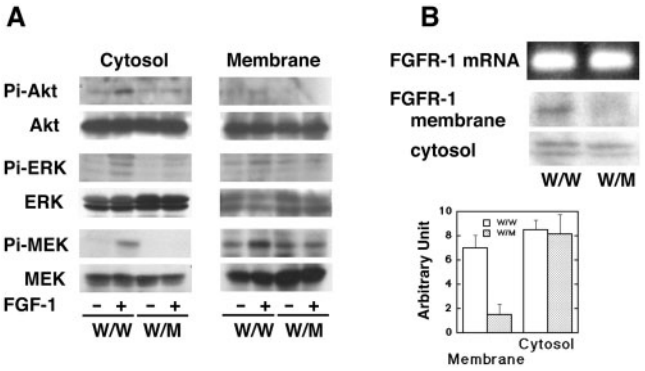

Fig. 4. **Analysis of the signalling pathways in rat astrocytes.** (A) Phosphorylation of signal-related proteins in W/W and W/M cells. The cells were washed and incubated in 0.1% BSA/F-10 for 16 h. After washing, the cells were treated with FGF-1 (50 ng/ml) for 5 min, and the cytosol and membrane fractions were prepared and analysed by 10% SDS–PAGE and western blotting as described in the text by using antibodies against Akt, ERK and MEK proteins and phosphorylated form (Pi) of each of these proteins. (B) Analysis of FGFR-1. Expression of FGFR-1 mRNA in W/W and W/M cells was analysed by RT–PCR for 30 cycles. FGFR-1 protein was also analysed in the membrane and cytosol fractions of W/W and W/M cells by western blotting using specific antibody. The graph indicates the results of scanning of the western blotting gels as mean ± SE for three independent experiments. For cytosolic protein, both bands were analysed together.

culture for 1 week (W/M/W cells). Figure 5A shows induction of phosphorylation of Akt, MEK and ERK by FGF-1 in W/W and W/M/W cells but not in W/M cells. Figure 5B and C show the same change in reactivity to FGF-1 of the lipid biosynthesis and the apoE release.

DISCUSSION

Cholesterol homoeostasis in animals involves intra- and extra-cellular regulation of its metabolism (*18*) and extracellular transport of cholesterol in vertebrates is carried by plasma lipoprotein system. However, the blood–brain barrier prevents central nervous system from accessing to this system, so that it operates a unique and independent specific lipoprotein system for extra-cellular

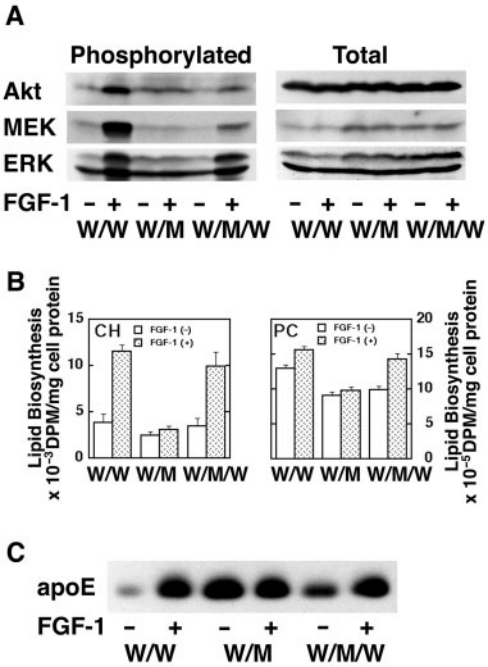

Fig. 5. **Recovery of reactivity of astrocytes to FGF-1 by short tertiary culture.** W/M cells were transferred to the tertiary culture of 1 week and analysed for the reactivity to FGF-1. The experiments were conducted in the same manner as those in Figs 3 and 4. (A) Phosphorylation of signalling proteins induced by FGF-1. (B) Increase of biosynthesis of cholesterol and phosphatidylcholine. (C) Secretion of apoE into the medium.

cholesterol transport. HDL is an exclusively found lipoprotein in cerebrospinal fluid that contains mainly apoE and apoA-I (*19*). While apoA-I is not synthesized by neural cells and its origin is uncertain (*20*, *21*), apoE is known to be synthesized at least in astrocytes and microglias to generate apoE-HDL (*1*, *2*). Many reports suggest that apoE-HDL delivers cholesterol to neurons and this lipoprotein seems to increase in the lesions of injury of brain and nerves (*3–12*).

We have reported that FGF-1 is a key factor for astrocytes to produce apoE-HDL in response to damage of nerve system, perhaps for both acute and chronic, major and minor injuries. In mouse brain, production of FGF-1 by the astrocytes in the peri-injury regions is observed prior to the appearance of apoE in the same cells, and the lack of apoE production retarded the recovery of the injury (*13*). Being consistent with this finding, astrocytes prepared by long primary culture and 1-week secondary culture (M/W cells) produce and release FGF-1, which stimulates these astrocytes themselves in an autocrine manner for production of apoE-HDL (*14*, *15*). FGF-1 also stimulates the astrocytes prepared by a conventional method of 1-week primary and 1-week secondary cultures (*15*).

When the secondary culture was prolonged for 1 month (W/M cells), the astrocytes produced apoE-HDL more than conventionally prepared W/W cells, but much less than M/W cells. However, the conditioned medium contained the activity to stimulate W/W cells for production of apoE-HDL and related reactions such as increase of lipid biosynthesis. W/M cells showed the loss of their reactivity to FGF-1 including signals activated *via* the FGFR-1 (*16*) and this seemed due to the decrease of FGFR-1 in the membrane fraction.

An exact reason and mechanism for this change are not known. Long-time primary culture does not induce such change in astrocytes, and long secondary culture resulted in loss of the reactivity of the cells to FGF-1. The primary culture cell population contains many types of cells other than astrocytes such as neurons, oligodendroglias and microglias at least at the beginning of the culture, and neurons gradually disappear in the 3-week primary culture before the cells are transferred to the secondary culture. The secondary culture starts after loss or decrease of the other types of cells almost exclusively with astrocytes. Thus, the presence of other types of neural cells for the long-time culture was one of the potential reasons to protect astrocytes from loss of the cellular reactivity to FGF-1. However, the finding that transferring the cells to the tertiary culture recovered the reactivity to FGF-1 excluded such possibility. This result rather indicated that the difference in microenvironment in relation to stage of cellular proliferation might cause some specific change in astrocytes with respect to expression of FGFR-1.

Although an exact mechanism for astrocytes to lose their FGF-1-reactivity is unknown, we cannot exclude the possibility that such change may be induced also *in vivo* when the astrocytes are exposed to a kind of specific stress. If this takes place, FGF-1 induces apoE-HDL biogenesis and excretion not only by autocrine reactions, but also by paracrine reactions with healthy astrocytes.

It is thus important to carry out further investigation on the mechanism by which the brain injury triggers production and release of FGF-1 by astrocytes in the peri-injury regions, and how FGF-1 stimulates the astrocytes in the regions. This process is a key for the recovery of the grain from the injury, so that the detailed information for the mechanism of these reactions will provide the base of development of technology to expedite this healing process.

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