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EXPRESSION OF APOLIPOPROTEIN E IN CHOLESTEROL-LOADED MACROPHAGES OF EXTRAHEPATIC TISSUES DURING EXPERIMENTAL HYPERCHOLESTEROLEMIA

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Summary

To study the expression of extrahepatic apolipoprotein E (apoE) under hypercholesterolemic conditions, apoE mRNA levels were evaluated in 14 tissues of rabbits fed on a cholesterol rich diet and compared to age-matched control animals. In hypercholesterolemic rabbits apoE expression was significantly induced in adipose tissue, adrenals, aorta, lung, and spleen. The increase in apoE mRNA levels in lung and spleen was associated with the presence of cholesterol-loaded macrophages. These cells were found to express high levels of apoE mRNA as demonstrated by *in situ* mRNA hybridization. Our results suggest that extrahepatic tissues would be partially responsible for the rise in serum apoE levels detected under hypercholesterolemic conditions.

Key Words: apolipoprotein E, hypercholesterolemia, macrophage

Apolipoprotein E (apoE) is a 35,000 Da protein which is involved in cholesterol transport pathways found in most classes of plasma lipoproteins (1). Like other apolipoproteins, apoE is predominantly synthesized in liver (2). However, synthesis of apoE in tissues other than liver or intestine has been widely documented, and this feature makes apoE unique among apolipoproteins. In monkeys, extrahepatic apoE mRNA accounts for 20-40% of total body mRNA and is mainly found in brain and adrenals (3,4). In humans apoE synthesis has been detected in kidney and

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adrenals (5) and in various fetal tissues (6). Extrahepatic apoE synthesis has also been detected in rats, mice, guinea pigs and rabbits (4,7-10). In rabbits and humans, serum apoE levels increase by to 20-fold under hypercholesterolemic conditions. This increase has been mainly attributed to an impairment of apoE catabolism due to saturation and downregulation of hepatic lipoprotein receptors (13,14) rather than to overproduction, since apoE hepatic synthesis does not change (15-17). We have reported the induction of apoE expression in human and rabbit atheromas and in spleen of hypercholesterolemic rabbits (17,18). However, there is no evidence of how extrahepatic apoE expression varies under hypercholesterolemic conditions in other tissues. To address this question we have studied the variations in apoE mRNA levels in 14 tissues from hypercholesterolemic rabbits. Our results show that there is a marked increase in extrahepatic apoE mRNA, mainly in those tissues in which a large number of cholesterol-loaded macrophages accumulate, and that these cells are responsible for the increase in apoE expression.

Materials and Methods

RNA analysis by Northern blots. Female New Zealand White rabbits (n=6), 3 months old and 2-2.5 Kg in weight, were fed ad libitum on either standard chow or chow supplemented with 2% (w/w) cholesterol for 8 weeks. RNA from the indicated tissues was isolated by the guanidine thiocyanate-CsCl cushion procedure (19). RNA samples (18 μg/sample) were electrophoresed in 1% agarose-formaldehyde gel and transferred to nitrocellulose membranes (20). Blots were hybridized in the presence of 40% formamide at 42°C for 48 h and washed at 65°C to a final stringency of 0.25 x SSC (1x SSC is 150 mM NaCl, 15 mM sodium citrate). Filters were autoradiographed with Amersham MP films at -70°C. Signals of the films were quantified by densitometry (Quantiscan program, Biosoft). Probes were labelled with [α-^{32}P]dCTP by random priming (Boehringer Mannheim kit). The probes used were the following: apoE, a 0.9 Kb PstI human cDNA fragment from plasmid pE-301 (American Type Culture Collection, ATCC); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 0.8 Kb XbaI-PstI human cDNA fragment from plasmid pHcGAP (ATCC).

Histology and immunocytochemistry. Tissue fragments from control (n=3) and hypercholesterolemic (n=3) rabbits were fixed by immersion in either 4% paraformaldehyde in 0.12 M phosphate buffer for 3 h or in 3% glutaraldehyde in 0.12 M phosphate buffer for 6 h at 4°C. To analyze cholesterol-rich cells, cryostat sections (10 μm thick) from paraformaldehyde fixed tissues were mounted with 1:1 glycerol:phosphate buffer and examined under polarized light (21). To obtain semithin sections (1 μm thick), tissue fragments were fixed in glutaraldehyde, dehydrated, embedded in araldite and stained with toluidine blue. For immunocytochemistry, cryostat sections (10 μm thick) of paraformalehyde fixed tissues were used. Macrophages were identified with the monoclonal antibody RAM-11 (Dako) (23) using indirect immunofluorescence. Slides were incubated for 12 h at 4°C with the monoclonal antibody diluted 1:50 in phosphate buffered saline (PBS) for 12 h at 4°C. The secondary antibody was a FITC-conjugated goat anti-mouse immunoglobulin (Sigma).

mRNA in situ hybridizations: Fresh tissues were fixed in 4% paraformaldehyde for 4 h at 4°C. Cryostat sections 10 μm thick were set on poly-L-lysine coated slides and stored at -70°C until used. Slides were quickly warmed to room temperature, rinsed in PBS, treated with proteinase K (5 μg/ml) in 100 mM Tris, 50 mM EDTA pH 7.5 for 10 min, rinsed in water, acetylated in acetic anhydride 0.25%-0.1 M triethanolamine for 5 min and rinsed in 2x SSC. Prehybridization mixture (50% formamide, 20% dextran sulphate, 5x Denhardt solution, 10 mM sodium phosphate pH 6.8, 5x SSC and 200 μg/ml sonicated herring sperm DNA) was added and preparations were prehybridized for 4 h at 50°C in a wet chamber. DNA probes, described above, were labelled with digoxigenine-dUTP by random priming (Boehringer Mannheim kit) following the manufacturer's instructions except that three times more hexamere mixture was used. Hybridization was performed at 50°C for 12 h. Slides were sequentially washed in 2x SSC-50% formamide, 2x SSC, 4x SSC:PBT (1:1) (PBT is phosphate buffer saline-1% Tween 20) and PBT at 48°C for 30 min each wash. Slides were then incubated with anti-digoxigenine antibody diluted 1:500 in PBT (Boehringer Mannheim) at room temperature for 2 h with gentle shaking and washed with PBT for 10 min. Slides were rinsed in 100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5 and developing was carried out for 16 h with nitroblue tetrazolium and 5-bromo, 4-chloro, 3-indolyl phosphate in the presence of 1 mM levamisole (Sigma) and 0.1% Tween 20. Developing was stopped by washing in PBT and preparations were dehydrated in increasing alcohols, in 100% xylene and then fixed with Eukitt.

Serum determinations. Serum cholesterol and triglyceride levels were determined by enzymatic methods (Boehringer Mannheim). Total plasma was subjected to denaturing electrophoresis in 4-22% SDS-polyacrylamide gradient gels as described in (24) and apoE was quantified by immunoblotting using a polyclonal anti-human apoE antibody raised in goat.

Results

Rabbits fed on cholesterol-rich diet for 8 weeks showed an increase in serum cholesterol levels up to 1266 $\pm$ 63 mg/dl as compared to 136 $\pm$ 43 mg/dl in age-matched controls. Triglyceride levels were similar in both sets of animals (92 $\pm$ 26 versus 87 $\pm$ 17 mg/dl). At this time, atherosclerotic lesions were detectable in hypercholesterolemic animals (not shown). A 7-fold increase in serum apoE levels was detected in cholesterol-fed animals (118 $\pm$ 20 mg/dl) compared to controls (16 $\pm$ 2 mg/dl). To determine how extrahepatic apoE expression varied under hypercholesterolemic conditions, Northern blots were performed with RNA samples extracted from 14 different tissues of hypercholesterolemic rabbits and age-matched controls (Fig. 1). In all cases RNAs from experimental and control animals were hybridized and exposed under the same conditions and for the same time. In control rabbits, the maximal apoE mRNA expression was observed in liver, as expected. ApoE mRNA was also readily detected in brain and spleen and to a lesser extent in lung. In hypercholesterolemic rabbits apoE expression was greatly induced in adipose tissue (pericardial fat), adrenals, aorta, lung and spleen. ApoE mRNA signals in these tissues were 20 to 45% of those observed in liver apoE mRNA as determined by film densitometry (Fig. 1). The other tissues showed no expression or smaller increases. The

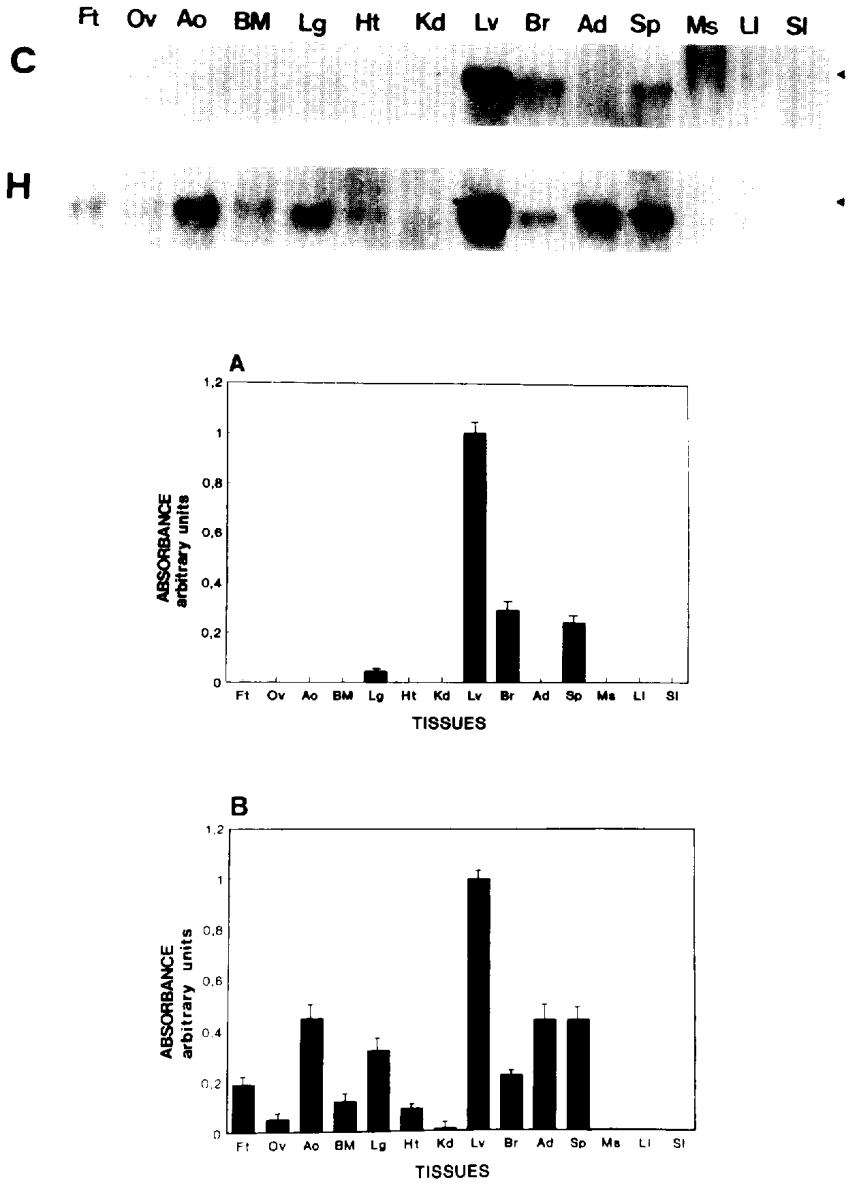

Fig. 1

Upper panel: ApoE mRNA levels in tissues from control (C) and hypercholesterolemic rabbits (H). Northern blot hybridization was performed with RNA from the different tissues. Arrowheads show the position of 18S RNA. Lower panel: Bar charts showing mRNA levels in the different tissues from control (A) and hypercholesterolemic (B) rabbits, measured relative to the levels found in liver (100%). Bars show standard errors. Ft, adipose tissue (pericardial fat). Ov, ovaries. Ao, aorta. BM, bone marrow. Lg, lung. Ht, heart. Kd, kidney. Lv, liver. Br, brain. Ad, adrenals. Sp, spleen. Ms, skeletal muscle. LI, large intestine. SI, small intestine.

comparison of apoE mRNA levels in adrenals, brain, heart, lung and adipose tissue of control and hypercholesterolemic rabbits showed a clear induction of apoE expression in adrenals, heart and adipose tissue in hypercholesterolemic animals (Fig. 2). Some apoE expression was observed in lungs of control animals, but a 5-fold increase in apoE mRNA was detected under hypercholesterolemia. No significant variations were found in brain apoE mRNA levels. In agreement with our previous studies (17,18), apoE mRNA levels did not vary significantly in liver, and were remarkably induced in aorta and spleen.

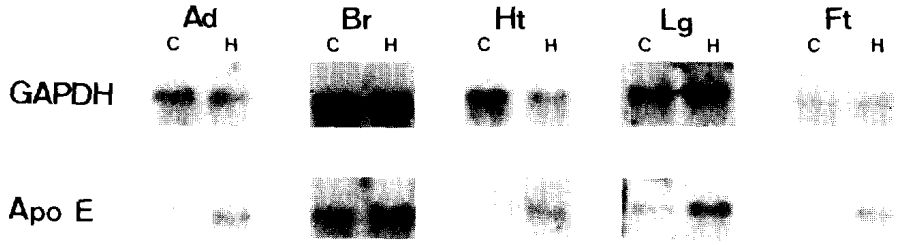

Fig. 2

Northern blots showing apoE and GAPDH mRNA levels in tissues from control (C) and hypercholesterolemic (H) rabbits. The same blot was consecutively hybridized to both probes. The tissues tested were adrenals (Ad), brain (Br), heart (Ht), lung (Lg) and adipose tissue (Ft).

Intracellular deposits of cholesterol exhibit strong birefringence when cryostat tissue sections are examined under polarized light (21). We used this histological technique to determine if there was any relationship between induction of apoE expression and the content of cholesterol-loaded cells in the tissue. We focused on the adrenals, because a previous report had not shown any increase in apoE expression in this tissue with cholesterol feeding (22). Adrenal glands from control rabbits showed lipid-loaded cells in the cortex while the adrenal medulla appeared negative (Fig. 3A). A cholesterol-rich diet led to an increase in cholesterol accumulation in all steroid secreting cells of the cortex, with cholesterol loading also extending to the adrenal medulla, as evidenced by the presence of lipid droplets in chromafin cells (Fig. 3B). Spindle-shaped cholesterol crystals were frequently observed in the zona fasciculata of adrenal cortex. These results were confirmed by light microscopy analysis of semithin sections. Normal cortical cells of the fasciculate zone exhibited a round pale nucleus and some fat droplets were found localized in the cytoplasm (Fig. 3C). In contrast, after 8 weeks of the hypercholesterolemic diet, cortical cells revealed a hypertrophied cytoplasm with a great accumulation of lipid droplets (Fig. 3D). Cholesterol loading was also observed in lung and spleen. Under hypercholesterolemia, lungs showed a prominent speckled birefringent pattern with numerous clusters of macrophage-derived foam cells (Fig. 4A). The red pulp of the spleen was another region where the density of macrophage-derived foam cells reached high levels with numerous clusters of bright cells distributed throughout the spongy network of splenic cords in hypercholesterolemic rabbits (Fig.

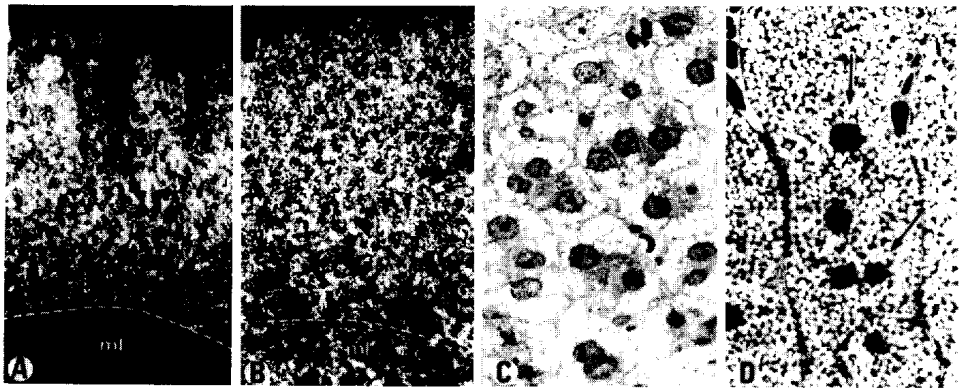

Fig. 3

Micrographs of adrenal glands under polarized light, exhibiting birefringent cholesterol deposits. A) Control rabbit. Note the accumulation of lipid-loaded cells in the cortex while the medulla layer (ml) appears negative. x 150. B) Hypercholesterolemic rabbit. Cholesterol-loaded cells increase in the cortex and extend to the medulla. C) Control adrenal showing cells with round pale nucleus and some lipid droplets in the cytoplasm. x800. D) Hypercholesterolemic gland. Cortical cells reveal hypertrophied cytoplasm with great accumulation of lipid droplets (indicated by arrows). Magnifications: A and B, x150; C and D, x800.

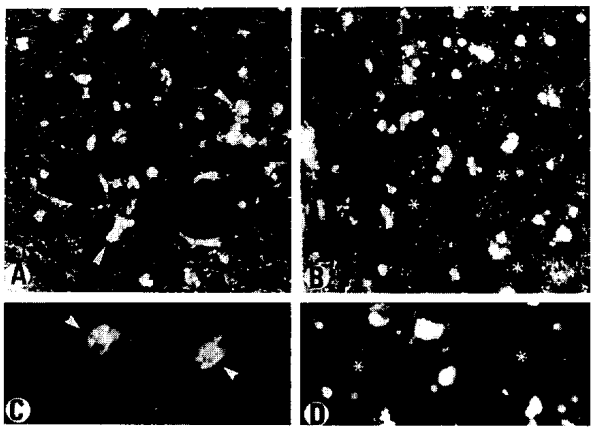

Fig. 4

Optic microphotographs under polarized light (A,B) and RAM-11 immunofluorescence (C,D). A) Lung of hypercholesterolemic rabbit showing numerous clusters of macrophage-derived foam cells (arrowheads). B) Splenic red pulp of a hypercholesterolemic rabbit showing numerous macrophage-derived foam cells distributed among splenic cords and venous sinusoids (asterisks). C) Distribution of the RAM-11 positive macrophages in the lung of a hypercholesterolemic rabbit. Two fluorescent clusters of macrophage-derived foam cells (arrowheads) appear in the alveolar septa. D) Distribution of RAM-11 positive macrophages in the red pulp of the spleen of a hypercholesterolemic rabbit. Asterisks indicate venous sinusoids. Magnifications: A and B, x200; C and D, x400.

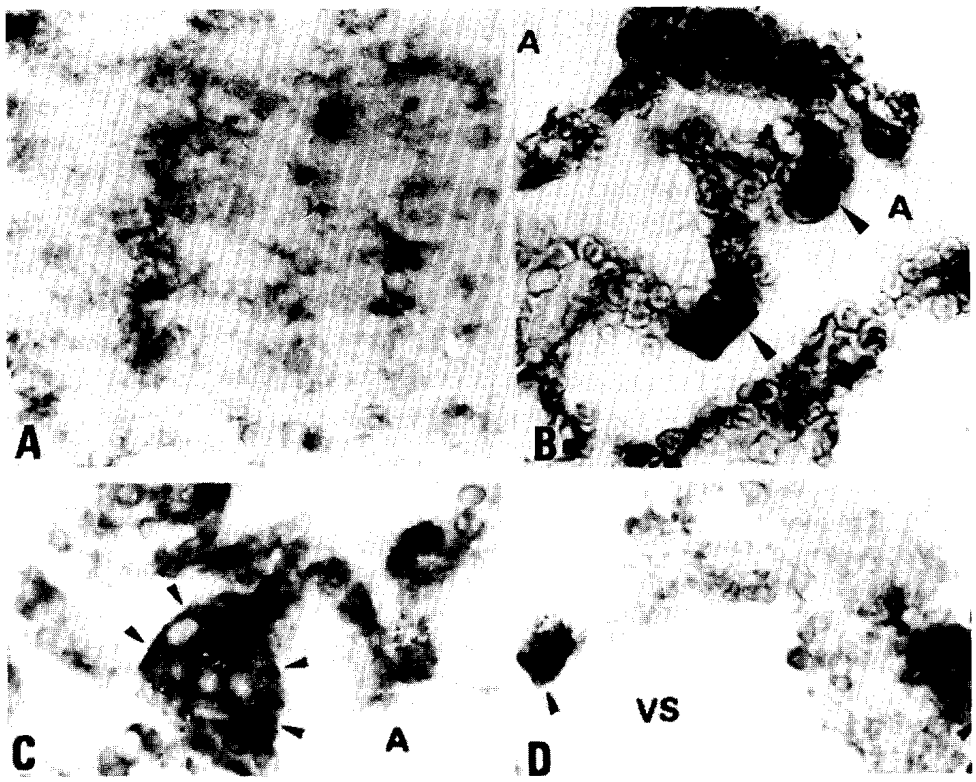

Fig. 5

In situ hybridization of apoE mRNA in adrenal cortex (A), lung (B and C) and spleen (D) from hypercholesterolemic rabbits. Hybridizations to digoxigenin-labelled apoE probes were carried out as described in Materials and Methods. (A) Cords of steroidogenic cells in adrenal cortex containing apoE mRNA. (B) Lung alveolar septa with macrophages containing apoE mRNA (arrowheads). Numerous unstained erythrocytes appear in the septa A, alveolus. (C) Detail of a cluster of alveolar macrophages illustrating selective staining of apoE mRNA in the cytoplasm (arrowheads). A, alveolus. (D) Red pulp of the spleen. Macrophages with a positive signal for apoE mRNA (arrowheads) are visible in the splenic cords and associated to the walls of the venous sinusoids (VS). Magnifications: A and B, x200; C and D, x400.

4B). No cholesterol deposition was detected in heart, kidney, brain, muscle or small and large intestine (not shown). Immunocytochemical analysis with the anti-rabbit macrophage monoclonal antibody RAM-11 (23) was carried out to confirm the macrophagic identity of cholesterol-loaded cells. The distribution pattern of the RAM-11 positive cells in lung (Fig. 4C) and red pulp of the

spleen (Fig. 4D) from hypercholesterolemic rabbits was similar to that of cholesterol-loaded foam cells observed with polarizing microscopy.

To investigate whether the cholesterol-loaded cells detected above were actively engaged in apoE expression, we performed *in situ* apoE mRNA hybridization, which allows the identification of apoE-expressing cells. In adrenal glands from hypercholesterolemic rabbits, apoE mRNA was preferentially detected in the steroidogenic cells of the cortex. In these cells the hybridization signal appeared in the perinuclear cytoplasm and also in the narrow cytoplasmic areas free of lipid droplets. As expected, no signal was detected in the nuclei (Fig. 5A). This result confirmed the increase in apoE mRNA levels detected by Northern analysis (Figs. 1 and 2). ApoE mRNA was also detected in macrophage-derived foam cells present in lung alveoli and spleen. In the lung, apoE positive macrophages were present in the alveolar septa, while endothelial cells were negative (Fig. 5B and 5C). In the spleen, apoE mRNA usually appeared in the cells of splenic cords, particularly associated with the endothelium of the venous sinuses (Fig. 5D). In all cases, hybridization with plasmid pBR322 (negative control probe) gave no signal.

Discussion

Since apoE is exported out of the cell once it is synthesized, determination of its mRNA levels offers the most accurate method of studying its expression. We have compared the tissue distribution of apoE mRNA under hypercholesterolemic and normal conditions. In control rabbits we have found that extrahepatic apoE is only expressed in brain, spleen and, to a minor extent, in lung. In hypercholesterolemic rabbits, an increase in apoE expression was observed in a number of tissues. The greatest increase occurred in adipose tissue, adrenals, aorta, lung and spleen, tissues where cholesterol has been shown to accumulate (25). As reported previously, hepatic apoE expression did not show significant variation upon hypercholesterolemia (17). We have found that in lung and spleen cholesterol accumulated in cells identified as macrophages. This is in agreement with an earlier study in which we detected the macrophage-specific RAM-11 antigen in cholesterol-loaded cells from both tissues (23).

Previous reports have shown that apoE is overexpressed in rabbit and human atheromas (17,26-29) and our finding that cholesterol-loaded foam cells are responsible for the enhanced apoE mRNA levels (18) have recently been confirmed by other researchers (30). This association has now also been demonstrated by *in situ* mRNA hybridization in lung and spleen, further indicating that apoE overexpression is a phenotypic characteristic of macrophage-derived foam cells. Increased apoE expression was also found in adrenals of hypercholesterolemic animals, which became highly hypertrophic, with cortical cells having a foamy appearance and expressing high levels of apoE mRNA, as shown by *in situ* hybridization. Elevated levels of apoE mRNA were also detected in adipose tissue of hypercholesterolemic rabbits, a result consistent with the correlation found between apoE expression and intracellular cholesterol in 3T3-L1 adipocytes (31). In conclusion, it has beeb shown that apoE is overexpressed in those cells in which there is marked lipid deposition.

Serum apoE levels are elevated under hypercholesterolemic conditions in humans and rabbits (1,11,32,33 and this work), a fact that has been related to arteriosclerosis. Data from apoE-deficient patients (1) and more recently from apoE mutant mice obtained by targeted gene disruption indicate that apoE protects against atherosclerosis (34-36). However, the origin of the increased plasmatic apoE found in hypercholesterolemic conditions is unclear. Although we have reported changes in liver expression for several apolipoproteins (37), hepatic apoE expression does not change significantly during hypercholesterolemia (15,16,17). Thus, it seems unlikely that the elevation in serum apoE levels is due to an increase in apoE secretion by liver. On the other hand, we have demonstrated a marked increase in extrahepatic apoE expression in hypercholesterolemic animals. Although it is unknown to what extent there is a correlation between apoE mRNA levels and apoE secretion into the bloodstream by each organ, the results reported here suggest that extrahepatic apoE could account for a significant fraction of circulating apoE.

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