

Effects of apolipoprotein E, β -very low density lipoproteins, and cholesterol on the extension of neurites by rabbit dorsal root ganglion neurons in vitro

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Abstract Previous studies suggest that during nerve regeneration apoE acts as a lipid transport protein that assists in the rapid initial extension of axons and then in their myelination. To determine whether apoE and/or apoE-containing lipoproteins can modulate axon growth, we assessed their effect on the outgrowth of neurites from neurons in mixed cultures of fetal rabbit dorsal root ganglion cells in vitro. Incubation with β -very low density lipoprotein (β -VLDL) particles, which are rich in apoE and cholesterol, increased neurite outgrowth and branching. Unesterified cholesterol added to the cultures had a similar, but less pronounced, effect. These data suggest that cholesterol might be the component responsible for the enhanced neurite growth. In contrast, purified, lipid-free apoE added to the cultures reduced neurite branching. Neurite branching was also reduced when purified apoE was added along with β -VLDL or cholesterol; however, the striking finding was that under these conditions the neurites extended farther from the neuronal cell body. Dorsal root ganglion cells were examined for the presence of receptors for native and apoE-enriched β -VLDL. Immunocytochemistry, ligand blots, ⁴⁵Ca²⁺ blots, and studies of the interaction of the cells with fluorescent lipoproteins provided evidence of two types of receptors for apoE-containing lipoproteins on neurons: the low density lipoprotein (LDL) receptor, which binds native β -VLDL, and the LDL receptor-related protein, which binds apoE-enriched β -VLDL. These findings indicate that apoE may play two complementary roles in neurite outgrowth. When complexed with lipoproteins, apoE stimulates neurite growth by the receptor-mediated delivery of cholesterol and perhaps other components necessary for neurite outgrowth. When apoE as a free protein is added together with apoE-containing lipoproteins, apoE decreases neurite branching and promotes neurite extension away from the cell body. These actions, which would be complementary in promoting target-directed nerve growth in vivo, provide the first direct evidence that apoE and apoE-containing lipoproteins can modulate the outgrowth of neuronal processes.—**Handelsmann, G. E., J. K. Boyles, K. H. Weisgraber, R. W. Mahley, and R. E. Pitas.** Effects of apolipoprotein E, β -very low density lipoproteins, and cholesterol on the extension of neurites by rabbit dorsal root ganglion neurons in vitro. *J. Lipid Res.* 1992. 33: 1677–1688.

Supplementary key words apolipoprotein E • dorsal root ganglia • nerve regeneration • lipoproteins • cholesterol

Apolipoprotein (apo) E is a lipid transport protein that helps to redistribute cholesterol and other lipids in various tissues (for review, see references 1–3). Recent work from several laboratories has suggested that apoE plays a role in peripheral nerve regeneration. Although the concentration of apoE in peripheral nerves normally is low, a denervating crush injury leads to apoE secretion by macrophages and to its accumulation in the nerve to very high concentrations (4–8). Much of this apoE, which accumulates extracellularly between nerve fibers, is associated with lipid and forms cholesterol- and phospholipid-rich lipoprotein particles (6). Some of the apoE is also present as a free protein (7). In vitro experiments using a neuronal cell line (PC12 cells) have shown that apoE-containing lipoproteins obtained from regenerating nerves are internalized by nerve cells via specific receptor-mediated endocytosis (9). In vivo experiments have shown that the low density lipoprotein (LDL) receptor, which recognizes apoE-containing lipoproteins, is present at high concentrations in regenerating nerve tips (6). These results have been the basis for proposing that apoE-containing lipoproteins mediate the delivery of lipids to regenerating axons.

This hypothesis suggests that the accumulation of apoE-containing lipoproteins and the expression of lipoprotein receptors by regenerating axons promote their

Abbreviations: apo, apolipoprotein; LDL, low density lipoproteins; β -VLDL, β -very low density lipoproteins; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine; PBS, phosphate-buffered saline; LRP, LDL receptor-related protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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rapid elongation and regeneration through the delivery of cholesterol and other lipids needed for membrane synthesis. As a first step toward establishing this possibility, we asked whether apoE or apoE-containing lipoproteins could alter the pattern of neurite outgrowth by dorsal root ganglion neurons, which give rise in vivo to many of the axons contained in peripheral nerves such as the sciatic nerve. We assessed the outgrowth of neurites by neurons in fetal rabbit dorsal root ganglion cells grown in tissue culture, where their environment could be readily controlled and lipoproteins could be added or omitted. Apolipoprotein E-containing lipoproteins (β -very low density lipoproteins, β -VLDL), purified apoE, and cholesterol were each evaluated for their effect on neurite outgrowth. In addition, because apoE-containing lipoproteins can be internalized by neurons (9), we determined which of the receptors known to be capable of specifically recognizing apoE are present on dorsal root ganglion neurons.

MATERIALS AND METHODS

Materials

Apolipoprotein E was isolated from cholesterol-fed rabbit plasma as previously described for human apoE (10) and dialyzed against tissue culture medium before use. Human apoC-I and C-III were isolated by high-performance liquid chromatography as previously described (11). The β -VLDL were isolated from the serum of cholesterol-fed rabbits according to the procedures of Kowal et al. (12). The β -VLDL were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetra-methylindocarbocyanine (DiI) by the method of Pitas et al. (13). Cholesterol, obtained from Supelco, Inc. (Bellefonte, PA), was dissolved in ethanol (10 mg/ml) and diluted with tissue culture medium (14) containing fatty acid-free albumin (1 mg/ml; Sigma, St. Louis, MO).

Tissue culture

Pregnant New Zealand White rabbits were given an overdose of pentobarbital (180 mg intravenously), and the fetuses (26–28 days of gestation) were removed under aseptic conditions. Each fetus was rapidly decapitated before dissection. The dorsal root ganglia were removed and placed in N2 medium, a serum- and lipid-free growth-promoting medium for neurons (14), containing penicillin and streptomycin (50 IU/ml and 50 μ g/ml, respectively). The roots and other adherent tissue were removed, but the capsule was left intact. The cleaned ganglia were incubated in 0.25% collagenase in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DME/F12; Sigma) for 30 min, washed with N2 medium, and dissociated by trituration about 20 times

with a sterile long-glass Pasteur pipette. The cell suspension was then filtered through sterile cotton gauze and passed through a 27-gauge needle. The suspension was next diluted with N2 medium and plated (1500 cells in 100 μ l) in the center of a 35-mm tissue culture dish, which was coated with bovine aortic endothelial cell matrix (Accurate Chemical and Scientific Corp., Westbury, NY) or type I collagen (approximately 6 μ g/cm²; Sigma). The cultures were maintained in a humidified incubator at 37°C and 5% CO₂. Two hours after plating and once the cells had adhered, 1.4 ml of N2 medium was added to each dish. At this time, test reagents, as required by the different experiments, were also added. Cultures grown in N2 medium alone served as the controls. All cultures prepared together on the same date are referred to as a batch. In preliminary studies 2.5 S nerve growth factor (25 ng/ml) was added to the medium. Compared to controls there was no difference in either neuronal survival or neurite extension when the cells were examined 48 h after addition of the nerve growth factor (data not shown). Nerve growth factor was therefore not used in any of the studies reported.

Immunocytochemistry

To assess neurite outgrowth after 48 h of incubation, the cultures were washed once with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 16 to 24 h. Neurite outgrowth was visualized by immunocytochemical detection of GAP-43, a neuron-specific growth-associated phosphoprotein (15). The specific monoclonal antibody against GAP-43 (9 E12), generously provided by Drs. P. Skene and D. Schreyer, has been described previously (16). We found that neurons grown in the presence of purified apoE did not adhere well to the extracellular matrix during the immunocytochemical procedure. For this reason, all fixed cultures were coated with a thin layer of gelatin (1% in distilled water) before the start of the immunocytochemical procedure. Approximately 1 ml of the gelatin solution at room temperature was added to each dish and immediately removed with a Pasteur pipette. The dish was then placed on ice briefly to set the gelatin. The immunocytochemical techniques were similar to those used by Boyles et al. (6, 7, 17).

The presence and distribution of LDL receptors were determined by immunocytochemistry using a specific polyclonal antiserum against rabbit LDL receptors (18). The presence and distribution of the LDL receptor-related protein (LRP), a receptor that binds apoE-enriched lipoproteins, were determined using monoclonal antibodies against human LRP (19), generously provided by Dr. S. Moestrup. The binding of these primary antibodies to the cultured cells was detected by using the ABC peroxidase (Vector Laboratories, Burlingame, CA) technique described previously (17).

Neuronal growth

The outgrowth of neurites stained immunocytochemically for GAP-43 was analyzed using a confocal laser scanning imaging system (MRC-600, Bio-Rad, Richmond, CA) at a magnification of 160 \times . To quantitate neurite outgrowth, the following variables were measured for each cell: the number of neurites, the radial distance from the cell body to the end of each neurite (neurite extension), the combined length of all neurite branches, and the number of branch points (neurite branching). The number of branch points probably also includes some neurite crossings, as these occasionally are difficult to distinguish from true branch points. The neurons to be measured were chosen at random, except that neurons whose neurites were not clearly visible and distinguishable from those of other neurons were excluded. For each neuron measured, we used phase-contrast microscopy to verify that GAP-43 was present along the entire length of the neuron. Because the neurons were chosen randomly, their size varied considerably, and therefore neurite length also varied. To make meaningful comparisons, it was necessary to convert the actual measured lengths to a corrected value, by dividing the measured neurite lengths by the diameter of the area covered by neurites. If the area covered by the neurites was not circular, several cross-sections were measured through the cell body to the end of the neurites, and these measurements were then averaged. For each treatment condition, at least 20 neurons chosen from three to six culture dishes were measured. Each treatment condition was repeated in three or four tissue culture batches.

Internalization of DiI-labeled β -very low density lipoproteins

To assess the internalization of DiI-labeled β -VLDL by neurons, the neurons were grown in either N2 medium or in N2 medium supplemented with 10% fetal calf serum for 48 h. Then, after the cultures were washed with N2 medium, one of the following reagent mixtures, previously incubated for 30 min at 37°C, was added: DiI-labeled β -VLDL (100 μ g of cholesterol/ml), β -VLDL (100 μ g of cholesterol/ml), and apoE (10 μ g/ml), or β -VLDL (100 μ g of cholesterol/ml) and apoE (10 μ g/ml) together with either apoC-I (10 μ g/ml) or apoC-III (10 μ g/ml). The internalization of apoE-enriched β -VLDL by LRP is known to be blocked by apoC-I but not apoC-III at these concentrations (12). The cultures were incubated for 2 h at 37°C, washed with PBS, and fixed with 4% paraformaldehyde for 16–24 h. Uptake of DiI-labeled β -VLDL was visualized by confocal fluorescent microscopy.

Demonstration of the LRP in dorsal root ganglion membranes by Western blot

Plasma membranes were isolated from freshly dissected fetal rabbit dorsal root ganglia and examined for the

presence of LRP. The ganglia were prepared and dissociated as described above. After dissociation, the ganglion cells were pelleted by centrifugation and homogenized on ice by sonication (several brief pulses) in buffer (5 ml/ml of packed ganglion cells) containing 20 mM Tris (pH 8.0), 0.15 M NaCl, 1 mM CaCl₂, 0.1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 800 *g* for 10 min at 4°C and the supernatant was saved. The pellet was briefly resonicated in additional buffer and centrifuged again. The two supernatants were combined and centrifuged at 100,000 *g* for 1 h at 4°C. The pellet was washed with buffer and suspended in 0.5 ml of half-strength gel application buffer (3% sodium dodecyl sulfate, 62.5 mM Tris, 10% glycerol, pH 6.8). Two hundred microliters of solubilized membranes was immediately subjected to non-reduced 5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (3-mm-thick gel) at 30 mA for 3–4 h in a cold room (4°C) and then transferred to nitrocellulose.

Detection of the LRP on the nitrocellulose was performed by immunoblot and calcium blot techniques. For the immunoblots, the nitrocellulose was incubated in a blocking buffer (PBS containing 5% nonfat dry milk and 5% bovine serum albumin) for 5 h. The nitrocellulose was then washed in PBS and transferred to a solution containing a polyclonal antibody (obtained from Dr. S. Moestrup) raised in rabbit against denatured human LRP (diluted 1:500 in PBS containing 5% albumin and 0.1% nonfat dry milk). The nitrocellulose was incubated for 2 h in the antibody solution, washed, and then incubated for 2 h in the secondary antibody solution (biotinylated donkey anti-rabbit immunoglobulin G (Amersham, Arlington Heights, IL)), diluted 1:250 in PBS containing 5% albumin and 0.1% dry milk). The nitrocellulose was again washed and incubated for 1 h in a solution containing avidin–biotin–horseradish peroxidase complex (Vector) diluted 1:250 in PBS containing 0.1% dry milk. After a final wash, the nitrocellulose was incubated in a solution containing diaminobenzidine (0.2 mg/ml; Sigma) to detect the presence of peroxidase. For the calcium blots, the method of Maruyama, Mikawa, and Ebashi (20) was used with modifications. Briefly, the nitrocellulose was washed three times for 20 min each in buffer containing 60 mM KCl, 5 mM MgCl₂, and 10 mM imidazole, pH 6.8. The nitrocellulose was then incubated in the same buffer containing ⁴⁵Ca²⁺ (1 μ Ci/ml) for 10 min. The nitrocellulose was then rinsed in 50% ethanol, washed in 50% ethanol for 5 min, dried, and subjected to autoradiography.

Statistical analysis

The data for each indicator of neurite outgrowth were analyzed by two-factor analysis of variance; the factors were the dose and the batch. Least squares tests for main and interaction effects of these factors were used. Control versus dose contrasts and their (pooled variance) standard

errors were obtained. From these results, 95% confidence intervals between control and dose were computed. Because the analysis indicated significant variability among batches, the reagent-treated neurons were compared only with the control neurons in the same batch to derive the percentages shown in the figures.

RESULTS

Neurite outgrowth

To evaluate the role of apoE, and the lipids with which it associates, in neurite outgrowth, we incubated rabbit

dorsal root ganglion cells with β -VLDL (66 μ g of cholesterol/ml), an apoE- and cholesterol-rich lipoprotein. The outgrowth of neurites was monitored at 48 h. The β -VLDL appeared to increase the neurite outgrowth, specifically by increasing neurite branching (Fig. 1). To determine whether the effects of β -VLDL on neurite outgrowth were due to the cholesterol or to the apoE component of the lipoprotein, we added either cholesterol (suspended in tissue culture medium with albumin as described above, 66 μ g/ml) or lipid-free, purified apoE (66 μ g/ml) to the cultures. Like β -VLDL, cholesterol appeared to increase neurite branching (Fig. 1), whereas purified apoE ap-

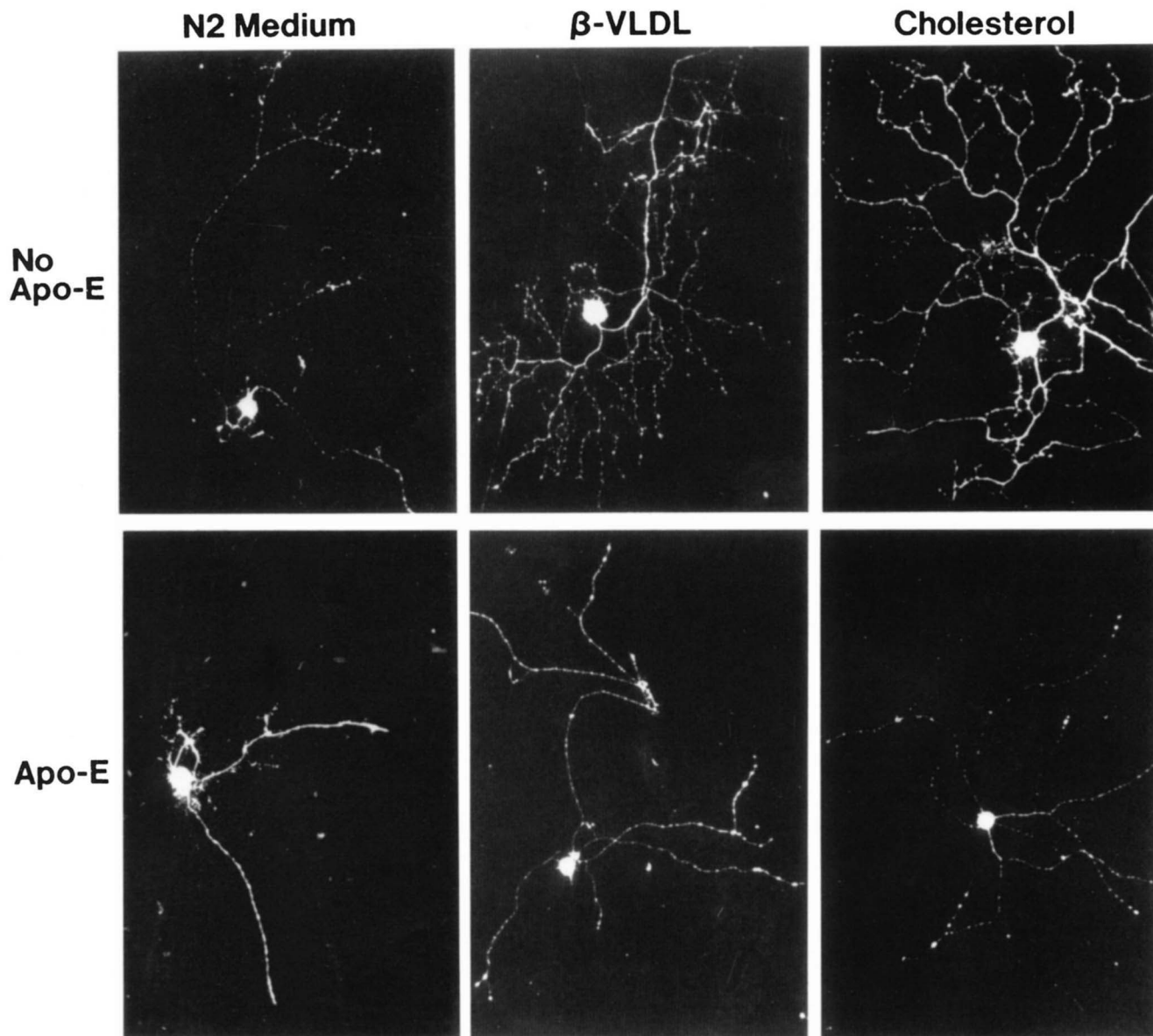


Fig. 1. The effects of β -VLDL, cholesterol, and apoE on the appearance of cultured dorsal root ganglion neurons grown on extracellular matrix. The top panels show representative neurons grown in N2 medium alone or with β -VLDL or cholesterol (66 μ g/ml). Note the extensive branching induced by β -VLDL or cholesterol. The bottom panels show representative neurons grown with the same reagents, to which apoE has been added (66 μ g/ml). Branching was reduced by apoE. The neurons were stained by immunofluorescence using specific antibodies against the neuron-specific phosphoprotein, GAP-43.

peared to decrease branching. To determine what effect the addition of free apoE might have on neurite outgrowth induced by β -VLDL or cholesterol, we added purified apoE together with either β -VLDL or cholesterol. The addition of apoE reduced the amount of neurite branching induced by β -VLDL or cholesterol (Fig. 1).

These differential effects were observed when the cells were grown either on extracellular matrix (Fig. 1) or on collagen (Fig. 2), although neurons grown on collagen had a different morphological appearance (compare Fig. 1 with Fig. 2) and neurite outgrowth was not as rapid. Despite these differences in neurite outgrowth, the effect

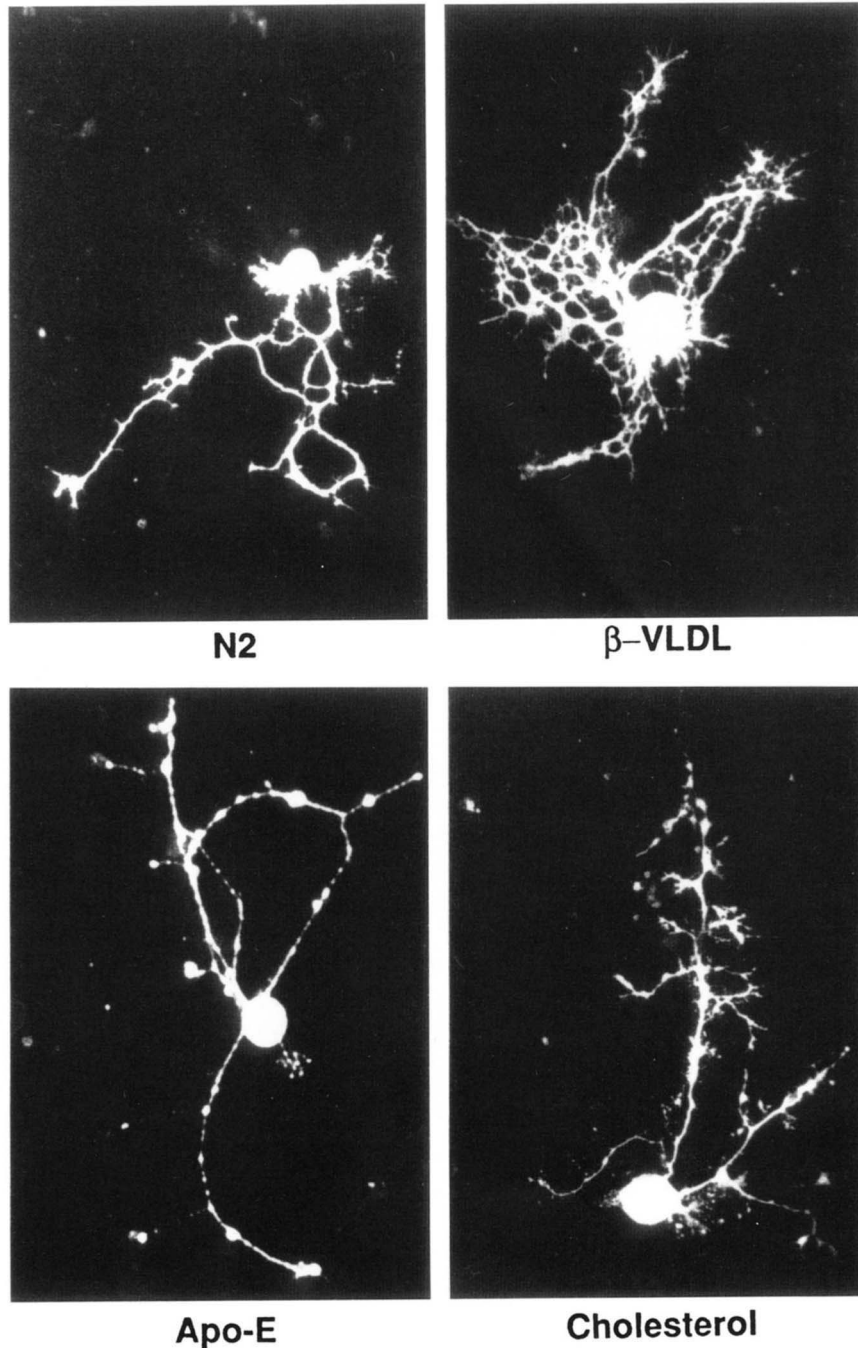


Fig. 2. The effects of β -VLDL, cholesterol, and apoE on the appearance of cultured dorsal root ganglion neurons grown on collagen. Note the extensive branching induced by β -VLDL and cholesterol (both at $66 \mu\text{g/ml}$), whereas branching was reduced by apoE ($66 \mu\text{g/ml}$). The neurons were stained by immunofluorescence using specific antibodies against the neuron-specific phosphoprotein, GAP-43. Neurons grown on collagen have a different appearance from those grown on extracellular matrix, probably because collagen does not support the same rate of neurite outgrowth as extracellular matrix.

of each reagent was the same. Because neurites grew more rapidly on the extracellular matrix, matrix-coated plates were used for all experiments.

The effect of β -VLDL, cholesterol, or apoE on neurite outgrowth from dorsal root ganglion neurons cultured on extracellular matrix is summarized in Fig. 3. Our measurements confirmed that β -VLDL, cholesterol, and apoE significantly affected neurite outgrowth (Fig. 3). The β -VLDL increased neurite outgrowth in two ways: by increasing the amount of neurite branching and by increasing the total combined length of the neurite processes extended by a single cell. Both increases were dose-dependent. At the highest concentration (66 μ g of cholesterol/ml), the combined neurite length increased to 170% of the control and neurite branching increased to 218% of the control (both significantly different from the control, $P < 0.05$). The β -VLDL did not affect the distance that these neurite processes extended away from the cell body (Fig. 3), or the number of neurites per neuron (data not shown).

Like β -VLDL, cholesterol significantly increased neurite branching, though cholesterol was less potent [142% of control for 66 μ g/ml (Fig. 3)]. Cholesterol did not significantly affect either the extension of these neurites away from the cell body or the total combined length of these neurites. Because cholesterol was added in a solution containing albumin, we also examined the effects of albumin added alone. Albumin (66 μ g/ml) had no effect on any of the measures of neurite growth (data not shown).

In contrast to β -VLDL or cholesterol, purified apoE decreased branching in a dose-dependent manner and also slightly decreased combined neurite length (Fig. 3). It did not significantly affect neurite extension or the number of neurites per neuron. Apolipoprotein E also appeared to decrease the adhesion of the neurons and neurites to the extracellular matrix, as neurons in cultures treated with apoE were easily washed off the substrate during the immunocytochemical procedures and had to be held in place by a layer of gelatin.

To determine what effect apoE might have on neurite outgrowth induced by β -VLDL or cholesterol, apoE was added to dorsal root ganglion cultures together with either β -VLDL or cholesterol (66 μ g/ml). The addition of apoE dramatically reduced the amount of neurite branching induced by β -VLDL or cholesterol and resulted in levels below that of the controls (Fig. 4). The combined length, however, changed little, and as a result, neurite extension away from the neuronal cell body was increased (Fig. 4).

Identification of receptors specific for apolipoprotein E-containing lipoproteins

Two types of receptors are known to bind β -VLDL: the LDL receptor and the LRP, which is identical to the receptor for α_2 -macroglobulin (21, 22). The LRP binds only β -VLDL that have been enriched in apoE (12, 23). To determine whether dorsal root ganglion neurons express these receptors and whether they may mediate the internalization of lipoproteins, we examined neurons by

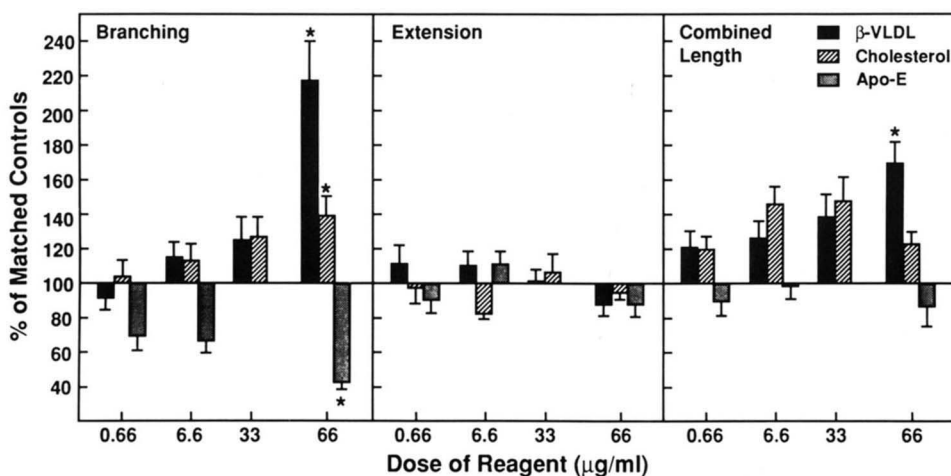


Fig. 3. The effects of various doses of β -VLDL, cholesterol, and apoE on neurite branching, extension, and combined length of cultured dorsal root ganglion neurons from fetal rabbits. The cells were grown on extracellular matrix and visualized by immunofluorescent staining with GAP-43 as shown in Fig. 1. β -Very low density lipoproteins and cholesterol increased branching in a dose-dependent manner, whereas apoE decreased branching. None of the reagents significantly affected neurite extension. β -Very low density lipoproteins significantly increased the combined length of the neurites at 66 μ g/ml. For each variable measured, the data were calculated as the percent difference between each treatment group and the matched control group for each batch. The percent differences for the different batches were then combined. Data are the mean \pm SEM; *, significantly different from the control group, i.e., 100%, $P < 0.05$.

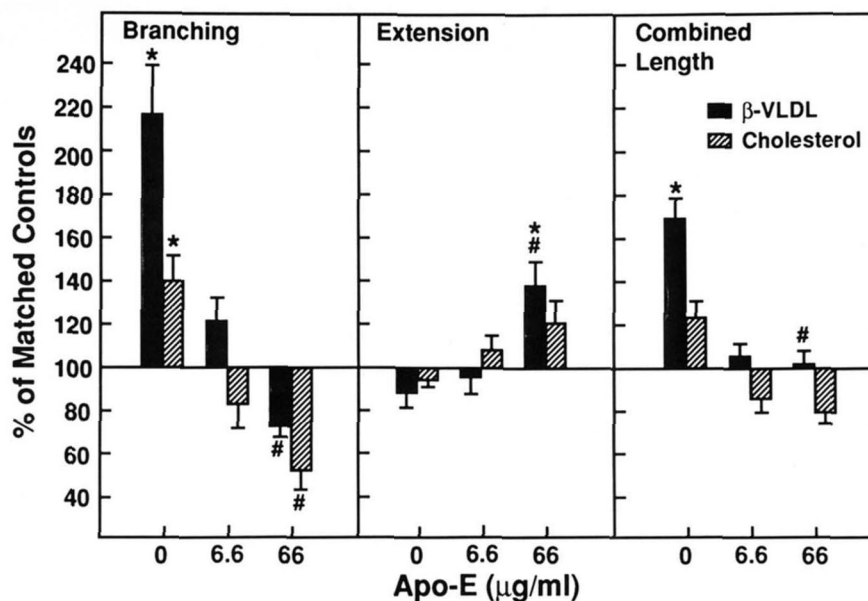


Fig. 4. The effects of various doses of apoE on neurite branching, extension, and combined length in the presence of β -VLDL or cholesterol (66 $\mu\text{g/ml}$). The cells were grown on extracellular matrix and visualized as in Fig. 1. Apolipoprotein E significantly decreased the neurite branching induced by β -VLDL and cholesterol, and also the combined neurite length induced by β -VLDL. In addition, it significantly increased neurite extension when added with β -VLDL. For each variable measured, the data were calculated as the percent difference between each treatment group and the matched control group for each batch. The percent differences for the different batches were then combined. Data are the mean \pm SEM; *, significantly different from the control group, i.e., 100%, $P < 0.05$; #, significantly different from 0 μg of apoE, $P < 0.05$.

immunocytochemistry using antibodies specific to each type of receptor. Immunoreactive LDL receptors were found on both neurons and the other cells of dorsal root ganglion cultures (Fig. 5A). On the neurons, the LDL receptors were distributed on both the cell bodies and the neurites. The LRP was also found on both neurons and other cells in the cultures. Unlike the LDL receptors, the LRP was detected only on the neuronal cell bodies, not on the neurites (Fig. 5B).

To confirm that the LRP is present in cells of the dorsal root ganglia, we isolated dorsal root ganglion membranes, subjected them to SDS-PAGE, and transferred the proteins to nitrocellulose. We then probed the blots with $^{45}\text{Ca}^{2+}$ and with both the monoclonal antibody used for immunocytochemistry and a rabbit antiserum that recognizes the denatured receptor. One of the calcium-binding proteins present in these membranes has a molecular weight of $\sim 600,000$ (Fig. 6), consistent with the properties of the LRP. The same protein is present in rat liver membranes, which are known to have a high concentration of LRP. Immunoblots indicated that the rabbit antiserum against denatured human LRP and the monoclonal antibody (data not shown) recognized the same protein in both fetal dorsal root ganglion and adult liver membranes (Fig. 7). The antibodies also recognized a second protein (molecular weight $\sim 115,000$) in the dorsal root ganglion membranes. This protein is probably a degradation product of LRP.

To demonstrate that the dorsal root ganglion neurons can bind and internalize β -VLDL and, therefore, that β -VLDL can deliver lipids to these cells, we studied the uptake of DiI-labeled β -VLDL by dorsal root ganglion cells grown in serum-free or serum-containing medium. Serum down-regulates the expression of the LDL receptors by many cells (24), but is not known to regulate the LRP (25). In our experiments, both neurons and glial cells internalized more DiI-labeled β -VLDL when grown in serum-free medium than in serum-containing medium (Fig. 8A), suggesting that at least part of the uptake was mediated by LDL receptors. The addition of apoE to the DiI-labeled β -VLDL, which was expected to promote binding by the LRP (12, 23), resulted in an increased internalization of β -VLDL by the cells. This increased internalization occurred both in serum-free and serum-containing medium. These data are consistent with a role for the LRP in the internalization of the apoE-enriched β -VLDL. The increased internalization that was induced by apoE was blocked by the addition of apoC-I, but not by the addition of apoC-III (Fig. 8B). These effects of apoC-I and apoC-III are also expected if β -VLDL bind to the LRP (11).

DISCUSSION

It has been hypothesized that apoE plays a role in peripheral nerve regeneration. Several findings have

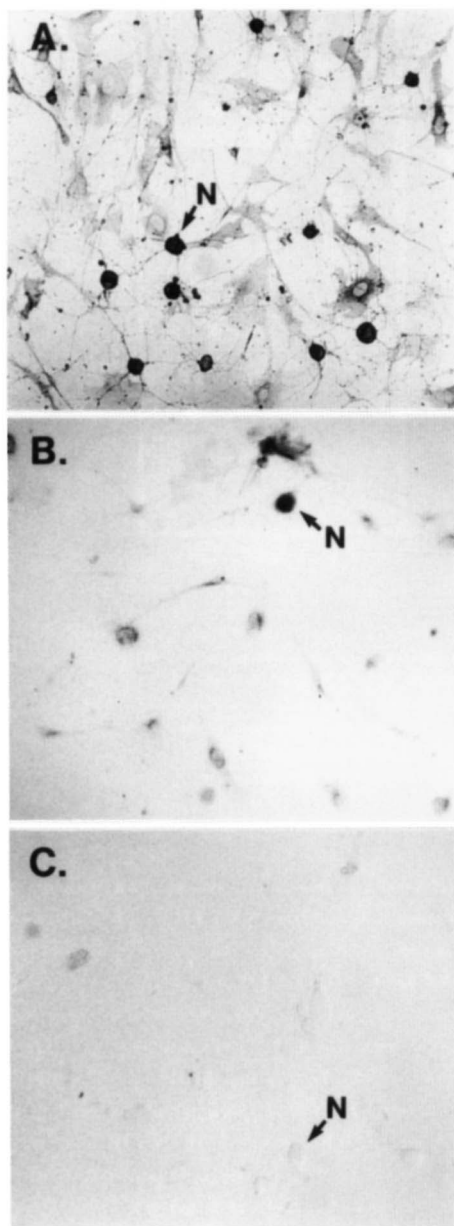


Fig. 5. Immunocytochemical detection of receptors for apoE-containing lipoproteins in cultures of dorsal root ganglion cells. A: Staining using a specific antiserum against rabbit LDL receptors and an ABC peroxidase reaction product to detect the binding of antiserum. All cell types in the culture appeared to be immunoreactive. On the neurons (N) the immunoreactivity, which was particularly intense, was located on both the cell bodies and neurites. B: Staining using specific antibodies against the LDL receptor-related protein (LRP). The neurons were more immunoreactive than other cell types, and very little immunoreactivity was present along the neurites. C: Staining using non-immune serum.

provided support for this hypothesis: the rapid induction of apoE synthesis after a denervating injury to the nerve, the massive accumulation of apoE-containing lipoproteins and free apoE in the regenerating nerve, and the expression of a receptor for apoE, the LDL receptor, on regenerating axons (4–8). The data from the present study pro-

vide the first direct evidence that apoE and apoE-containing lipoproteins can modulate the outgrowth of neuronal processes.

Using dorsal root ganglion cells in culture, we have provided experimental evidence that apoE plays two complementary roles in neurite outgrowth (shown schematically in Fig. 9). One role is to facilitate the receptor-mediated uptake of lipoproteins. Delivery of lipids to dorsal root ganglion cells via the uptake of lipoproteins results in an increase in the branching of neurites and in the total combined length of the neurite processes extended by a single cell. Specifically, we found that the incubation of dorsal root ganglion cells with β -VLDL, an apoE- and cholesterol-rich lipoprotein, increased the branching of neurites and increased the total combined length of neurite processes. Adding unesterified cholesterol alone to the culture medium had a similar, but less

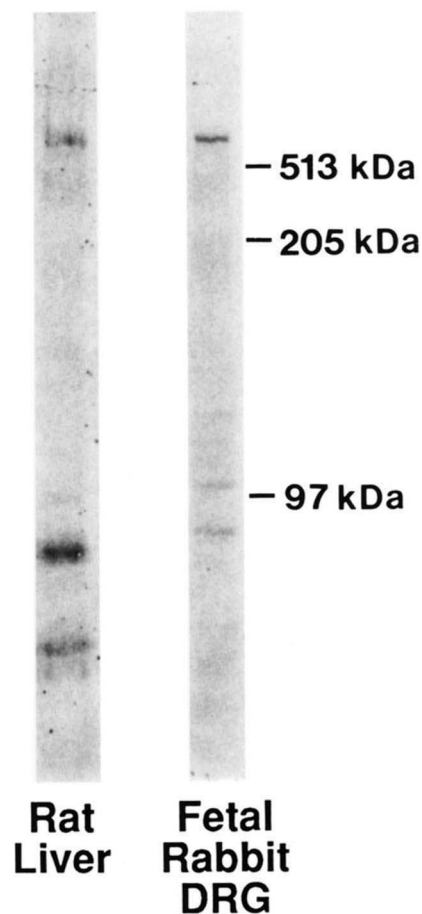


Fig. 6. The $^{45}\text{Ca}^{2+}$ blot showing the presence of a 600-kDa protein corresponding to the LRP in membranes isolated from fetal rabbit dorsal root ganglion (DRG) cells or from adult rat liver. The SDS-PAGE gradient gel (3.5–7.5%) was run under nonreducing conditions, electrophoretically transferred to nitrocellulose, and probed with $^{45}\text{Ca}^{2+}$ as described in Materials and Methods. The positions of the molecular weight markers (human apoB, myosin, and phosphorylase b) are indicated from top to bottom, respectively.

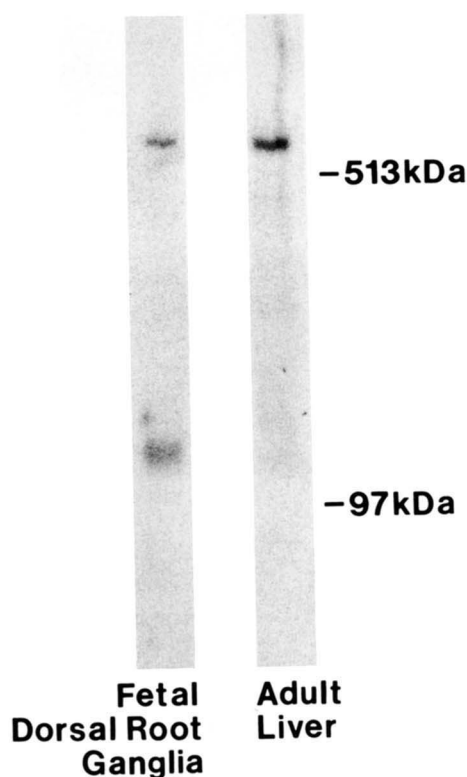


Fig. 7. Immunoblot demonstrating the presence of LRP in membranes isolated from fetal rabbit dorsal root ganglion cells or from adult rabbit liver. The SDS-PAGE gradient gel (3.5–7.5%) was run under nonreducing conditions, electrophoretically transferred to nitrocellulose, and probed with a specific polyclonal antibody against denatured LRP, as described in Materials and Methods. The positions for the molecular weight markers (human apoB and phosphorylase b) are indicated.

pronounced, effect. This smaller effect of cholesterol alone suggests that the receptor-mediated internalization of β -VLDL provides a more effective means of delivering cholesterol to the cells and that the availability of cholesterol for membrane production may be limiting for the projection of neurites. The possibility that other components of β -VLDL contribute to its effect on neurite outgrowth cannot be excluded.

Apolipoprotein E may serve as the major protein delivering cholesterol to neurons. We have demonstrated that two receptors that recognize apoE-containing lipoproteins, the LDL receptor and the LRP (which binds apoE-enriched lipoproteins), are both present on dorsal root ganglion neurons. In addition, both of these receptors appear to mediate the internalization of β -VLDL by dorsal root ganglion neurons. In addition, both of these receptors appear to mediate the internalization of β -VLDL by dorsal root ganglion neurons *in vitro*. The LDL receptor has already been implicated in the regrowth of injured axons in the regenerating sciatic nerve, where these receptors have been shown to accumulate (8). Whether the

LRP also accumulates in regenerating axons is unknown. However, because of the high concentration of apoE present in regenerating nerves (4–8), the LRP, which recognizes lipoproteins enriched in apoE, might be expected to play an important role in peripheral nerve regeneration.

The increase in neurite branching induced by β -VLDL or cholesterol was unexpected, and the mechanism by which these agents increase neurite branching remains unexplained. Presumably, it is the cholesterol of the β -VLDL that is the active agent. Cholesterol might directly or indirectly influence the membrane properties of neurites to increase neurite adhesion to the extracellular matrix, or induce an increase in the numbers of growth cones, both of which would influence neurite branching. In addition, our data suggest that the availability of cholesterol may limit neurite outgrowth.

It is also possible that the non-neuronal cells in our tissue cultures played a role in the β -VLDL-induced enhancement of neurite outgrowth. Glial cells and fibroblasts also internalized β -VLDL in our experiments. The β -VLDL, cholesterol, or apoE might regulate the secretion of factors by non-neuronal cells, which could in turn influence the growth and branching of neurites. In future experiments, we will explore the issue of whether the effects of β -VLDL, cholesterol, and apoE on neurite growth are due to direct influences on neurons, indirect influences on non-neuronal cells, or a combination of the two.

The second role that apoE may play in neuronal growth is to modulate the interaction of the neurite and the extracellular matrix by decreasing neurite adhesiveness. We found that the branching of neurites induced by β -VLDL or cholesterol in the culture medium was decreased by the addition of purified apoE, and that the extension of these neurites away from the neuronal cell body increased. Thus, although β -VLDL or cholesterol stimulated neurite outgrowth, the pattern of that outgrowth was altered by the presence of additional purified apoE. This decrease in the branching of neurites, coupled with an increase in the extension of these neurites induced by cholesterol or β -VLDL, may be explained by either of two possible effects of adding apoE to the culture medium. One possibility is that added apoE decreases branching by stimulating cholesterol efflux from neurons. Support for this possibility comes from experiments on fibroblasts in tissue culture. Adding purified apoE increased cholesterol efflux from these cells (26). However, this mechanism would not account for the increased extension of neurites away from the cell body that was observed when purified apoE was added together with cholesterol or β -VLDL, because none of these three agents alone affected neurite extension. A second, and perhaps better, explanation is that apoE modulates the interactions of neurites with extracellular

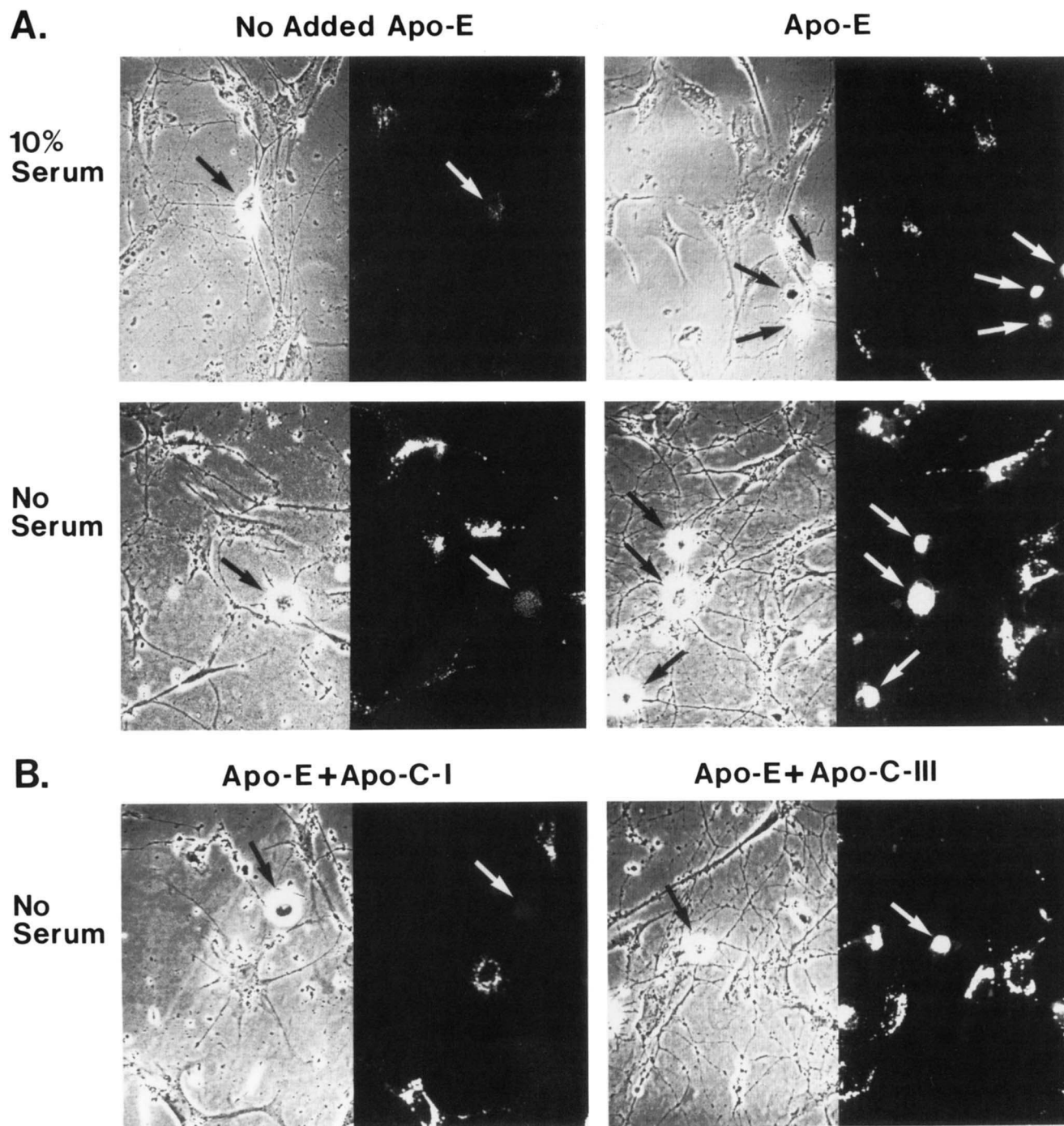


Fig. 8. Internalization of DiI-labeled β -VLDL in cultures of dorsal root ganglion cells. **A:** The cultures were grown in either N2 medium containing serum (top panels) or N2 medium alone (bottom panels). When the DiI-labeled β -VLDL were preincubated with apoE (right panels), the neurons (indicated by arrows) internalized more β -VLDL. The cells were photographed using a confocal laser scanning imaging system at a magnification of 160 \times . The non-neuronal cells internalizing DiI-labeled β -VLDL were primarily Schwann cells. **B:** The effects of apoC-I and apoC-III on the internalization of DiI-labeled β -VLDL. The increased internalization induced by apoE in neurons (indicated by arrows) was inhibited by apoC-I, but not by apoC-III.

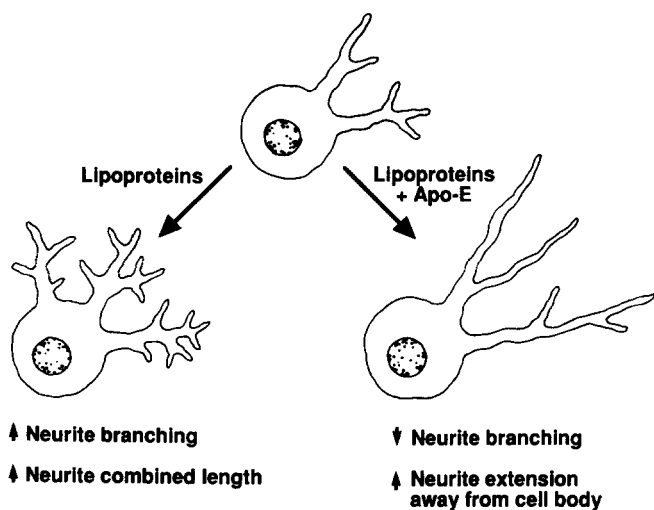


Fig. 9. Schematic representation of the effect of lipoproteins alone or of lipoproteins and added apoE on the outgrowth of neurites.

matrix by decreasing neurite adhesion to the substrate. This mechanism is suggested by the finding that apoE accumulates extracellularly within the injured sciatic nerve, where it may associate with extracellular matrix or cell-surface proteins (6, 27). This association might decrease the adhesion between the growing neurites and the extracellular matrix and help to direct neurite growth down Schwann cell tubes. In support of decreased adhesion of neurites in the presence of added apoE is our observation that both neurons and their neurites treated with apoE did not adhere to the substrate through the many washings of the immunocytochemical procedure unless held in place by a coat of gelatin. In a previous report (28), the adhesiveness of the substrate on which neurons are grown was shown to correlate with the degree of neurite branching: the more adhesive the substrate, the greater the branching of neurites. Neurons adhere more strongly to astrocytes than to fibroblasts, and their neurites are more highly branched on astrocytes. Apolipoprotein E, by decreasing the adhesion between the neurites and the extracellular matrix, may decrease neurite branching; therefore, when neurite outgrowth is stimulated, apoE may promote the increased extension of neurites away from the neuronal cell body. This combination of effects by apoE, receptor-mediated delivery of lipids and decreasing neurite adhesion, could facilitate rapid, target-directed axon growth in vivo. ■

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