Apolipoprotein E: Cholesterol Transport Protein with Expanding Role in Cell Biology

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Apolipoprotein E is a plasma protein that serves as a ligand for low density lipoprotein receptors and, through its interaction with these receptors, participates in the transport of cholesterol and other lipids among various cells of the body. A mutant form of apolipoprotein E that is defective in binding to low density lipoprotein receptors is associated with familial type III hyperlipoproteinemia, a genetic disorder characterized by elevated plasma cholesterol levels and accelerated coronary artery disease. Apolipoprotein E is synthesized in various organs, including liver, brain, spleen, and kidney, and is present in high concentrations in interstitial fluid, where it appears to participate in cholesterol redistribution from cells with excess cholesterol to those requiring cholesterol. Apolipoprotein E also appears to be involved in the repair response to tissue injury; for example, markedly increased amounts of apolipoprotein E are found at sites of peripheral nerve injury and regeneration. Other functions of apolipoprotein E, unrelated to lipid transport, are becoming known, including immunoregulation and modulation of cell growth and differentiation.

APOLIPOPROTEIN E (APO-E) IS ONE OF ALMOST A DOZEN protein constituents of plasma lipoproteins that serve various functions, including maintenance of the structure of the lipoprotein particles and regulation of the metabolism of several different lipoproteins [for review, see (*1, 2*)]. Apolipoprotein E, a protein with a relative molecular mass (M_r) of 34,000, is a constituent of liver-synthesized very low density lipoproteins (VLDL), which function primarily in the transport of triglyceride from the liver to peripheral tissues, and of a subclass of high density lipoproteins (HDL), which participate in cholesterol redistribution among cells. In addition, apo-E becomes a major protein constituent of intestinally synthesized chylomicrons, which transport dietary triglyceride and cholesterol. A major physiological role for apo-E in lipoprotein metabolism is its ability to mediate high-affinity binding of apo-E–containing lipoproteins to the low density lipoprotein (LDL) receptor, also referred to as the apo-B,E(LDL) receptor (*1, 3, 4*). Lipoprotein binding to the receptors initiates the cellular uptake and degradation of the lipoproteins, which leads to the use of the lipoprotein cholesterol in the regulation of intracellular cholesterol metabolism. Apolipoprotein E shares this function with apo-B, the protein constituent of plasma LDL. Furthermore, apo-E mediates the binding of chylomicron remnants to a second, postulated, hepatic receptor (the so-called chylomicron remnant or apo-E receptor) (*1, 4*). The precise mechanisms involved in the interaction of apo-E with the lipoprotein receptors and in the regulation of lipoprotein metabolism (and other metabolic roles) will be discussed later.

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Apolipoprotein E, initially termed the "arginine-rich apoprotein," was first identified as a lipoprotein constituent of VLDL in 1973 by Shore and Shore (*5*) and was extensively characterized in several animal species after it was realized that dietary cholesterol altered its distribution in plasma. Apolipoprotein E becomes a major protein constituent of several cholesterol-enriched lipoproteins that accumulate in the plasma of rabbits, dogs, swine, rats, and monkeys fed high levels of fat and cholesterol (*6*). It is now known that these cholesterol-enriched, apo-E–containing lipoproteins are chylomicron and VLDL remnants (referred to collectively as β-VLDL) and a subclass of HDL (referred to as HDL_1, HDL_c, or simply HDL–with apo-E) [for review, see (*7, 8*)]. As mentioned previously, apo-E is also present in chylomicrons, VLDL, and HDL in normolipidemic humans and is approximately equally distributed between VLDL and HDL in plasma that is devoid of chylomicrons (*9*). The normal plasma concentration of this protein is 5 mg/dl [for review, see (*1*)].

Considerable impetus to understanding the role of apo-E in lipoprotein metabolism came from the observation of Havel and Kane (*10*) that apo-E–enriched β-VLDL accumulate in the plasma of patients with type III hyperlipoproteinemia, a genetic disorder. This observation, in association with those obtained with cholesterol-fed animals, suggested that apo-E played a key role in cholesterol metabolism and specifically in the metabolism of chylomicrons and VLDL. When high levels of cholesterol are consumed, the alterations in lipoprotein metabolism leading to hypercholesterolemia are induced by lipoprotein overproduction and a secondary impairment of normal plasma clearance or catabolism, whereas in type III hyperlipoproteinemia the metabolic defect is secondary to the occurrence of an abnormal variant form of apo-E that binds poorly to the lipoprotein receptors (to be discussed in detail later).

Biochemical Characterization of Apolipoprotein E

Polymorphism. The polymorphic nature of apo-E was established by Utermann and his associates (*11*), using isoelectric focusing, and further clarified by Zannis and Breslow (*12*), using two-dimensional electrophoresis. The three major isoforms of apo-E, referred to as apo-E2, E3, and E4, are products of three alleles ($\epsilon 2$, $\epsilon 3$, $\epsilon 4$) at a single gene locus. Three homozygous phenotypes (apo-E2/2, E3/3, and E4/4) and three heterozygous phenotypes (apo-E3/2, E4/3, and E4/2) arise from the expression of any two of the three alleles. The distribution of the phenotypes in two populations is summarized

Table 1. Prevalence of apolipoprotein E phenotypes in two selected population studies that include a thousand or more individuals who were phenotyped.

Phenotype	Prevalence (%) by study	
	Utermann *et al.* (*11*)	Menzel *et al.* (*14*)
E4/4	3	2
E3/3	60	63
E2/2	1	1
E4/3	23	20
E4/2	2	3
E3/2	12	11
No. of subjects	1031	1000

in Table 1 (*11, 13, 14*) [for a clarification of the nomenclature concerning the polymorphic forms of apo-E, see (*15*)]. The most common phenotype is apo-E3/3 and the most common allele is $\epsilon 3$; therefore apo-E3 is considered to be the parent form of the protein, and apo-E4 and E2 are variants. Apolipoprotein E2 is the most common form of apo-E associated with type III hyperlipoproteinemia and is defective in receptor binding (*1, 13*). Apolipoprotein E4 displays normal binding but is associated with elevated plasma cholesterol and LDL [for review, see (*16*)].

The molecular basis for apo-E polymorphism was elucidated by analysis of the amino acid sequences of the three isoforms (*17*). Amino acid substitutions accounted for the differences among apo-E4, E3, and E2. Apolipoprotein E4 differs from apo-E3 in that in apo-E4 arginine is substituted for the normally occurring cysteine at amino acid residue 112. The most common form of apo-E2 differs from apo-E3 at residue 158, where cysteine is substituted for the normally occurring arginine. The charge differences among the three isoforms detected by isoelectric focusing is explained by the single amino acid substitutions. A secondary form of apo-E polymorphism is explained by posttranslational glycosylation (*15*). The glycosylated (sialylated) isoforms arise from the attachment of a carbohydrate moiety, which is variably sialylated, at a single site in apo-E (threonine residue 194) (*18*).

Gene regulation and biosynthesis. The apo-E gene occurs on chromosome 19 (*19, 20*) and is linked to another apolipoprotein, apo-C-I, and an apo-C-I pseudogene (*21*). The LDL receptor and apo-C-II have also been mapped to this chromosome (*22*), but apparently they are not closely linked to each other or to apo-E. The apo-E gene is 3.7 kilobases in length and contains four exons (*20, 23*). The promoter sequence TATAATT occurs approximately 30 base pairs (bp) upstream from the transcriptional initiation site. Other promoter and enhancer elements important in regulating apo-E biosynthesis have also been identified (*23*). The apo-E messenger RNA (mRNA) is 1163 bp in length (*24, 25*). The primary translation product is composed of 317 amino acids, with the 18 amino-terminal amino acids serving as a signal peptide. The mature apo-E is secreted as a 299–amino acid protein with a M_r of 34,200.

Sites of synthesis. Apolipoprotein E is produced in most organs. Significant quantities of apo-E mRNA are detected in the liver, brain, spleen, lung, adrenal, ovary, kidney, and muscle in several different species (*26–29*). One notable exception is the epithelium of the intestine, which does not appear to participate in apo-E production. The largest quantity of apo-E mRNA is found in the liver, which is the major source of apo-E, probably accounting for two-thirds to three-fourths of the plasma apo-E. The second largest concentration of apo-E mRNA is found in the brain (approximately one-third the level seen in the liver) (*26*). Several cell types within organs produce apo-E and thus are probably responsible for the mRNA seen in these organs. The participation of several cell types in the widespread production of apo-E indicates the importance of apo-E in lipid transport and possibly in roles unrelated to lipid transport.

Hepatic parenchymal cells are primarily responsible for apo-E production within the liver (*28*). It appears that apo-E is secreted from the hepatocytes primarily as a component of VLDL. However, it is possible that liver-synthesized apo-E is released independently of VLDL as discoidal particles that contain primarily phospholipids. The hepatic carcinoma cell line HepG2 has also been shown to synthesize and secrete apo-E (*30*).

Macrophages derived from the peritoneal cavity of mice or from human blood monocytes also produce large quantities of apo-E (*31–33*). Apolipoprotein E synthesis and secretion can be induced to very high levels by loading mouse peritoneal macrophages with cholesterol (*31*). In fact, under in vitro culture conditions, apo-E represents 5% to 10% of the newly synthesized proteins produced by the cholesterol-loaded macrophages. The apo-E is released from the cells in combination with phospholipid and occurs in the form of apo-E–phospholipid disks that float at a density of approximately 1.08 g/ml. Cholesterol can also be released from the macrophages; however, its release from the cell appears to be independent of apo-E secretion. The cholesterol is released to acceptors, such as HDL, in the extracellular fluid, and apo-E combines with these HDL to form HDL–with apo-E (*34, 35*). As will be discussed later, the HDL–with apo-E, which can interact with lipoprotein receptors by virtue of the presence of apo-E, participate in the redistribution of cholesterol to cells expressing LDL receptors.

Apolipoprotein E production can also be modulated by the state of activation of macrophages (*32, 33*). For example, endotoxin stimulation markedly decreases apo-E production (*33*). In addition to its role in lipid transport, the apo-E synthesized and secreted by macrophages may convey the state of activation or inactivation of the macrophages to other cells within the local environment. This concept will be expanded upon in considering possible immunoregulatory roles for apo-E. It is possible that macrophages are responsible for the apo-E mRNA seen in the spleen and lung. However, the involvement of other cell types in apo-E synthesis has not been excluded.

In the brain, astrocytes are the cell type responsible for producing apo-E (*36, 37*). These cells perform various functions in the brain, from providing a structural framework to regulating ion and metabolite concentrations. In addition, astrocytes in culture synthe-

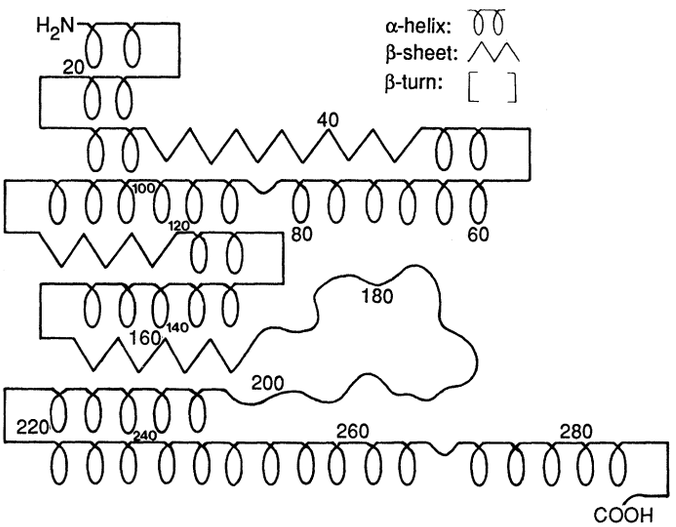

Fig. 1. Predicted secondary structure of apo-E, showing α-helices, β-sheet structure, and β-turns. The remainder of the molecule is predicted to have a random structure.

size and secrete large quantities of apo-E (*37*), whereas other glial cells and neurons produce none. It is noteworthy that apo-E is a major apolipoprotein of cerebrospinal fluid (CSF) in humans and dogs (*38*). Apolipoprotein E exists in the CSF as small spherical and discoidal lipoproteins that transport cholesterol and phospholipid. Unlike the plasma, in which apo-B–containing LDL are major lipoproteins involved in cholesterol transport, the CSF lacks apo-B and LDL; presumably apo-E assumes the major role for lipid transport in the CSF.

Smooth muscle cells obtained from the rhesus monkey or rat aorta and grown in culture also produce apo-E (*29, 39*). The apo-E represented 5% of the total protein secreted from these cells. Furthermore, several lines of evidence suggest that apo-E is involved in modulating smooth muscle cell proliferation and differentiation (*39*).

Structure. The predicted secondary structure of apo-E, based on the Chou-Fasman algorithm, is shown in Fig. 1. The α-helix, β-sheet, β-turn, and random structure are predicted to make up 62%, 9%, 11%, and 18% of the protein, respectively. The α-helical content has been determined experimentally for human and rabbit apo-E to be ~65% and ~70%, respectively (*40, 41*). An interesting structural feature of all the apolipoproteins is the amphipathic α-helix, characterized by one face composed of apolar residues and the other of polar residues (*42*). This structure has been postulated to be important in lipid binding (*42*). There are several such sequences within apo-E. The region with the strongest potential to be involved in lipid binding is the carboxyl-terminal third of apo-E, residues 202 to 299. However, other regions of the apo-E molecule have lipid-binding capability. Cyanogen bromide fragments, encompassing various regions of apo-E, all bind to phospholipid and float by ultracentrifugation (*43*).

An interesting feature of the apo-E structure can be easily appreciated by inspection of Fig. 1; a region of random structure appears to divide the apo-E molecule into two separate domains. The molecule is predicted to have a highly ordered structure throughout the amino-terminal half of the molecule. Then, beginning at about residue 165, a long stretch of predicted random structure extends for about 35 residues. The random structure is followed by a highly ordered structure representing approximately the carboxyl-terminal third of the molecule.

In fact, physicochemical studies have identified two distinct structural domains within the apo-E molecule (*41*). Limited enzymatic hydrolysis of apo-E in aqueous solution with a number of proteases, including thrombin, elastase, *Staphylococcus aureus* V8 protease, subtilisin, trypsin, and chymotrypsin, consistently resulted in the generation of two major classes of fragments. One class, extending from amino acid residues 20 to 165–180, represented an amino-terminal domain of apo-E. The second class of fragments, extending approximately from residues 225 to 299, represented the carboxyl-terminal domain of apo-E. These two regions of the apo-E molecule displayed strikingly different physical properties, as determined by guanidine-HCl denaturation. For example, an amino-terminal model domain (residues 1 to 191) behaved much more like a typical globular protein, having high free energy of denaturation. In contrast, a carboxyl-terminal model domain (residues 216 to 299) behaved more like a typical apolipoprotein, having low free energy of denaturation.

There is evidence that the two structural domains of apo-E actually represent functional domains (*41*). Hydrodynamic studies revealed that the tetrameric structure displayed by apo-E in aqueous solution was mediated through protein-protein interactions of the carboxyl-terminal domains. Furthermore, since the carboxyl-terminal domain contains the best examples of amphipathic α-helices, it very likely represents one of the major regions of apo-E mediating lipid binding. On the other hand, the amino-terminal 22-kilodalton (kD) thrombolytic fragment (residues 1 to 191) of the apo-E molecule contains the receptor binding domain, and the 22-kD fragment displays full binding to the LDL receptor. The 22-kD fragment of apo-E has recently been crystallized, and the three-dimensional structure of this region may be helpful in defining more precisely the nature of the interaction of apo-E with lipoprotein receptors (*44*).

The receptor binding domain of apo-E has been mapped in detail [for review, see (*1, 43, 45*)]. Initially, it was established that a limited number of arginine and lysine residues within apo-E (and also apo-B) were essential for binding to the LDL receptor. Selective chemical modification of either arginine or lysine residues completely inhibited apo-E (and apo-B) binding to the LDL receptors in vitro (*46*). Furthermore, modification of the arginine or lysine residues of either apo-E– or apo-B–containing lipoproteins markedly retarded their plasma clearance in vivo, further establishing the key role of these residues of the ligands in mediating specific lipoprotein catabolism via the LDL receptor pathway (*47*).

The specific amino acid residues in apo-E involved in mediating receptor binding have been identified by using four complementary experimental approaches: (i) identifying and sequencing natural apo-E mutants defective in receptor binding, (ii) generating apo-E fragments and testing their receptor binding activity, (iii) mapping the epitope of an apo-E monoclonal antibody that blocked binding of apo-E–containing lipoproteins, and (iv) producing site-directed mutant forms of apo-E.

As noted, apo-E variants associated with type III hyperlipoproteinemia do not bind normally to the lipoprotein receptors (*1, 8, 13, 48*). The most common variant is apo-E2(Arg^{158}→Cys), in which cysteine replaces the normally occurring arginine at residue 158 (Table 2). However, several other rare apo-E variants (usually apo-E2) associated with this disorder also bind defectively (Table 2) (*17, 24, 49, 50*). Sequencing showed that single amino acid substitutions in the defective mutants are clustered near residues 140 to 160 (Fig. 2), the middle of the molecule. In all of these natural mutants, neutral amino acids substitute for the basic residues arginine or

Table 2. Receptor binding activity of apolipoprotein E variants expressed as a percentage of the activity of plasma apo-E3 as compared with the natural variants, or of *E. coli*–produced apo-E3 as compared with the variants produced by site-directed mutagenesis, and calculated from the concentration required to displace 50% of the ^{125}I-labeled LDL from fibroblast LDL receptors. See (*1*) and (*53*) for a detailed discussion of these variants.

Substitution	Percent of normal receptor binding activity
Natural variants	
Cys^{112}→Arg (apo-E4)	100
Arg^{158}→Cys (most common apo-E2 variant)*	<2
Arg^{145}→Cys*	45
Lys^{146}→Gln*	40
Cys^{112}→Arg, Arg^{142}→Cys*	<20
Gly^{127}→Asp, Arg^{158}→Cys*	4
Ala^{99}→Thr, Ala^{152}→Pro	Unknown
Site-directed mutagenesis variants	
Arg^{136}→Ser†	40
His^{140}→Ala	52
Lys^{143}→Ala	9
Leu^{144}→Pro	13
Arg^{150}→Ala	24
Ala^{152}→Pro‡	27
Ser^{139}→Arg, Leu^{149}→Ala	154

*The defect is associated with type III hyperlipoproteinemia (the presence of β-VLDL in the plasma). †The 136 variant has been seen in a patient with type III hyperlipoproteinemia (*98*). ‡The 152 variant has been seen in a family with hyperlipidemia (*24*).

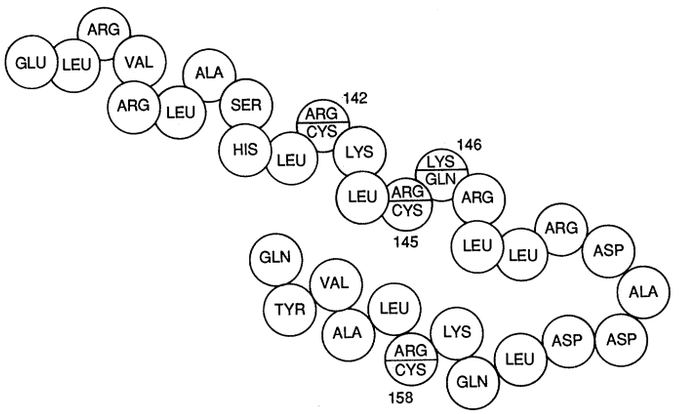

Fig. 2. Diagram of amino acid residues of apo-E in the vicinity of the receptor binding domain. Residues in the vicinity of 140 to 160 constitute the site responsible for the binding of apo-E to lipoprotein receptors. Single natural amino acid substitutions known to affect binding occur at residues 142, 145, 146, and 158. [From (*48*), courtesy of The New York Academy of Sciences]

LDL receptor (consensus) -Cys-Asp-X-X-X-Asp-Cys-X-Asp-Gly-Ser-Asp-Glu-

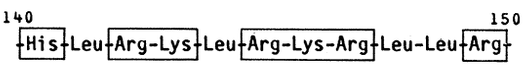

Fig. 3. Sequences postulated to mediate receptor-ligand interaction. Basic amino acid residues of apo-E that may be involved in mediating binding to the receptor are within the boxes. An ionic interaction between the basic residues of apo-E and the acidic residues (aspartic and glutamic acids) of the receptor is postulated.

lysine in this region of the molecule. These data focused our attention on this region of apo-E as being the putative receptor binding domain.

In a second series of studies, apo-E was cleaved into smaller fragments by two different procedures: thrombin and cyanogen bromide (*43*). Thrombin produced two major fragments—the amino-terminal residues 1 to 191 (22 kD) and the carboxyl-terminal residues 216 to 299 (10 kD). The amino-terminal fragment possessed full receptor binding activity, whereas the carboxyl-terminal fragment had none. The only cyanogen bromide fragment with receptor binding activity encompassed residues 126 to 218 (*43*). A third line of evidence also highlighted this same region of apo-E. The epitope of a monoclonal antibody to apo-E that blocked receptor binding was localized to residues 140 to 150 (*45*).

The receptor binding region of apo-E (Fig. 2) is rich in basic amino acids; only here do the arginine and lysine residues occur in doublets and triplets. The region from residue 131 to 150, predicted to be an α-helix, contains three sites at which substitutions disrupt receptor binding; hence the α-helix residues are thought to be directly involved in mediating binding. Residues 151 to 154 are predicted to form a β-turn; they are followed by a β-sheet encompassing residues 155 to 164. The variant at residue 158 appears to reduce binding by altering the local molecular conformation (*51*).

The role of other specific amino acid residues in receptor binding has been elucidated by a fourth approach, site-directed mutagenesis. Recombinant techniques can be used to produce an apo-E3 in *Escherichia coli* that displays normal binding and plasma clearance (*52*); therefore, these techniques were used to produce apo-E mutants with alterations at specific sites in this region to examine the effect of other basic residues and of local conformation on receptor binding (*53*). Table 2 lists the mutant forms of apo-E that were produced; their binding activity is compared with that of the recombinant apo-E3 (which is identical to plasma apo-E3). Basic amino acids converted to neutral residues reduced binding to approximately 10% to 50% of normal, about the same range as is obtained with the naturally occurring variants (Table 2). The substitution of proline for leucine-144 or for alanine-152 decreased receptor binding significantly (Table 2), probably by altering the conformation of the α-helix or β-turn, respectively (see Fig. 1). However, not all substitutions in this region reduce receptor binding. A double mutation (at both residues 139 and 149) not only failed to decrease receptor binding activity, it apparently increased binding (Table 2) (*53*).

The remarkable consistency of all the foregoing data indicates that the basic amino acids arginine and lysine (and histidine) in the vicinity of residues 140 to 160 are important in mediating the binding of apo-E to the LDL receptor. The molecular conformation in this region is important for normal receptor binding, but substitutions outside this immediate region also could have an effect on the binding domain by altering the conformation of the protein. Additional mutants created by alteration of specific residues will be used to test this postulate. However, as shown in Fig. 3, the receptor binding of apo-E may represent an ionic interaction between the basic residues in this region and acidic residues of the postulated ligand binding domain of the LDL receptor (*3, 54*).

The key role that apo-E plays in normal lipoprotein metabolism is highlighted by the association between the abnormal apo-E2 and type III hyperlipoproteinemia. Type III hyperlipoproteinemia has also been seen in a kindred who have little or no apo-E (*55*). The molecular defect is a mutation in the acceptor splice site of the third intron of the apo-E gene that prevents production of a normal apo-E mRNA, which in turn prevents protein synthesis (*56*).

Metabolic Pathways Involving Apolipoprotein E

The various metabolic pathways involving apo-E can be divided into three different categories. Two of these include very well established pathways that involve lipid transport functions of apo-E [for review, see (*57*)], whereas the third is much more speculative and apparently involves mechanisms other than, or in addition to, the lipid transport function.

1) Redistribution of lipids among cells of different organs. Apolipoprotein E has an "endocrine-like" function in that it is synthesized in one or more sites and participates in the transport of cholesterol and other lipids from sites of lipid synthesis or absorption to cells of other organs where the lipid is used, stored, or excreted. Examples of this type of function for apo-E include the metabolism of chylomicrons and chylomicron remnants, VLDL and VLDL remnants, and a subclass of HDL (HDL–with apo-E).

2) Redistribution of lipids among cells within an organ or tissue. Apolipoprotein E also has a "paracrine-like" function, being produced by various cells within a tissue and participating in the transport of cholesterol and other lipids from one cell to another. As a general mechanism, macrophages or other cells capable of storing and releasing lipids to acceptors in the interstitial fluid (apo-E–lipid complexes or HDL) play a key role in this redistribution. Apolipoprotein E, because of its ability to bind with high affinity to LDL receptors, targets the lipid complexes or HDL–with apo-E for uptake by cells in the local environment. The cholesterol can be used by the cells for biosynthetic processes, including membrane repair or cell proliferation. In the macrophages that synthesize and secrete apo-E, the apo-E may have an "autocrine-like" function. The

secreted apo-E captures lipids from the environment and delivers them back to the macrophage, where they are stored. An excellent example of the involvement of apo-E in autocrine-like and paracrine-like functions is in peripheral nerve injury and regeneration. However, it is likely that this mechanism is applicable generally in local areas of tissue injury and repair and may also be involved in the progression and regression of atherosclerotic lesions (classically described as an injury-repair response in the artery wall).

3) Functions of apolipoprotein E unrelated to lipid transport. As will be discussed, apo-E inhibits mitogenic stimulation of lymphocytes by binding to specific sites on the surface of the lymphocytes. These observations suggest that apo-E could have a broader role in immunology than is now understood. In addition, apo-E produced by smooth muscle cells may be involved in modulating cell proliferation and differentiation.

Apolipoprotein E in lipid redistribution among cells of different organs.

1) Transport of dietary lipids from the intestine to the liver and peripheral tissues. Chylomicrons synthesized by the intestine in response to dietary fat and cholesterol enter the mesenteric and thoracic duct lymph, where they acquire apo-E (Fig. 4) [for review, see (*57, 58*)]. In the plasma, lipoprotein lipase catalyzes the hydrolysis of the triglyceride of the chylomicrons and the free fatty acids generated are taken up primarily by adipocytes, where they are stored in triglyceride droplets. Upon hydrolysis of the triglyceride, the chylomicrons become smaller and more enriched in cholesterol and are referred to as chylomicron remnants. Chylomicron remnants are rapidly cleared from the plasma by the liver, where the cholesterol is either used in membrane or lipoprotein biosynthesis or excreted as free cholesterol or bile acids.

Apolipoprotein E is responsible for mediating the uptake of the chylomicron remnants [for review, see (*1, 4*)]. There appears to be a unique receptor [distinct from the apo-B,E(LDL) receptor] on hepatocytes capable of mediating the uptake of chylomicron remnants. This putative receptor has been referred to as the chylomicron remnant or apo-E receptor (*1, 4*). There are several reasons for postulating the existence of this receptor; one reason is that individuals with familial hypercholesterolemia—that is, patients with absent or defective LDL receptors—are capable of clearing chylomicron remnants from their plasma (*59*). This does not mean that the LDL receptor does not normally play a role in chylomicron remnant uptake. In vitro, chylomicron remnants do, in fact, bind with high affinity to the LDL receptors. However, liver membranes have distinct apo-E–binding proteins (unrelated to the LDL receptor) (*60, 61*). It remains to be determined whether they represent physiologically important proteins mediating remnant binding, uptake, or both, by the liver in vivo.

Chylomicron remnant uptake by the liver may be a multistep process involving the binding of chylomicron remnants in the space of Disse and their subsequent uptake by hepatocytes (*61*). Further hydrolysis of lipids and their transfer into hepatocytes may even occur while the particles reside in the space of Disse. As is evident from this discussion, the precise mechanism for chylomicron remnant uptake needs to be elucidated; however, it is known that the uptake of chylomicron remnants is mediated by apo-E.

2) Transport of lipids from the liver to peripheral tissues. Very low density lipoproteins are triglyceride-rich particles containing apo-E and apo-B100 that are synthesized and secreted from hepatocytes (Fig. 4) [for review, see (*57*)]. In the plasma they are acted upon by lipoprotein lipase, which catalyzes the release of free fatty acids. Like chylomicrons, the VLDL become progressively smaller and more cholesterol-enriched. These are the VLDL remnants that progress through a conversion cascade to become intermediate density lipoproteins (IDL) and finally to become LDL, the final step in the cascade. However, during this lipolytic processing, a fraction

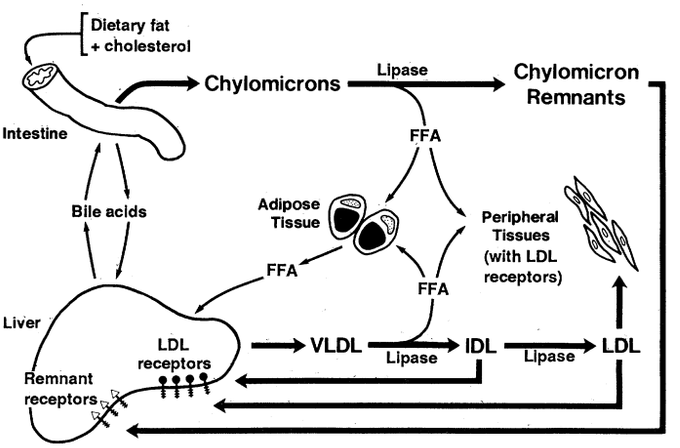

Fig. 4. General scheme summarizing the metabolism of chylomicrons, VLDL, IDL, LDL, and chylomicron remnants (FFA, free fatty acids). [From (*58*), courtesy of J. B. Lippincott Company]

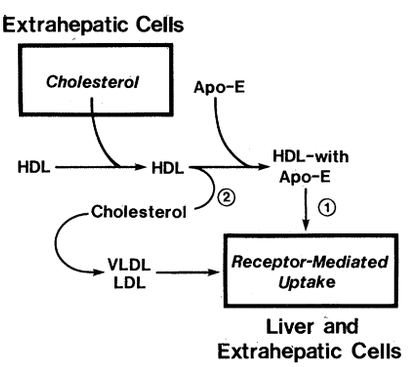

Fig. 5. Scheme showing the role of HDL in acquiring cholesterol from cells containing excess cholesterol and redistributing it to cells requiring cholesterol for steroid hormone production and membrane biosynthesis or to the liver for elimination from the body. ① The presence of apo-E on the HDL targets these lipoproteins to cells with lipoprotein receptors. ② Alternatively, the cholesterol on HDL may be transferred to other lipoproteins, which are then taken up by receptor-mediated endocytosis. [From (*8*), courtesy of *Circulation*]

of the remnants also are cleared from the plasma via LDL receptors. The cellular uptake of the VLDL remnants and IDL is mediated primarily by apo-E. As the particles become more LDL-like (progressively smaller and more cholesterol-rich), they lose their apo-E, and apo-B100 becomes the ligand responsible for LDL binding to the LDL receptors.

3) Transport of cholesterol from peripheral tissues to the liver. Elimination of cholesterol from the body occurs primarily through the liver via bile formation [for review, see (*57*)]. Therefore, it is essential that cholesterol in peripheral tissues be transported back to the liver. A process referred to as reverse cholesterol transport was postulated several years ago and was envisioned to involve HDL as the means of transport (*62*). This process is diagrammed schematically in Fig. 5.

Reverse cholesterol transport has several key elements [for review, see (*1, 7, 63*)]. Cholesterol-loaded cells, such as macrophages, can release their cholesterol to acceptors in the interstitial fluid. An avid cholesterol acceptor is HDL, especially the phospholipid-rich, non-apo-E–containing HDL (HDL–without apo-E). As the HDL become cholesterol-enriched, they acquire apo-E. As noted, apo-E is available in the interstitial fluid as a product secreted by various cell types, including macrophages, smooth muscle cells, and others, and has an avidity for HDL as they become cholesterol-enriched. As shown by in vitro studies, the presence of apo-E actually facilitates the acquisition of cholesterol by HDL (*34, 35*). In fact, when excess apo-E is added to a system that has a source of cholesterol plus HDL and an enzyme that esterifies cholesterol (lecithin:cholesterol acyl-

transferase), the HDL become very cholesterol-enriched, their diameter doubling in the process (*34*). The cholesteryl esters are arranged in concentric layers in the core of the particle (*64*), and apparently there is an increase in the free cholesterol content of the surface of the HDL particles. Such particles have been shown to bind avidly to LDL receptors by virtue of the presence of apo-E on the HDL (*35*). Typical HDL lacking apo-E do not bind to the LDL receptors.

Thus, the cholesterol-rich HDL–with apo-E formed in the interstitial fluid of various tissues could transport cholesterol from peripheral tissues to the liver, where they are taken up by LDL receptors (Fig. 5). Such cholesterol-rich HDL–with apo-E are abundant in the plasma of many animals, including rats, dogs, and swine [for review, see (*7, 8, 63*)], and have been shown to be present in the interstitial fluid (peripheral lymph) in dogs (*65*). In humans, HDL–with apo-E are present in lower concentrations (*66*), and reverse cholesterol transport appears to involve an additional process. Humans and certain animals, such as rabbits, have the cholesteryl ester transfer protein, which transfers cholesteryl esters from one lipoprotein to another (*67*). It is envisioned that HDL cholesteryl esters are transferred to lower density lipoproteins, such as VLDL, IDL, or LDL, which then are taken up by the liver. However, it is likely that even in humans some proportion of reverse cholesterol transport occurs via HDL–with apo-E delivery of cholesterol directly to the liver.

Apolipoprotein E in lipid redistribution among cells within an organ or tissue. It is easy to envision how the HDL–with apo-E or apo-E–lipid complexes could function in the delivery of cholesterol to cells in the local environment where they are formed. Thus, apo-E could participate in the redistribution of cholesterol from cells with excess cholesterol to others requiring cholesterol. Cells requiring cholesterol can express high levels of LDL receptors and take up the apo-E–containing particles.

An interesting model involving the coordinated storage and redistribution of cholesterol among cells of injured and regenerating peripheral nerves has been described (*68–70*). The role of apo-E in this process is shown diagrammatically in Fig. 6. After a crush or cut injury to the rat sciatic nerve, apo-E is produced and accumulates to levels 100- to 200-fold greater than that in uninjured nerve. The extracellular apo-E can attain levels representing 5% of the total soluble protein in the regenerating nerve segment. The production of apo-E peaks by 7 to 10 days after injury and slowly returns to baseline levels by 8 weeks, when regeneration of the rat sciatic nerve is largely complete.

The cell type responsible for the production of apo-E in this situation is the macrophage (*70*). Immediately after injury, resident macrophages within the sciatic nerve begin secreting apo-E. In addition, as part of the inflammatory reaction, monocytes rapidly enter the site of injury, become macrophages, and begin producing apo-E. Axon degeneration and myelin destruction proceed rapidly. Most of the cholesterol, and possibly other key lipids, are retained within the area of degeneration and accumulate within Schwann cells and macrophages. It is likely that the apo-E produced within the lesion scavenges cholesterol from the cellular and myelin debris and delivers the lipids to the macrophages, where they are stored.

Within 1 or 2 days, regeneration begins with the proximal stump of the nerve sending out numerous sprouts (neurites); usually, only one will survive and become a regenerated axon. The tips of the neurites express high levels of LDL receptors (*70*). As the regenerating axons grow down the neurolemmal tube, they continue to express high levels of LDL receptors on their growing tips (*70*). Presumably the receptors participate in taking up cholesterol to be used in membrane biosynthesis. Cholesterol may be provided by local macrophages that are loaded with lipids or from cellular debris. It is likely that apo-E–lipid complexes present in the interstitial fluid deliver cholesterol to the neurites via this receptor-mediated process. Apolipoprotein E–lipid complexes have been isolated from the injured nerve segment, and they are capable of interacting with LDL receptors of neurites on PC12 cells (a pheochromocytoma cell line) maintained in culture (*71*). The growing tips of the PC12 cells have LDL receptors that bind and internalize HDL–with apo-E–lipid complexes.

Within 1 to 2 weeks, remyelinization of the axons by the Schwann cells begins. The lipids stored in the Schwann cells are depleted, and then the Schwann cells begin expressing LDL receptors (*70*), presumably to mediate the uptake of cholesterol for myelin formation. It is likely that the macrophage cholesterol is released to the apo-E–lipid complexes or to the HDL in the interstitial fluid and that apo-E mediates the uptake of the cholesterol via the LDL receptors on the Schwann cells.

The nerve model may describe a more general process that occurs to a greater or lesser extent in various tissues in response to injury and repair. Teleologically speaking, it would appear that nature has gone to great lengths to develop a system for capturing and storing cholesterol (and possibly other lipids) and for redistributing it to cells requiring cholesterol for biosynthesis. Apolipoprotein E and the LDL receptor play key roles in this process.

Functions of apolipoprotein E unrelated to lipid transport. Apolipoprotein E and peripheral nerve regeneration. The correlation between the extremely high levels of apo-E production by macrophages in the distal stump of the sciatic nerve and the expression of LDL receptors on the growing tips of the neurites and the Schwann cells strongly suggests that one of the roles of apo-E in nerve regeneration is lipid transport. More speculative roles for apo-E in nerve regeneration, unrelated to lipid transport, include the possibility that apo-E could be a neurotrophic or neurotropic factor involved in one of several events required for nerve survival and

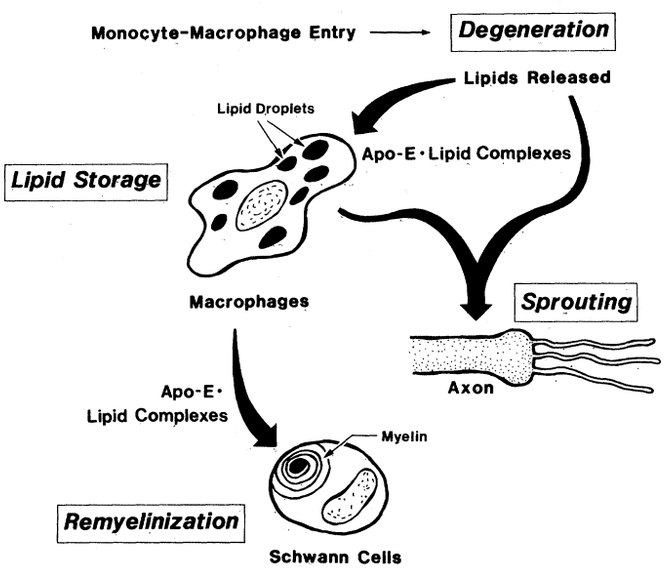

Fig. 6. Scheme showing the role of apo-E in the redistribution of lipids among cells within the injured sciatic nerve. After injury (crush or cut), monocyte-macrophages enter the nerve and degeneration begins. Lipids (especially cholesterol) are stored within macrophages. Some of this cholesterol is derived from the degenerating tissue and may be delivered to the macrophages via apo-E–mediated uptake. Sprouts rapidly begin to appear, and they express LDL receptors on their growing tips (presumably to acquire cholesterol for membrane biosynthesis). Later, remyelinization of the regenerated axon by Schwann cells occurs. The Schwann cells express LDL receptors during this phase of regeneration. Apolipoprotein E–lipid complexes or HDL–with apo-E appears to participate in the redistribution of the cholesterol to the Schwann cells and the sprouting neurites.

repair. One possible role may relate to the high level of apo-E apparently localized to the extracellular matrix after nerve injury (*70*). It was shown earlier that heparin-binding growth factors (for example, the basic fibroblast growth factor) stimulate neurite extension in the nerve cell line PC12 (*72*). This effect may be mediated, at least in part, by the binding of the factor to the heparin-like extracellular matrix (glycosaminoglycans) and by influencing the way the cells interact with the matrix (*72*). Increased adhesion of cells to the matrix accelerates cell growth and division (*73*). In fact, neurons use heparin binding sites in matrix proteins for adhesion and axon extension (*74*). Apolipoprotein E, which is also a heparin-binding protein (*75*), could promote cell-matrix interactions and stimulate axon extension. On the other hand, the binding of apo-E to extracellular glycosaminoglycans could alter the interaction of various growth factors with the matrix. It is possible that apo-E displaces the growth factors from the matrix, thus modulating their mitogenic activity. Clearly, it is possible to envision other mechanisms whereby apo-E could have roles unrelated to lipid transport.

Apolipoprotein E and smooth muscle cell proliferation and differentiation. Modulation of smooth muscle cell proliferation and migration is of particular importance in arterial wall pathophysiology (*76*). For example, a characteristic of atherosclerotic vascular disease is the proliferation and migration of smooth muscle cells into the intima, where the disease process occurs. Various factors regulating these activities, including matrix glycoproteins, glycosaminoglycans, and growth factors, are produced by cells (including smooth muscle cells, endothelial cells, and macrophages) within the local environment, and these factors may modulate the phenotype of the smooth muscle cells [for review, see (*77*)]. For example, heparin and heparin-like glycosaminoglycans, added exogenously or produced by cells within the artery wall, are potent inhibitors of smooth muscle cell proliferation and motility (*78*). Majack *et al.* (*79*) and Cochran *et al.* (*80*) analyzed the changes in protein production by smooth muscle cells after treatment of the cells with heparin in vitro. Majack *et al.* (*39*) identified a protein of M_r 35,000 to 38,000 that accumulated in the culture medium as apo-E. The apo-E accumulates in the medium, apparently bound to the heparin (*39*).

Although this is speculative, apo-E could play a role in smooth muscle cell biology because of its ability to bind to heparin (*75*) and heparin-like glycosaminoglycans (*81*) that are present in the matrix of the arterial wall. The formation of apo-E–heparin-like complexes could modify the interaction of smooth muscle cells with the matrix. Such complexes could enhance cell-matrix interaction, with apo-E bridging the ground substance and the cells and thus stimulating cell proliferation, mobility, or both. On the other hand, apo-E could interfere with normal cell-matrix interaction and inhibit these processes. Furthermore, as discussed above with respect to neurite extension, it is possible that apo-E could modify the activity of certain growth factors by competing with them for binding to heparin-like glycosaminoglycans in the matrix. These represent testable hypotheses.

Apolipoprotein E production may also participate in smooth muscle cell differentiation. Apolipoprotein E synthesis and secretion are enhanced in growth-arrested smooth muscle cells maintained in 0.5% fetal calf serum (*39*). These quiescent cells continue to produce elevated apo-E even after they are provided with 10% fetal calf serum or platelet-derived growth factor. Owens *et al.* (*82*) showed that growth arrest promotes differentiation of smooth muscle cells in vitro. Furthermore, in studies that may be applicable in ascertaining a role for apo-E in smooth muscle cell differentiation, Millis *et al.* (*83*) demonstrated that there is a marked increase in the production of an unidentified protein with M_r ≈38,000 when smooth muscle cells progress from a monolayer culture to form multiple layers of differentiated cells (a so-called nodular culture). This protein, which binds to heparin, is increased 15- to 30-fold in the medium during smooth muscle cell nodulation. While further data are clearly needed to establish that the protein of M_r ≈38,000 observed in the studies of Millis *et al.* (*83*) is apo-E, the studies of Majack *et al.* (*39*) clearly demonstrated the increased production of apo-E in growth-arrested smooth muscle cells. Therefore, enhanced synthesis of apo-E could occur as a consequence of smooth muscle cell differentiation and be a phenotypic marker (or possibly a mediator) of such differentiation.

Apolipoprotein E and immunoregulation. A variety of cells (including various classes of lymphocytes and monocyte-macrophages) and several different soluble factors (such as lymphokines, monokines, and immunoglobulins) are key elements of the immune system and interact to modulate immunoreactivity. One class of macromolecules that has immunoregulatory activity includes specific lipoproteins [for review, see (*1, 84*)]. Low density lipoproteins, as well as apo-E–containing lipoproteins, have the capacity to inhibit or stimulate antigen- and mitogen-induced T lymphocyte activation and proliferation. These opposing effects appear to be mediated by the interaction of lipoproteins with two different cell-surface receptors or binding sites—namely, the LDL receptor and the so-called immunosuppressive receptor. The metabolic state of the cell may determine which of the two receptors and which of the two responses are expressed by the lymphocytes.

Lymphocytes express LDL receptors (*85*), and Cuthbert and Lipsky (*86*) showed that at low concentrations lipoprotein binding to the LDL receptor resulted in an enhanced responsiveness of lymphocytes to mitogens (determined by thymidine incorporation in phytohemagglutinin-stimulated lymphocytes). The proliferative effect appears to be due to the delivery of fatty acids and other nutrients to the lymphocytes via the LDL receptor pathway (*87*).

In contrast, the binding of the lipoproteins to the immunosuppressive receptor or binding site on the lymphocytes renders the lymphocytes resistant to mitogenic stimulation and inhibits early transformation events [such as phosphatidylinositol turnover, calcium uptake, and cyclic nucleotide metabolism (*88*)] that are required for lymphocyte activation. Curtiss and Edgington (*89, 90*) first demonstrated that a subclass of LDL containing both apo-E and apo-B100 (LDL-In) could suppress lymphocyte proliferation and activation. Furthermore, Curtiss and associates demonstrated that removal of the neutral lipids does not alter the activity of the LDL-In (*91*). These studies were extended by Hui *et al.* (*92*), who showed that inhibition was mediated by apo-E–containing lipoproteins and to a lesser extent by apo-B–containing LDL binding to specific low-affinity sites on the surface of the lymphocytes. The binding of the lipoproteins to the lymphocyte binding site seems to be sufficient for inhibition; internalization of the lipoproteins is not required (*93*). Inhibition was achieved only in the presence of high concentrations of the lipoproteins, and the inhibitory effects could be readily reversed by the addition of transferrin to the incubation medium (*94*). The role of the transferrin is unclear; however, it does appear that the inhibitory effects of the lipoproteins are independent of the LDL receptor (*86*).

The nature and specificity of the putative immunosuppressive receptors or binding sites remain unclear. They resemble the LDL receptors on fibroblasts in a number of ways. The binding sites on lymphocytes are specific for apo-B– and apo-E–containing lipoproteins (*92*), are calcium-dependent, and are Pronase-sensitive (*90*). On the other hand, the interaction of lipoproteins with the immunoregulatory receptors is of low affinity [dissociation constant (K_d) = $2 \times 10^{-7} M$ and $9 \times 10^{-8} M$ for LDL and apo-E HDL_c, respectively], while the binding of these lipoproteins to LDL receptors is of high affinity (*92*). Furthermore, lymphocytes from

patients with homozygous familial hypercholesterolemia, which lack or have defective LDL receptors, are also subject to lipoprotein regulation by mitogenic stimuli (*86, 90, 95*). However, it is now known that a variety of mutations affect LDL receptors; all mutations result in defective LDL binding, whereas certain mutant receptors are still capable of binding apo-E–containing lipoproteins with high affinity (*96*). In addition, apo-E isolated from patients with type III hyperlipoproteinemia is as capable of immunosuppression as normal apo-E (*97*), whereas the mutant apo-E binds poorly to the LDL receptor. These earlier studies need to be extended with apo-E in which the precise mutation has been defined and with defective cells in which the receptor defect is known.

It is difficult, given our present state of knowledge, to develop a scheme demonstrating how apo-E (as well as LDL) might be involved in modulating the immune function. Two sets of facts undoubtedly provide clues and need to be considered further. First, apo-E–containing lipoproteins and LDL bind to specific sites on the surface of lymphocytes and modulate various intracellular events. Second, macrophages, an additional cellular component of the immune response, can produce large quantities of apo-E. Furthermore, macrophage production of apo-E can be suppressed by agents known to activate antimicrobial and antitumor functions of these cells (*32, 33*). Clearly, more data are required to determine how apo-E may be involved in lymphocyte or macrophage function, or both, in the immune response.

Conclusions

Apolipoprotein E, a M_r ≈34,000 protein constituent of several plasma lipoproteins, serves as a high-affinity ligand for the LDL receptor. Genetic variability of apo-E, resulting from different alleles coding for proteins with single amino acid substitutions, has been most informative in defining at a cellular and molecular level the role of this protein in normal and abnormal lipoprotein metabolism. The genetic disorder type III hyperlipoproteinemia, which is associated with premature atherosclerotic disease, is secondary to the presence of a variant apo-E that does not bind normally to the lipoprotein receptors. Characterization of the mutants, in association with several other biochemical approaches, has localized the receptor binding domain of apo-E to an arginine- and lysine-rich region in the middle of the apo-E molecule (in the vicinity of residues 140 to 160).

A major function of apo-E is the transport of lipids (especially cholesterol) among various cells of the body from sites of synthesis or absorption to sites of utilization (peripheral tissues) or excretion (liver). Equally important is its role in the local redistribution of lipid within a tissue during normal cholesterol homeostasis and especially during injury and repair. The elaborate system for storing, releasing, and reutilizing cholesterol depends on a coordinated regulation of apo-E synthesis and LDL receptor expression.

The production of apo-E by several cell types, such as hepatocytes, macrophages, astrocytes, and smooth muscle cells, in most tissues of the body emphasizes the centrality of this apolipoprotein in lipid transport and suggests its importance in roles other than lipid transport. The pronounced production and accumulation of apo-E in response to peripheral nerve injury and during the regenerative process are astounding. It is apparent that apo-E plays a prominent role in the redistribution of cholesterol to the neurites for membrane biosynthesis during axon elongation and to the Schwann cells for myelin formation. However, it is possible that the high levels of apo-E in the regenerating nerve reflect an additional role, unrelated to lipid transport. Clues to the additional role may come from further investigation of the possible involvement of apo-E in smooth muscle cell proliferation, differentiation, or both. The interaction of apo-E with heparin-like molecules in the extracellular matrix may modify the matrix, thereby altering cell-matrix or growth factor–matrix interactions. This is, of course, highly speculative. In addition, the possibility that apo-E modulates the immune response is intriguing and may provide information as to the nature of the effects of lipoproteins on tumorigenesis or immunoreactivity.

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