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Stable Expression and Secretion of Apolipoproteins E3 and E4 in Mouse Neuroblastoma Cells Produces Differential Effects on Neurite Outgrowth*

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Previously, we demonstrated in cultured dorsal root ganglion neurons that, in the presence of β-migrating very low density lipoproteins (β-VLDL), apolipoprotein (apo) E4, but not apoE3, suppresses neurite outgrowth. In the current studies, murine neuroblastoma cells (Neuro-2a) were stably transfected with human apoE3 or apoE4 cDNA, and the effect on neurite outgrowth was examined. The stably transfected cells secreted nanogram quantities of apoE (44–89 ng/mg of cell protein in 48 h). In the absence of lipoproteins, neurite outgrowth was similar in the apoE3- and apoE4-secreting cells. The apoE4-secreting cells, when incubated with β-VLDL, VLDL, cerebrospinal fluid lipoproteins (d < 1.21 g/ml), or with triglyceride/phospholipid (2.7:1 (w/w)) emulsions, showed a reduction in the number of neurites/cell, a decrease in neurite branching, and an inhibition of neurite extension, whereas in the apoE3-secreting cells in the presence of a lipid source, neurite extension was increased. Uptake of β-VLDL occurred to a similar extent in both the apoE3- and apoE4-secreting cells. With low density lipoproteins or with dimyristoylphosphatidylcholine emulsions, either alone or complexed with cholesterol, no differential effect on neurite outgrowth was observed. A slight differential effect was observed with apoE-containing high density lipoproteins. The differential effect of apoE3 and apoE4 in the presence of β-VLDL was blocked by incubation of the cells with heparinase and chlorate, with lactoferrin, or with receptor-associated protein, all of which prevent the uptake of lipoproteins by the low density lipoprotein receptor-related protein (LRP). The data suggest that the secreted and/or cell surface-bound apoE interact with the lipoproteins and facilitate their internalization via the heparan sulfate proteoglycan-LRP pathway. The mechanism by which apoE3 and apoE4 exert differential effects on neurite outgrowth remains speculative. However, the data suggest that apoE4, which has been shown to be associated with late onset familial and sporadic Alzheimer's disease, may inhibit neuronal remodeling and contribute to the progression of the disease.

Apolipoprotein (apo)[1] E, a 34-kDa protein coded for by a gene on chromosome 19, plays a prominent role in the transport and metabolism of plasma cholesterol and triglyceride through its ability to interact with the low density lipoprotein (LDL) receptor and the LDL receptor-related protein (LRP) (1, 2). Apolipoprotein E also may play an important role in the redistribution of lipids within the central nervous system (3). More than three-fourths of the apoE in the plasma is synthesized by the liver (4), whereas apoE in cerebrospinal fluid (CSF) is produced primarily by astrocytes within the brain (5–7). Three common isoforms of apoE, distinguished by differing mobility on isoelectric focusing gels, differ in amino acids at positions 112 and 158 (1). The most common isoform, apoE3, has cysteine at position 112 and arginine at 158, whereas apoE2 has cysteine at both positions, and apoE4 has arginine at both. The isoforms are encoded by three alleles at the same gene locus. Apolipoprotein E4 has recently been shown to be associated with Alzheimer's disease (AD) (8–18).

Late onset familial AD is linked to the proximal long arm of chromosome 19 (10), which contains the apoE locus (1). Saunders and co-workers at Duke University (14) were the first to demonstrate that the apoE4 allele is associated with both late onset familial and sporadic forms of AD, which together account for 90% of cases (14). The association of apoE4 with AD has been confirmed by several groups (9, 12, 19, 20). Whereas the allele frequency for apoE4 is ~0.143 in the general population (21), it is 0.36–0.57 in various populations of patients with late onset AD (9, 12, 14, 19, 20). In families with late onset AD, the risk for AD increases from 20 to 90%, and the mean age of onset decreases from 84 to 68 years with an increasing number of apoE4 alleles. Homozygosity for apoE4 is virtually certain to result in AD by age 80 (11). The defining neuropathological features of AD are the presence of extracellular deposits of β-amyloid in the cerebral cortex and vascular amyloid around blood vessels as well as intracellular deposits of hyperphosphorylated τ in the form of neurofibrillary tangles in neurons of the cortex, hippocampus, and amygdala (22). Apolipoprotein E is associated with both the senile plaques and neurofibrillary tangles present in the brains of AD patients (10).

The mechanism by which apoE4 is related to AD is unknown

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[1] The abbreviations used are: apo, apolipoprotein; AD, Alzheimer's disease; β-VLDL, β-migrating very low density lipoproteins; CSF, cerebrospinal fluid; LDL, low density lipoproteins; LRP, LDL receptor-related protein; DRG, dorsal root ganglion; VLDL, very low density lipoproteins; HSPG, heparin sulfate proteoglycan; DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F-12; FBS, fetal bovine serum; DMPC, dimyristoylphosphatidylcholine; PBS, phosphate-buffered saline; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; HDL, high density lipoproteins; RAP, receptor-associated protein.

and could involve a direct effect of apoE on neurons. We therefore examined the effect of apoE3 and apoE4 on neurons *in vitro*. When fetal rabbit dorsal root ganglion (DRG) neurons were incubated with β-migrating very low density lipoproteins (β-VLDL), which are rich in apoE and cholesterol, neurite outgrowth and branching were increased (23). Addition of rabbit apoE (structurally similar to human apoE3) together with the β-VLDL reduced neurite branching but very significantly increased neurite extension (23). In an extension of these studies, we found that human apoE3 and apoE4, when added together with β-VLDL, have differential effects on the outgrowth of neurites from DRG neurons in culture (24). Compared with cells incubated with β-VLDL alone, cells treated with human apoE3 plus β-VLDL showed decreased branching and increased neurite extension, whereas cells treated with apoE4 plus β-VLDL had decreased neurite outgrowth (24). Similar observations showing an isoform-specific effect of exogenous apoE3 and apoE4 in the presence of a source of lipid have been described using a murine neuroblastoma cell line (Neuro-2a) in culture (25).

In the present studies, we stably transfected the Neuro-2a cells with human apoE3 or apoE4 cDNA and then examined the effect of endogenously produced apoE on neurite outgrowth. When incubated with β-VLDL, VLDL, or CSF lipoproteins, these cells, expressing small amounts of endogenous apoE3 and apoE4, displayed differences in neurite outgrowth. The enhanced neurite outgrowth in the apoE3-secreting cells and inhibition of neurite outgrowth in the apoE4-secreting cells were abolished by reagents known to block binding and internalization of apoE-enriched lipoproteins by the heparan sulfate proteoglycan (HSPG)-LRP pathway (26–28).

EXPERIMENTAL PROCEDURES

Materials—Dimyristoylphosphatidylcholine (DMPC), Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (F12) (1:1), media supplements (progesterone, putrescine, selenite, and transferrin), sodium chlorate, heparinase, lactoferrin, triolein, and egg yolk phosphatidylcholine (type XI-E) were purchased from Sigma; fetal bovine serum (FBS) and insulin were from Life Technologies, Inc. Suramin was from Miles Inc. (FBA Pharmaceuticals, West Haven, CT), and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) was from Molecular Probes Inc. (Eugene, OR). The murine neuroblastoma cell line (Neuro-2a) was purchased from American Type Culture Collection (Rockville, MD). Bovine CSF was obtained from Pel-Freez, Inc. (Fayetteville, AR).

Preparation of Lipoproteins and Liposomes—Rabbit β-VLDL ($d <$ 1.006 g/ml) were isolated from the plasma of New Zealand White rabbits fed a high fat, high cholesterol diet for 4 days (29). Rabbit VLDL ($d <$ 1.006 g/ml) were isolated by ultracentrifugation from fasting plasma obtained from rabbits fed a normal rabbit chow. The VLDL were washed once by ultracentrifugation at d = 1.006 g/ml. Bovine CSF lipoproteins ($d <$ 1.21 g/ml) were isolated by ultracentrifugation as described previously (3). They were washed once by recentrifugation through a solution of d = 1.21 g/ml. Canine apoE HDL_c (d = 1.006–1.02 g/ml) were isolated by ultracentrifugation and Pevikon electrophoresis from the plasma of foxhounds fed a semisynthetic diet containing hydrogenated coconut oil and cholesterol (30). The β-VLDL were iodinated by the method of Bilheimer *et al.* (31), and free iodine was removed by PD10 column chromatography. The DMPC vesicles were prepared essentially as described previously (32). The DMPC alone (90 mg) or with the addition of cholesterol (10 mg) was dissolved in benzene and dried by lyophilization. The lyophilized material was then resuspended in 3 ml of 0.15 M NaCl, 10 mM Tris-Cl, and 1 mM EDTA (pH 7.6) and sonicated for 30 min at 37 °C using a sonifier cell disrupter (Branson 450, Danbury, CT) equipped with a microtip and full setting at 7 (50 watts) (32). The material was centrifuged for 10 min at 2,000 rpm (37 °C), and the supernatant was used for additions to cells. The lipid emulsion A was prepared as described previously (33, 34). Briefly, the lipids were mixed together in the ratio, 100 mg of triolein and 25 mg of egg yolk phosphatidylcholine, and then dried under a stream of nitrogen. The pellet was then resuspended in 5 ml of 10 mM Tris-Cl, 0.1 M KCl, and 1 mM EDTA (pH 8.0) buffer and sonicated as described previously (34). The material was then centrifuged for 10 min at 2,000 rpm. The composition of the final emulsion was 2.7:1 for triolein/phosphatidylcholine (w/w). The size and morphology of the emulsion particles were determined by negative staining electron microscopy.

Preparation of Expression Vectors—The expression vectors were assembled in the pBSSK plasmid (Stratagene, La Jolla, CA). The constructs contained the rat neuron-specific enolase promoter (kindly provided by Dr. J. G. Sutcliffe, Scripps Clinic and Research Foundation, La Jolla, CA), which has been previously used to direct neuron-specific expression of the human amyloid precursor protein and β-galactosidase in transgenic mice (35, 36). In addition, the construct contained the first exon (noncoding), the first intron, and the first six bases of the second exon (prior to the initiation methionine) of the human apoE gene, followed by the apoE cDNA. The apoE4 construct was identical except that it also contained the third intron. The noncoding region of the fourth exon was downstream from the cDNA, followed by 112 base pairs of the 3′-flanking sequence of the human apoE gene that contains the polyadenylation signal. The apoE constructs for insertion in these expression vectors were kindly provided by Drs. S. Lauer and J. Taylor of the J. David Gladstone Institutes. The orientation of the cDNAs was confirmed by sequencing, using an Applied Biosystems automated sequencer. The final constructs were referred to as neuron-specific enolase-E3 (for E3 cDNA) and neuron-specific enolase-E4 (for E4 cDNA). Plasmid DNA was purified by two rounds of cesium chloride gradient ultracentrifugation (37). To test the constructs, Chinese hamster ovary cells and human embryonic kidney 293 cells were transiently transfected (lipofectin-mediated), and the concentration of apoE in the medium was measured as described below. Similar levels of expression of apoE3 and apoE4 were achieved (data not shown).

Production of Stably Transfected Neuro-2a Cell Lines—Cells at 20–30% confluence were cotransfected with pSV2 *neo* and either neuron-specific enolase-E3 or neuron-specific enolase-E4 using a calcium phosphate precipitation protocol (38). Control cells were transfected with pSV2 *neo* alone following the same protocol (38). Stably transfected cells were selected by growth in DMEM/F12 media containing 10% FBS and 400 μg/ml G418 (Geneticin, Life Technologies, Inc.). Individual G418-resistant colonies were selected and expanded. Secretion of human apoE3 or apoE4 by the transfected cells was verified by Western blotting of the conditioned media as described below.

Apolipoprotein E Quantitation—Intracellular, cell-surface-bound, and secreted apoE were quantitated in cells maintained for 96 h in N2 medium, a serum- and lipid-free medium (DMEM/F12 containing growth supplements (39) with or without added β-VLDL (40 μg of cholesterol/ml)). The medium was changed once at 48 h. The secreted apoE reported is that present in the medium following the second 48-h incubation. The media were collected and, after the addition of protease inhibitors, centrifuged to eliminate suspended cells. The cell monolayers were washed with PBS and incubated for 1 h at 4 °C with 2 ml of DMEM/F12 containing 25 mM Hepes and 10 mM suramin, a polyanion that is able to release apoE bound to the cell surface (27). The apoE was precipitated from the medium and the suramin extract by addition of 50 μg/ml of fumed silica (Sigma) and centrifugation at 13,000 × g for 10 min.

Each pellet was washed 3 times with sterile water and dissolved in gel-loading buffer. Cellular apoE was extracted from the cells, following suramin removal of surface-bound apoE, using STEN buffer (50 mM Tris-Cl, pH 7.6, containing 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 20 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin). Samples were electrophoresed on 5–20% polyacrylamide gradient gels containing sodium dodecyl sulfate, as described previously (40). The proteins were transferred to nitrocellulose paper by blotting and treated with an anti-human apoE polyclonal antiserum (1:1,000 dilution) raised in rabbit (generously provided by Dr. K. H. Weisgraber, Gladstone Institutes). The nitrocellulose immunoblot then was incubated with donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5,000 dilution) (Amersham Corp.). After washing to remove unbound antibody, the immunocomplex was detected using an ECL kit (Amersham Corp.), according to the manufacturer's instructions. Quantitation of the level of apoE bound, internalized, and secreted by the cells was accomplished by densitometric scanning (Ambis Scanner, San Diego, CA) and based on a standard curve of purified human plasma apoE3 and apoE4.

Neurite Outgrowth—Cells were grown in DMEM/F12 containing 10% FBS and G418 (400 μg/ml). On the day the experiment was initiated, the cells were subcultured into 35-mm plates in DMEM/F12 with 10% FBS. The cells were allowed to adhere to the plastic plates for 2 h at 37 °C, and then the culture medium was changed to N2 medium with or

without increasing concentrations of lipoproteins. After 48 h at 37 °C, the media were replaced with the same medium (with or without lipoproteins), and the incubation was continued for an additional 48 h. (The CSF lipoproteins were dialyzed against N2 medium prior to addition to the cells.) The cells then were washed with DMEM/F12 containing 0.2% bovine serum albumin, nonspecifically stained for 1 h at 37 °C with DiI added in Me_2SO as described previously (24), and fixed with 2.5% glutaraldehyde in PBS (v/v). Neurons were imaged in fluorescence mode with a confocal laser scanning system (MRC-600, Bio-Rad), and the images were digitized with an Image-1/AT image analysis system (Universal Images, West Chester, PA). The neuronal images were coded before characterization, and the following variables were measured: 1) number of neurites (defined as cell surface projections at least one-half the cell diameter) on each neuron; 2) neurite branching (the number of branch points on each neurite); and 3) neurite extension (the length of the longest neurite, measured from the cell body). Typically, in each experiment the neurites of 20–40 neurons/plate were measured, and the results were presented as the mean ± S.E.

In studies on the effect of the inhibitors of lipoprotein binding to the LRP, cells were incubated for 1 h at 37 °C in N2 medium containing the indicated concentrations of either lactoferrin, chlorate, or heparinase or with the receptor-associated protein (RAP). Then the β-VLDL were added, and the incubation was continued for a total of 96 h. The reagents, except for β-VLDL, were re-added every 24 h. The media and β-VLDL were replaced after 48 h.

Cell Association and Degradation of ^{125}I-β-VLDL—The cells were grown for 24 h in 35-mm dishes in N2 medium alone. Then ^{125}I-β-VLDL (3 μg of protein/ml of medium) were added, and the incubation was continued for 16 h at 37 °C. The medium was analyzed for trichloroacetic acid-soluble lipoprotein degradation products as described previously (41). The cells were placed on ice, washed with PBS containing 0.2% bovine serum albumin, and dissolved in 0.1 N NaOH. Lipoprotein cell association was determined by measuring cellular radioactivity using a γ counter (Beckman Gamma 8000, Beckmann Instruments, Fullerton, CA) (41).

Cell Association of DiI-labeled β-VLDL—The cells were grown for 24 h in 35-mm dishes in N2 medium. Then DiI-labeled β-VLDL (4 μg of protein/ml of medium), prepared as described previously (42, 43), were added, and the incubation was continued for 5 h at 37 °C. The cells then were washed with PBS and fixed with 4% paraformaldehyde in PBS (v/v). Uptake of DiI-labeled β-VLDL was visualized by fluorescence microscopy. To quantitate the amount of DiI-labeled lipoprotein in the cells at the end of the incubation, the cells were scraped, using two 0.5-ml aliquots of PBS, and lyophilized. The DiI was extracted from the dried cell pellet with methanol and analyzed using a spectrofluorometer (excitation 520 nm, emission 570 nm) (42). Standards of DiI in methanol were used for quantitation.

Association of ApoE with Lipid Particles—Apolipoproteins E3 and E4 were iodinated using Bolton-Hunter reagent (DuPont NEN) as described previously (44) and then incubated with the lipid particles for 1 h at 37 °C. The samples then were fractionated by chromatography on a Superose 6 column (10/30 HR, Pharmacia Biotech Inc.) and eluted with 1 mM EDTA in PBS at a constant flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and analyzed for cholesterol and triglyceride, and the ^{125}I-apoE content was measured in a Beckmann 8000 counter (Beckmann Instruments) (45).

Statistical Analysis—Data were analyzed using a paired *t*-test.

RESULTS

The levels of apoE secreted into the medium, bound to the cell surface, and accumulating intracellularly by the stably transfected Neuro-2a cells expressing human apoE3 or apoE4 were assessed by Western blot analysis and quantitated by densitometry (Table I). The cells secreted 44–54 ng of apoE3 and 60–89 ng of apoE4/mg of cell protein in 48 h. The apoE3- and apoE4-secreting cells had similar amounts of apoE bound to the cell surface (releasable by suramin treatment), ranging from 4.9 to 8.0 ng of apoE/mg of cell protein. The intracellular content of apoE in the two apoE3-expressing cell lines was 140 and 259 ng of apoE/mg of cell protein. Similar amounts of intracellular apoE (111–215 ng/mg) were seen in the apoE4-expressing cell lines. The addition of β-VLDL to the cells did not have a significant effect on the amount of apoE secreted, surface-bound, or present within the apoE3- or apoE4-secreting cells (Table I).

TABLE I

ApoE3 or apoE4 secreted, releasable by suramin, or present inside cells stably transfected with apoE3 or apoE4 cDNA

Transfected cells were incubated for 96 h in medium with or without β-VLDL (40 μg cholesterol/ml). The medium was changed at 48 h. Apolipoprotein E secreted in the last 48 h, intracellular, and suramin-releasable (surface-bound) apoE were quantitated at the end of the 96 h of incubation as described under "Experimental Procedures." The data are the mean of two separate determinations. The duplicates did not differ by more than 12%.

Cells	Secreted	Releasable	Intracellular
	ng apoE/mg cell protein		
ApoE3-expressing			
Clone 1	54	6.2	140
+ β-VLDL	56	7.2	119
Clone 3	44	4.9	259
+ β-VLDL	45	4.3	251
ApoE4-expressing			
Clone 4	60	6.7	215
+ β-VLDL	63	5.3	231
Clone 5	69	8.0	135
+ β-VLDL	62	6.5	128
Clone 6	89	5.2	111
+ β-VLDL	87	5.6	105

In initial experiments, two Neuro-2a cell lines that secreted similar amounts of apoE3 (clone 1, 54 ng/mg of cell protein) and apoE4 (clone 4, 60 ng/mg of cell protein) (Table I) were used to examine the growth of neurites. When these cells were grown in N2 medium in the absence of β-VLDL, there were no apparent differences in neurite outgrowth between the apoE3- and apoE4-secreting cells. However, incubation of the cells in N2 medium containing β-VLDL resulted in a markedly different pattern in the outgrowth of neurites from these cells. Apolipoprotein E3-secreting cells incubated with β-VLDL developed long neurites (Fig. 1*A*), whereas in apoE4-secreting cells, neurite outgrowth was suppressed (Fig. 1*B*).

Differences in neurite outgrowth in the absence and presence of increasing concentrations of β-VLDL were quantitated by measuring the number of neurites per cell, neurite branching, and neurite extension (Figs. 2, *A–C*, respectively). The values for the non-apoE-transfected control cells incubated for 96 h in N2 medium in the absence of β-VLDL are set at 100%. The expression of either apoE3 or apoE4 by the transfected Neuro-2a cells did not influence neurite number, branching, or extension when the cells were grown in N2 medium in the absence of added lipoprotein (Figs. 2, *A–C*). However, as shown in Fig. 2*A*, the addition of β-VLDL resulted in an increase in the number of neurons in the control cells and in the cells secreting apoE3 (significantly increased at 40 μg of β-VLDL cholesterol/ml compared with apoE3-secreting cells in N2 medium). On the other hand, in the presence of high concentrations of β-VLDL, the Neuro-2a cells secreting apoE4 showed a significant reduction in the number of neurites/cell as compared with the apoE4-secreting cells in the N2 medium.

As described previously for DRG cells (23, 24), the addition of β-VLDL alone resulted in increased branching of neurites. As shown in Fig. 2*B*, addition of β-VLDL to the non-apoE-transfected cells resulted in a significant increase in neurite branching. In addition, at the highest concentration of β-VLDL cholesterol, the apoE3-secreting cells displayed enhanced branching by comparison with the apoE3-secreting cells grown in N2 medium alone. In contrast, the apoE4-secreting cells tended to show decreased branching when incubated with β-VLDL; however, this decrease did not reach statistical significance.

Neurite extension was increased in the Neuro-2a cells secreting apoE3 when they were incubated with the highest concen-

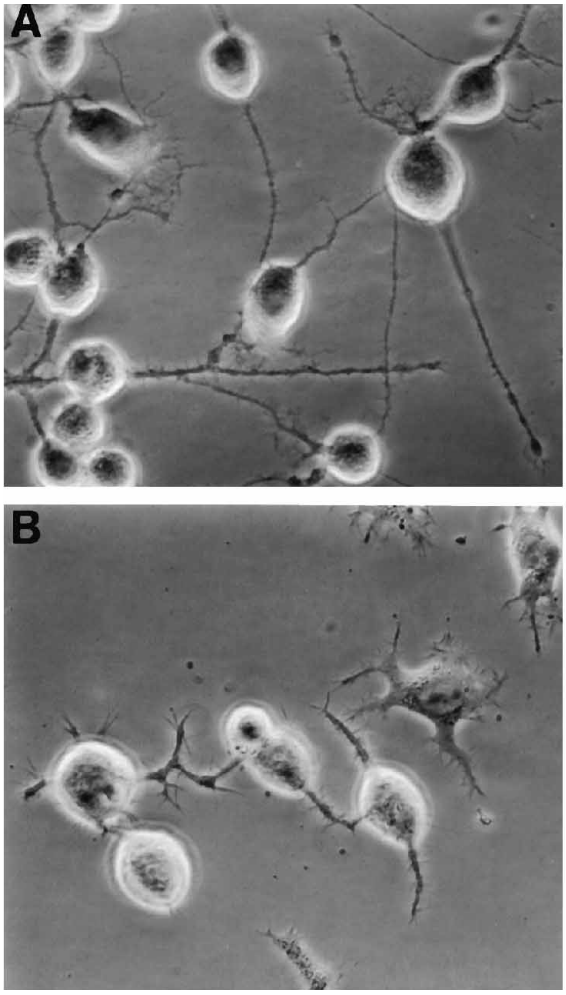

FIG. 1. **Photomicrographs of representative Neuro-2a cells stably transfected with apoE3 (*A*) or apoE4 (*B*) cDNA and grown for 96 h in N2 medium containing β-VLDL (40 μg of cholesterol/ml).**

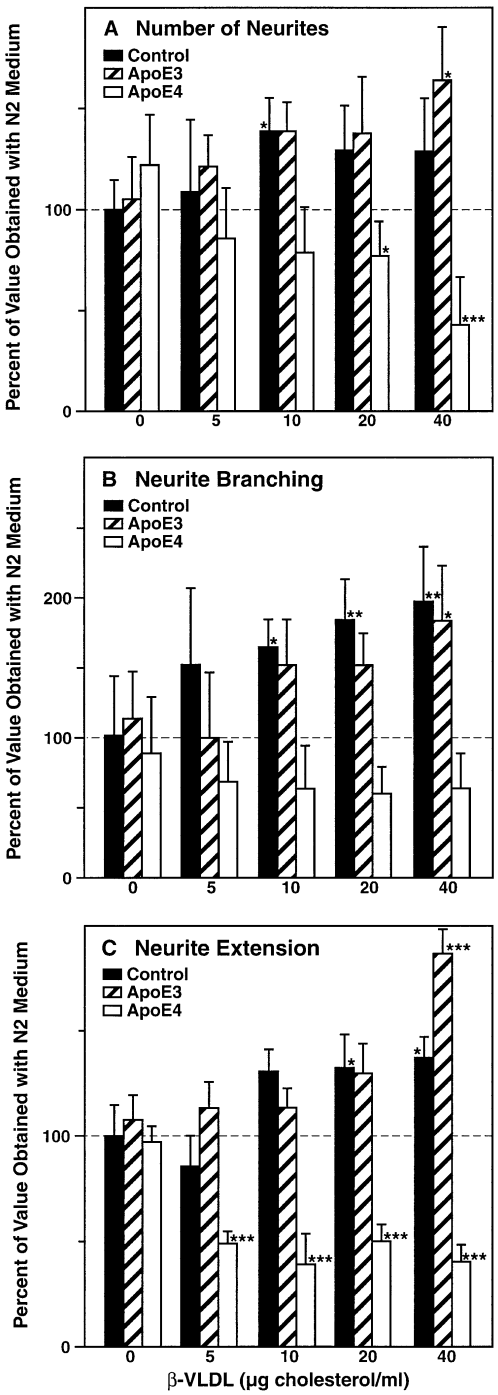

FIG. 2. **Effect of β-VLDL on the number of neurites/cell (*A*), neurite branching (*B*), and neurite extension (*C*) from control Neuro-2a cells and from cells stably transfected to express apoE3 or apoE4.** Cells (clone 1 for apoE3-expressing and clone 4 for apoE4-expressing) were incubated for 96 h in N2 medium alone or in medium containing increasing concentrations of β-VLDL. The media were changed at 48 h. The cells were stained with DiI and fixed, and the indicated parameters were measured. Each data point was obtained by the measurement of 20–50 cells expressing neurites in four separate experiments. The data are presented as the percentage of the value obtained with control cells with N2 medium alone. The data are the mean ± the S.E. The average values obtained with control cells incubated with N2 medium alone were as follows: *A*, neurites/cell = 3; *B*, branch points/neurite = 2; *C*, average neurite length = 155 μm. For calculation of the level of significance for the effect of added β-VLDL, the results in the presence of β-VLDL are compared with the data obtained with the same cells in the absence of β-VLDL (*i.e.* grown in N2 medium alone). *, $p < 0.025$; **, $p < 0.010$; ***, $p < 0.005$

trations of β-VLDL. In contrast, in the apoE4-secreting cells, neurite extension was very significantly suppressed, even at the lowest concentration of β-VLDL used (Fig. 2*C*).

The results described in Fig. 2 were based on a comparison of cells having neuritic outgrowths and did not take into account those Neuro-2a cells without neuritic extensions. Approximately 25–30% of the Neuro-2a cells in N2 medium possessed neurite extensions (defined as a cell-surface projection of at least one-half the cell diameter). However, as shown in Fig. 3, it was apparent that in the presence of β-VLDL, the number of apoE3-secreting cells developing neurites increased markedly to 60–70% of the total. On the other hand, the number of apoE4-secreting cells developing neuritic extensions was significantly reduced, compared with the control or apoE3-secreting cells. Thus, the apoE3-secreting cells incubated with β-VLDL not only had longer neuritic extensions but also showed an increase in the number of cells with neurites. The apoE4-secreting cells grown in the presence of β-VLDL showed fewer neurites, and those that were produced were much shorter.

To ensure that the differential effect of β-VLDL on neurite outgrowth in the apoE3- and apoE4-secreting cells was not due to clonal variation or to differences in the secretion or intracellular content of apoE in the various cell lines, additional experiments were performed with the other stably transfected cell lines secreting apoE3 or apoE4. Incubation of these cells

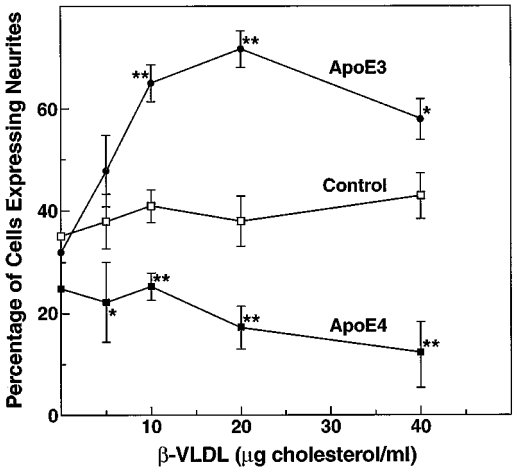

FIG. 3. **Effect of β-VLDL on the percentage of cells expressing neurites.** The cells were incubated as described in Fig. 2. Four different fields in each dish were selected, and the percentage of cells displaying neurites was measured. Data are the means of three different experiments performed in duplicate (± S.E.). The percentages of cells expressing neurites in the absence of β-VLDL were as follows: control cells (*Control*), 35 ± 11; apoE3-expressing cells (*ApoE3*), 32 ± 9; apoE4-expressing cells (*ApoE4*), 25 ± 13. *, $p < 0.025$ *versus* control; **, $p < 0.005$ *versus* control

TABLE II

Effect of β-VLDL (40 μg cholesterol/ml of medium) on the number of neurites/cell, neurite branching, and neurite extension from cells stably transfected with apoE3 or apoE4

The level of secretion of apoE by clones 1, 3, 4, 5, and 6 is described in Table I. Clone 2 secreted 36 ng of apoE3/mg of cell protein/48 h. Surface-bound and internalized apoE was not quantitated for clone 2. The conditions for incubation with β-VLDL are as described in the legend to Fig. 2. Each data point was obtained by the measurement of 25–40 cells. The data are the mean ± S.E. All values are percent of values obtained with control cells in N2 medium alone.

Cell type	No. of neurites	Branching	Extension
ApoE3-expressing			
Clone 1	165 ± 30	186 ± 39	186 ± 13
Clone 2	150 ± 25	180 ± 15	190 ± 23
Clone 3	170 ± 39	175 ± 20	180 ± 25
Apo E4-expressing			
Clone 4	43 ± 25	65 ± 26	41 ± 9
Clone 5	49 ± 15	70 ± 31	50 ± 15
Clone 6	53 ± 19	60 ± 25	45 ± 19

with β-VLDL also resulted in differential effects of apoE3 and apoE4 on neurite outgrowth. As summarized in Table II, in the presence of β-VLDL, all of the apoE4-secreting cells showed a significant reduction in the number of neurites expressed, branching, and neurite extension, whereas the apoE3-secreting cells displayed an increased number of neurites, increased branching, and increased extension as compared with cells grown in N2 medium lacking a source of lipoprotein.

To determine whether apoE4 blocks neurite extension in the presence of β-VLDL or whether it induces neurite retraction, the cells were incubated for 48 h in N2 medium alone to stimulate neurite outgrowth. The medium then was changed, and the cells were incubated for an additional 48 or 96 h in media containing β-VLDL (40 μg of cholesterol per ml). The addition of β-VLDL did not decrease the extension of neurites of apoE4-expressing cells compared with cells incubated in N2 medium alone (data not shown). Therefore, it appears that apoE4 in the presence of β-VLDL inhibits neurite extension directly and does not cause a retraction of neurites that have already extended.

Other lipoproteins were used to determine if any lipid vehicle carrying apoE would substitute for β-VLDL. Incubation of the apoE3- or apoE4-expressing cells with rabbit VLDL, a lipoprotein rich in triglyceride, resulted in similar effects on neurite extension as obtained with β-VLDL. As shown in Table III, when the Neuro-2a cells secreting apoE3 were incubated with VLDL, they showed an increase in neurite extension, whereas the apoE4-secreting cells in the presence of VLDL showed an inhibition of neurite extension. In other experiments, human LDL and canine apoE HDL_c, an apoE-enriched plasma HDL induced by cholesterol feeding and resembling apoE-containing lipoproteins in the CSF (3), also were used. The apoE3- and apoE4-secreting Neuro-2a cells did not respond to LDL (40 μg of cholesterol/ml) (*i.e.* there was no difference in neurite extension as compared with control cells grown in N2 medium alone (data not shown)). On the other hand, incubation of apoE HDL_c (40 μg of cholesterol/ml) with the apoE4-secreting or apoE3-secreting cells resulted in only a small reduction or increase in neurite extension, respectively (control cells in N2 medium, 100%; apoE4-secreting cells plus HDL_c, 85–90% of the value obtained with N2 medium; apoE3-secreting cells plus HDL_c, 110% of the value obtained with N2 medium).

Liposomes and lipid emulsions also were used in an attempt to define the type of lipid vehicle required for the delivery of the apoE. The DMPC emulsion alone or DMPC complexed with cholesterol were incubated with the apoE3- and apoE4-secreting cells for 96 h at increasing phospholipid concentrations of up to 45 μg of phospholipid and 5 μg of cholesterol/ml of medium (higher concentrations were toxic to the cells). In these studies, there was no effect on neurite outgrowth with either of the apoE-transfected Neuro-2a cells (data not shown). Previously, we have shown that apoE complexes with DMPC and mediates high affinity binding to the LDL receptor (46). On the other hand, a lipid emulsion particle (emulsion A in Table III), which was a triglyceride- and phospholipid-containing spherical particle (~35.8 nm), caused a significant enhancement of neurite extension in the apoE3-secreting cells and was associated with an inhibition of outgrowth in the apoE4-secreting cells.Thus, specific combinations of lipids and/or a unique particle size may be required to elicit the apoE isoform-specific effects on neurite outgrowth. It is interesting to note that the delivery of cholesterol to the cells does not appear to be required for the differential effect.

Additional studies using the lipoproteins from bovine CSF suggest that natural lipoproteins in the central nervous system may mediate the isoform-specific effects of apoE3 and apoE4. As shown in Fig. 4, addition of lipoproteins isolated from CSF ($d < 1.21$ g/ml) to the cells caused an inhibition of neurite outgrowth from the apoE4-expressing cells and an increase in outgrowth from the apoE3-expressing cells. When CSF lipoproteins were used at a concentration of 40 μg of lipoprotein cholesterol/ml, the effect was similar to that obtained using β-VLDL at the same concentration.

Cerebrospinal fluid lipoproteins ($d < 1.21$ g/ml) were analyzed for protein and cholesterol content and apolipoprotein composition. The ratio of cholesterol to protein was approximately 1:1, similar to data reported for canine CSF (3). The bovine CSF lipoproteins ($d < 1.21$ g/ml) contained only apoE and apoA-I when separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie Brilliant Blue staining. These results are similar to those reported previously for human and canine CSF lipoproteins (3, 47).

The ability of the neuroblastoma cells to bind, internalize, and degrade β-VLDL was examined to determine whether the

TABLE III

Effect of β-VLDL, VLDL, or lipid emulsions on neurite extension from cells stably transfected with apoE3 or apoE4 cDNA

Cells (clone 1 for apoE3-expressing and clone 4 for apoE4-expressing) were incubated for 96 h in N2 medium alone or containing the indicated concentrations of particles: β-VLDL, 40 μg of cholesterol/ml of medium (this corresponds to 5 μg of triglyceride/ml of medium); VLDL, 5 μg of triglyceride/ml of medium; emulsion A, 5 μg of triglyceride/ml of medium. CHOL, cholesterol; TG, triglyceride; PL, phospholipid. Each data point was obtained by the measurement of 30–40 cells expressing neurites in three separate experiments. The data are the mean ± S.E.

Treatment	Lipid composition	Mean size	Value obtained with control cells in N2 medium alone		
			Control	ApoE3-expressing	ApoE4-expressing
	w/w/t	*nm ± S.D.*			
N2 alone			100 ± 10	110 ± 15	115 ± 11
β-VLDL	CHOL/TG/PL (5.6:0.4:1)	43.7 ± 25.6	120 ± 15	160 ± 18[a]	60 ± 13[a]
VLDL	CHOL/TG/PL (1:7.4:1)	39.5 ± 18.7	110 ± 11	155 ± 21[a]	61 ± 19[a]
Emulsion A	TG/PL (2.7:1)	35.8 ± 14.9	95 ± 14	150 ± 12[a]	75 ± 12[a]

[a] $p < 0.010$ *versus* control.

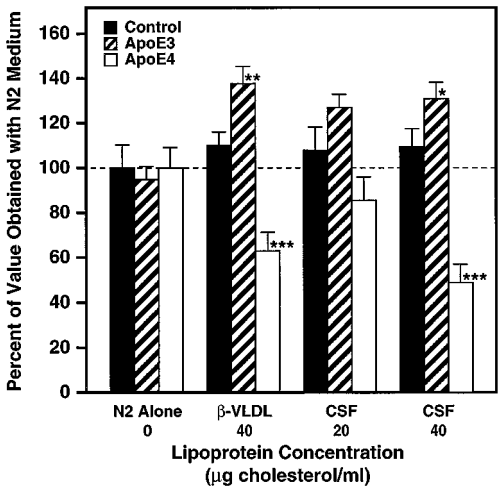

FIG. 4. **Effect of CSF lipoproteins on neurite extension from Neuro-2a cells stably transfected to express apoE3 or apoE4.** Cells were incubated with β-VLDL or bovine CSF lipoproteins ($d < 1.21$ g/ml) under the conditions described in the legend to Fig. 2. Each data point represents the measurement of 20–40 neurons. The data are reported as the mean ± S.E. The calculation of the level of significance of the differences observed was performed as described in the legend to Fig. 2. *, $p < 0.025$; **, $p < 0.01$; ***, $p < 0.005$

TABLE IV

Cell association and degradation of ^{125}I-β-VLDL by stably transfected and control Neuro-2a cells

Cells were incubated for 24 h in N2 medium alone. The ^{125}I-β-VLDL (3 μg of protein/ml of medium) were then added, and after 16 h at 37 °C the lipoprotein cell association (bound and internalized) and degradation by Neuro-2a cells were measured. The data reported are the mean of two separate experiments performed in duplicate (± S.D.). Control, cells transfected with pSV2 *neo* alone.

Cell type	^{125}I-β-VLDL	
	Cell association	Degradation
	ng lipoprotein protein/mg cell protein	
Control cells	750 ± 16	2,467 ± 331
ApoE3-expressing cells	671 ± 40[a]	1,945 ± 219
ApoE4-expressing cells	662 ± 50[a]	1,788 ± 188[b]

[a] $p < 0.05$ *versus* control.
[b] $p < 0.01$ *versus* control.

differences in neurite outgrowth in the apoE3- and apoE4-expressing cells were due to a different ability of the secreted apoE3 and apoE4 to stimulate the delivery of apoE and/or lipoprotein lipids to the cells. In these studies, ^{125}I-β-VLDL were used to quantitate the binding, uptake, and degradation of the lipoproteins in the Neuro-2a cells (Table IV). The total amount of cell-associated (bound and internalized) ^{125}I-β-VLDL was very similar in the apoE3- and apoE4-secreting cells (both were slightly lower than that seen in the non-apoE-transfected control cells). The degradation of ^{125}I-β-VLDL by the apoE3- and apoE4-secreting cells was similar. There was a small (but statistically significant) decrease in the degradation of ^{125}I-β-VLDL by the apoE4-secreting cells when compared with the non-apoE-transfected control Neuro-2a cells.

In a parallel experiment, the cells were incubated with DiI-labeled β-VLDL to visualize the internalization of the lipoproteins in the apoE3- and apoE4-secreting cells by fluorescence microscopy. Following internalization, DiI is trapped in the lysosomes, and the fluorescent intensity of the cells, therefore, is proportional to the total amount of lipoprotein internalized and degraded (42). In these studies, no difference in the uptake of DiI-labeled β-VLDL was observed in the apoE3- and apoE4-secreting cells. Extraction and quantitation of the DiI from cells incubated with DiI-labeled β-VLDL (40 μg of cholesterol/ml) for 16 h at 37 °C confirmed the visual impression that the uptake of DiI-labeled β-VLDL was similar in the apoE3- and apoE4-secreting cells. The control cells incorporated 8.9 ± 0.4 ng of DiI/mg of cell protein, while the apoE3- and apoE4-expressing cells incorporated 10.2 ± 1.0 and 10.8 ± 0.3 ng of DiI/mg of cell protein, respectively.

To demonstrate that apoE binds to the lipid particles when it is present at the concentrations secreted by the cells, we incubated radiolabeled apoE3 or apoE4 with the β-VLDL, VLDL, or emulsion A for 1 h at 37 °C (100 ng of apoE with 40 μg of β-VLDL cholesterol or 100 ng of apoE with either 5 μg of VLDL or emulsion A triglyceride) and fractionated them by FPLC. Approximately 70% of the apoE was associated with the β-VLDL and 50% with the VLDL and emulsion A. There was no difference in the amount of apoE3 or apoE4 associated with the lipid particles.

To determine which receptor was involved in mediating the differential effects of apoE3 and apoE4 on neurite outgrowth, we used inhibitors that block the binding and internalization of apoE-enriched lipoproteins by the HSPG·LRP pathway, but not by the LDL receptor pathway, and determined the effect on neurite outgrowth. Prior to the addition of β-VLDL, the cells were preincubated for 1 h with either heparinase (20 units/ml) and chlorate (20 mM), with the RAP (5 μg/ml), or with lactoferrin (10 μg/ml). The binding of apoE-enriched lipoproteins to the LRP requires their initial binding to cell-surface HSPG. Heparinase and chlorate cleave and reduce the sulfation of cell-surface HSPG, respectively (28, 48). Lactoferrin blocks binding of lipoproteins to both HSPG and LRP, whereas RAP primarily blocks the binding of apoE-enriched lipoproteins to the LRP. All of these reagents previously have been shown to inhibit the uptake of apoE-enriched β-VLDL by the LRP (26, 28, 49, 50). As previously shown in Fig. 2, β-VLDL alone stim-

TABLE V

Effect of chlorate, heparinase, RAP, and lactoferrin in the presence of β-VLDL on neurite extension from cells stably transfected with apoE3 or apoE4 cDNA

Cells were incubated for 1 h in N2 medium alone or containing the indicated concentrations of chlorate, heparinase, RAP, or lactoferrin. Then the β-VLDL were added, and the incubation was continued for a total of 96 h. The reagents, except for β-VLDL, were readded every 24 h. The media and β-VLDL were changed at 48 h. Each data point was obtained by measuring 30–40 neurons expressing neurites in two separate experiments. Data are the mean ± S.E. Values are percent of values obtained with control cells in N2 medium alone.

Treatment	Control	ApoE3-expressing	ApoE4-expressing
N2 alone	100 ± 8	105 ± 10	103 ± 9
β-VLDL (40 μg cholesterol/ml)	160 ± 13	209 ± 13[a]	70 ± 4[b]
β-VLDL + chlorate (20 mM) and heparinase (20 units/ml)	159 ± 14	163 ± 20[c]	138 ± 12[d]
β-VLDL + RAP (5 μg/ml)[e]	176 ± 11	179 ± 15	160 ± 16[d]
β-VLDL + lactoferrin (10 μg/ml)	128 ± 16	154 ± 19[c]	130 ± 12[d]

[a] $p < 0.05$ *versus* value obtained with control cells (non-apoE-expressing cells incubated with β-VLDL).
[b] $p < 0.01$ *versus* value obtained with control cells (non-apoE-expressing cells incubated with β-VLDL).
[c] $p < 0.05$ *versus* apoE3-expressing cells with β-VLDL alone.
[d] $p < 0.01$ *versus* apoE4-expressing cells with β-VLDL alone.
[e] In a parallel set of experiments, 5 μg/ml of RAP did not block the binding of DiI-labeled LDL to the Neuro-2a cells.

ulated the outgrowth of neurites. The stimulation of neurite outgrowth by β-VLDL was further enhanced in the apoE3-expressing cells and markedly inhibited in the apoE4-secreting cells (Fig. 2 and Table V). The addition of chlorate and heparinase or RAP did not block the stimulatory effect of β-VLDL on neurite outgrowth in the control cells (Neuro-2a cells not expressing apoE), suggesting that the effect of β-VLDL alone is mediated by the LDL receptor; however, these reagents blocked the isoform-specific effects in the cells secreting apoE (Table V). Chlorate and heparinase treatment of the cells or the addition of RAP prevented the stimulation of neurite extension in the apoE3-expressing cells incubated with β-VLDL (that is, significantly decreased the β-VLDL-induced neurite extension in the Neuro-2a cells secreting apoE3). Moreover, chlorate and heparinase or RAP blocked the inhibition of neurite extension seen in the apoE4-expressing cells (that is, the apoE4-expressing cells in the presence of β-VLDL did not demonstrate inhibition of neurite extension but, in fact, showed increased extension) (Table V). In the presence of heparinase and chlorate or RAP, in the apoE-secreting cells, neurite outgrowth was similar to that observed when β-VLDL were added to the control cells in the absence of apoE (Table V). Therefore, in the presence of these reagents, the LDL receptor-mediated effect of β-VLDL was not blocked. Lactoferrin also blocked the effects of apoE3 and apoE4 on neurite outgrowth; however, it also slightly suppressed the effect of β-VLDL on neurite extension in the control cells. These data show that inhibition of the interaction between β-VLDL and the HSPG·LRP pathway prevents the differential effects of apoE3 and apoE4 on neurite outgrowth (Table V).

DISCUSSION

We have previously shown that human apoE3 and apoE4 have a differential effect on the outgrowth of neurites from DRG neurons and Neuro-2a cells in culture when added exogenously to the cells together with β-VLDL (23–25). Compared with cells treated with β-VLDL alone, cells incubated with β-VLDL and apoE3 had decreased neurite branching and increased neurite extension, whereas cells incubated with apoE4 and β-VLDL showed a decrease in both neurite branching and extension (24). In the current studies, we have demonstrated that in the presence of β-VLDL, VLDL, CSF lipoproteins, or specific lipid emulsions (containing triglyceride and phospholipid), the endogenous synthesis and secretion of nanogram quantities of apoE3 and apoE4 by transfected murine neuroblastoma cells (Neuro-2a) had differential effects on neurite outgrowth. Stably transfected cells secreting comparable amounts of human apoE3 or apoE4 display similar patterns of neurite outgrowth when incubated in the absence of lipoproteins. However, the addition of as little as 5–10 μg of β-VLDL cholesterol/ml of medium or 5 μg of VLDL triglyceride/ml of medium caused a dramatic difference in the neurite extension and branching in the apoE3- and apoE4-secreting cells. Whereas both branching and extension were increased in the apoE3-producing cells, these processes were very significantly inhibited in the apoE4-producing cells. This differential effect was not due to clonal variation, because similar results were obtained using several different stably transfected lines that secreted different levels of apoE. The inhibitory effect on neurite outgrowth in the apoE4-secreting cells was dependent upon the presence of a source of lipid during the initiation of neurite formation, since β-VLDL added to apoE4-secreting cells after the neurites had already formed did not result in neurite retraction.

It has been shown that the addition of a lipid source (β-VLDL, VLDL, CSF lipoproteins, apoE-containing HDL, or specific lipid emulsions) to the apoE3- and apoE4-secreting cells is necessary for the isoform-specific effect on neurite outgrowth to occur. This pathway may involve a secretion-capture role for apoE, as has been postulated to occur with other cell types secreting apoE (1, 6, 27). We have shown that the apoE secreted by the transfected Neuro-2a cells interacts with the lipoproteins in the medium. In addition, the apoE may interact with cell-surface HSPG and, by either or both of these processes, may mediate lipoprotein or lipid emulsion uptake by the LDL receptor or the LRP (1, 26, 27, 51). Our data demonstrate that it is the HSPG·LRP pathway that is important for the differential effects of apoE3 and apoE4 on neurite outgrowth. These data are consistent with the studies of Holtzman *et al.* (52), in which the addition of RAP and anti-LRP antibody blocked the stimulatory effect of apoE3 on NGF-induced neurite extension in an immortalized central nervous system-derived neuronal cell line in culture. The data also support a role for the LDL receptor in mediating the stimulatory effect of β-VLDL alone on neurite outgrowth. The VLDL receptor that also binds apoE-containing lipoproteins and could be involved in mediating the uptake of the β-VLDL is not present in the Neuro-2a cells.[2]

It has been shown previously that apoE-enriched lipoproteins bind initially to cell-surface HSPG and are then transferred to LRP prior to internalization or that the HSPG·LRP complex itself is internalized by the cells (1, 6, 26–28). Lipid-

[2] B. P. Nathan, S. Bellosta, R. W. Mahley, and R. E. Pitas, unpublished results.

free apoE is a poor ligand for any of the lipoprotein receptors, including the LRP. Thus, a specific lipoprotein vehicle (*e.g.* β-VLDL, VLDL, CSF lipoproteins, specific lipid emulsions, or apoE-containing HDL), capable of being enriched in apoE, may be required to target apoE3 and apoE4 into a specific intracellular pathway to allow for the differential effects of the apoE isoforms. Once they are internalized, it is necessary to postulate that the apoE isoforms are handled differently by the cells in such a way that apoE3 has a stimulatory role and apoE4 has an inhibitory role in regulating neurite outgrowth.

There are several lines of evidence suggesting that the differential effects of the apoE isoforms on neurite outgrowth are secondary to an effect on the cytoskeleton, possibly by modulating microtubular assembly. In Neuro-2a cells, the addition of exogenous apoE3 plus β-VLDL results in an abundance of well formed microtubules in the presence of neurite extension, whereas cells incubated with apoE4 and β-VLDL primarily contain monomeric tubulin, and neurite outgrowth is impaired (25). *In vitro* biochemical studies have shown that apoE3 interacts avidly with the microtubular-associated proteins τ and MAP 2C, whereas apoE4 does not (53, 54). It has been postulated that apoE3 facilitates the ability of τ and MAP 2C to interact with and stabilize microtubules. These observations suggest one possible mechanism whereby apoE3 may be protective in preventing late onset AD and apoE4 may be detrimental (55, 56). If this is, in fact, the mechanism by which apoE3 and apoE4 affect neurite outgrowth, the question remains as to whether it is a direct or indirect effect of apoE on the cytoskeleton. If it is a direct effect, apoE must escape intracellular endosomes and enter the cytoplasm by an unknown mechanism. It has been suggested that apoE may occur in the cytoplasm (57). Alternatively, apoE3 and apoE4 may have an indirect effect on the cytoskeleton and signal changes in activity that support or suppress extension. This could occur following internalization of the apoE or through differential signaling events induced by apoE3 and apoE4 following binding to the HSPG·LRP on the cell surface. Interpretation of these data remains speculative at the present time.

The β-VLDL and VLDL, which in the presence of apoE3 or apoE4 give the maximum differential effect on neurite outgrowth in both the current studies and in those previously reported (23, 24), do not occur in the central nervous system. The CSF, however, does contain HDL. It has been shown previously that human and canine CSF contain separate populations of HDL containing either apoE or apoA-I (3, 47). The apoE-containing HDL in the CSF are similar to the apoE HDL_c used in our studies that were able to mediate a small inhibitory effect of apoE4 on neurite outgrowth. Moreover, the addition of CSF lipoproteins mimicked the effect of the β-VLDL. Thus, the CSF lipoproteins could serve as vehicles for the delivery of the apoE3 or apoE4 to neurons.

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