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Journal

Arteriosclerosis Thrombosis and Vascular Biology, 13(9)

ISSN

1079-5642

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Publication Date

1993-09-01

DOI

10.1161/01.atv.13.9.1382

Peer reviewed

Abundant Expression of Apoprotein E by Macrophages in Human and Rabbit Atherosclerotic Lesions

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Previous studies have demonstrated the presence of apoprotein (apo) E protein and message in arterial lesions. To determine the source of the synthesized apoE, we performed simultaneous in situ hybridization and immunocytochemistry on human and rabbit atherosclerotic tissue. Studies of serial sections of aortic atherosclerotic lesions from humans and hypercholesterolemic New Zealand White rabbits and Watanabe heritable hyperlipidemic rabbits revealed a similar pattern of macrophage-specific apoE expression in the rabbit and human lesions. In early lesions of rabbit atherosclerotic tissue, in which many macrophages were present, there was abundant expression of apoE mRNA. Northern blot analyses of total mRNA obtained from arterial macrophage-derived foam cells, freshly isolated from ballooned, cholesterol-fed New Zealand White rabbits, demonstrated positive hybridization with an apoE-specific riboprobe. Western blot analyses of conditioned media from the isolated foam cells placed in culture for up to 24 hours demonstrated the presence of secreted apoE. These studies demonstrated that in atherosclerotic lesions, arterial wall macrophages synthesize and secrete apoE and probably account for most of the apoE synthesized in the atherosclerotic artery. (*Arterioscler Thromb.* 1993;13:1382-1389.)

KEY WORDS • atherosclerosis • apoprotein E • macrophages • in situ hybridization • immunocytochemistry

Apoprotein (apo) E is one of the primary apoproteins bound to the surface of most lipoprotein particles and mediates receptor-dependent clearance of lipoproteins from plasma.[1] It is synthesized predominantly in the liver, but is also expressed in many other tissues,[2] including the artery wall.[3,4] Because many individuals with apoE deficiency or allelic variations leading to type III hyperlipoproteinemia develop premature atherosclerosis,[5-7] it has been speculated that a deficiency in apoE leads to an atherogenic phenotype. Indeed, two recent studies using targeted gene disruption of the apoE gene in mice have provided convincing evidence that the absence of apoE promotes the atherogenic process. Wild-type mice are extremely resistant to diet-induced hypercholesterolemia and the development of atherosclerosis. However, mice that do not express apoE are spontaneously hypercholesterolemic and develop atherosclerotic lesions by 3 months of age.[8,9] It is still unclear how the presence of apoE, either in mice or humans, protects against atherosclerotic lesion development. The ability of apoE to promote the clearance of atherogenic lipoproteins undoubtedly underlies much of its protective capacity.[2] However, the fact that arterial cells can express apoE suggests that apoE may also play a local role in the artery wall by regulating cellular events occurring in the developing atherosclerotic lesion.

Immunocytochemical studies with cell type–specific antibodies conducted over the past 10 years have demonstrated that macrophages are a significant cellular component of atherosclerotic lesions at all stages of lesion development in both humans and experimental animals.[10,11] However, with the exception of their capacity for taking up and storing large amounts of cholesterol (and thus becoming foam cells), there is limited information concerning the properties and functions of macrophages resident within the artery wall. Recent in situ studies from our laboratories have focused on the capacity of arterial macrophages to express proteins that may play a role in the atherogenic process.[12-14] Although it is known that macrophages in vitro synthesize and secrete apoE,[15] the specific expression of apoE by arterial macrophages in vivo has not yet been convincingly demonstrated. Previous immunocytochemical studies have demonstrated the presence of apoE in atherosclerotic lesions,[16-20] and recently Northern blot analysis[3] and the polymerase chain reaction (PCR)[4] have shown that apoE message is expressed by cells within the artery wall. However, these studies failed to demonstrate which cell types express apoE in vivo. The present study used in situ hybridization, immunocytochemistry, and direct study of macrophage-derived foam cells isolated from atherosclerotic lesions to demonstrate that macrophages within atherosclerotic lesions express and secrete apoE.

Methods

Procurement and Preparation of Tissue

Eight separate samples of human aorta were obtained from the University of California, San Diego organ transplant center. The tissue donors, both male and female, were trauma cases and ranged in age from 16 to 56 years. The aortic samples were placed in fixative (formal/sucrose) containing antioxidants (50 μmol/L butylated hydroxytoluene [BHT] and 2 mmol/L EDTA) immediately after removal from the donors and were immersion fixed for 24 hours as previously described.[12-14] The tissue was then rinsed, cut into smaller segments, dehydrated, and embedded in paraffin. Sections of each sample were stained with hematoxylin and eosin and characterized as to the type and severity of the atherosclerotic lesions. The lesions chosen for study included examples of diffuse intimal thickening, fatty streaks, and advanced, complex lesions. Aortic tissue was also obtained from New Zealand White rabbits that had been fed a high-cholesterol diet (2% added to standard rabbit chow) for 25 weeks and from Watanabe heritable hyperlipidemic rabbits between 8 and 36 months of age. In all cases, the aortas were perfusion fixed with formal/sucrose containing BHT and EDTA and the tissue prepared as described above. The rabbit lesions consisted predominantly of macrophage-rich advanced fatty streaks.

In Situ Hybridization and Immunocytochemistry

In situ hybridization was done according to our previously published methods.[12-14] Rabbit and human apoE cDNA fragments[21,22] were subcloned into an appropriate expression plasmid (pBluescript, Strategene). Templates for RNA synthesis were produced by linearizing the plasmids with appropriate restriction enzymes: for human apoE sense probe, *Sma* I; human apoE antisense probe, *Eco* RI; rabbit apoE sense probe, *Not* I; and rabbit apoE antisense probe, *Sst* I. Using a riboprobe transcription kit (Strategene), ^{35}S-uridine triphosphate riboprobes were then prepared (300 to 400 bases in length). The rabbit-specific cDNA was a generous gift of Dr Yu-sheng Chao (Merck Research Laboratories), and the human cDNA probe was obtained from the American Type Culture Collection repository. Before hybridization some of the aortic sections were pretreated with RNase as additional controls. All of the sections were then treated with proteinase K, acetylated, and incubated with the ^{35}S-labeled riboprobes overnight at 55°C. After hybridization, the sections were treated with RNase, washed, dipped in autoradiographic emulsion (Kodak, NTB-2), and incubated at 4°C for 2 to 4 weeks.[12-14]

Immunocytochemical staining of serial sections of both the human and rabbit tissue was also done according to previously published techniques.[23] In general, after blocking nonspecific binding sites with nonimmune serum, sections were incubated with the primary antibodies for 1 hour at room temperature, followed by biotinylated secondary antibodies and either avidin-biotin-horseradish peroxidase or avidin-biotin-alkaline phosphatase according to the manufacturer's specifications (Vector Labs). Control sections were stained with nonimmune serum or irrelevant antibodies of the same heavy-chain class as the primary antibodies. The primary antibodies used included HAM-56, a human macrophage–specific monoclonal antibody[10]; RAM-11, a rabbit macrophage–specific monoclo-

nal antibody[11]; HHF-35, a monoclonal antibody that recognizes muscle actin[10]; MB47, a monoclonal antibody that recognizes the receptor-binding domain of native apoB of both human and rabbit species[24]; MDA-2, a monoclonal antibody directed against malondialdehyde (MDA)-lysine[25]; and 1E, a monoclonal antibody specific for human apoE.[26] In addition, we used RE-4, a rabbit apoE–specific monoclonal antibody. This antibody was generated against apoE purified from rabbit β-very-low-density lipoprotein (β-VLDL) and shown to bind specifically to rabbit apoE by Western blotting techniques. In those cases in which in situ hybridization was done on sections that had been previously immunostained with the macrophage-specific antibodies, additional steps were taken to stabilize the RNA. These steps included blocking nonspecific binding sites with blocking buffer that included RNasin (4 units/mL; Promega) and intensifying the horseradish peroxidase reaction product with metal ions such as cobalt or nickel.[27] The immunostained sections were then stored in 95% ethanol before in situ hybridization. Both the in situ hybridizations and immunostained sections were analyzed and photographed using a Nikon Microphot microscope. The in situ hybridizations were photographed using epiluminescence or simultaneous bright-field illumination and epiluminescence as shown in Fig 1.

Isolation of Foam Cells

The isolation of foam cells was performed according to previously published techniques.[28] Briefly, the endothelial cells of the aorta and left iliac artery of young, male, New Zealand White rabbits were removed with a 4F Fogarty embolectomy catheter,[28] and the animals were placed on a high-cholesterol diet (2%) for 13 weeks (1 week before the catheterization and up to 12 weeks after the denudation procedure). For each preparation of foam cells, 4 to 6 rabbits were killed with a bolus injection of sodium pentobarbital, and the entire aorta and left iliac artery were removed and placed in cold Hanks' balanced salt solution made with pyrogen-free water and supplemented with glucose, amino acids, penicillin (100 IU/mL), and streptomycin (100 μg/mL). Under a sterile hood and on ice, the atherosclerotic lesions (intima) were dissected from the associated media and adventitia and minced. The foam cells were released from the minced intima by using an enzymatic digestion procedure[29] in which 1.0 g of intima was incubated with 10.0 mL Hanks' balanced salt solution containing 450 units collagenase (Sigma type VIIS), 4.7 units elastase (Sigma), and 1.0 mg/mL soybean trypsin inhibitor (Sigma) for three 1-hour intervals at 37°C (after each hour the released cells were collected and fresh enzymes were added to the remaining tissue). The released cells were pelleted, resuspended, and purified on a discontinuous density gradient of metrizamide (Sigma) (30% cushion, 10% top) that was centrifuged at 1200g in a swinging bucket rotor for 15 minutes at 10°C. Alveolar macrophages were isolated from the same ballooned, cholesterol-fed rabbits via saline lavage.[28]

RNA Extraction and Northern Blot Analysis

Northern blot analysis of apoE message in extracts of arterial foam cells was done as previously described.[12,13] Total RNA was extracted from freshly isolated foam cells and alveolar macrophages by using the technique of Chomczynski and Sacchi.[30] Briefly, the cells were treated with guanidinium isothiocyanate, and the RNA was ex-
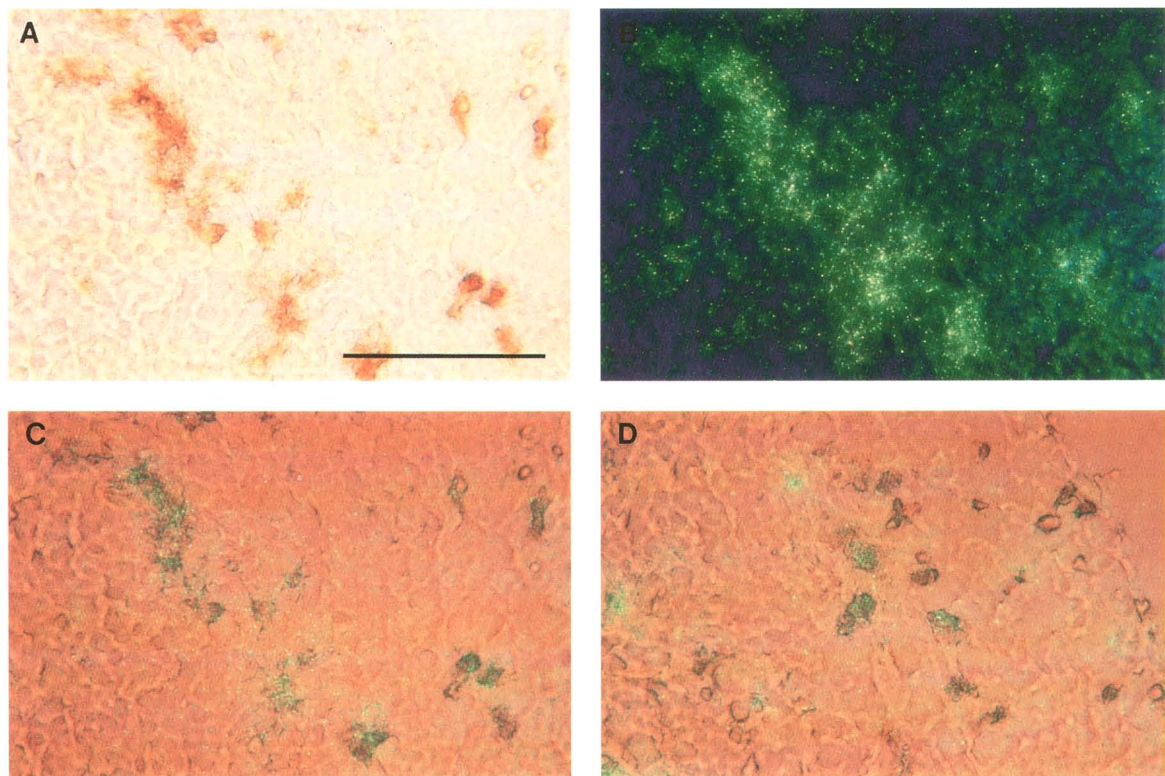
FIG 1.   *Photomicrographs of simultaneous immunocytochemistry and in situ hybridization showing macrophage expression of apoprotein (apo) E in an atherosclerotic lesion from a human abdominal aorta. A, Bright-field image showing immunocytochemical staining of macrophages with antibody HAM-56. B, Epiluminescence image of same area showing silver grains indicative of positive hybridization with human apoE–specific antisense probe. C, Combined bright-field and epiluminescence image of the same area showing localization of apoE mRNA in macrophages. D, Combined bright-field and epiluminescence image of a different area of the lesion. Note that there are immunostained macrophages that are positive and negative for hybridization with the apoE probe. The "brown" staining represents HAM-56 staining for macrophages, and the "green" indicates the in situ signal for apoE mRNA (original magnification ×266 [all panels]; bar=100 μm).*

tracted with phenol and chloroform/isoamyl alcohol and then precipitated with isopropanol. Gel electrophoresis was done using 10 μg/lane mRNA on 1.2% agarose-formaldehyde gels, the bands were transferred to nylon membranes (Amersham Hybond-N), and autoradiograms were produced using standard techniques.[12,13]

Collection of Conditioned Media and Western Blot Analysis

The freshly isolated macrophage-derived foam cells were plated at a density of 2.5×10^5 cells/well in 12-well tissue-culture plates in OptiMEM I medium (GIBCO) containing 0.5% heat-inactivated fetal calf serum and 20 μg/mL gentamicin. After an overnight incubation, cultures were washed to remove nonadherent cells, fresh medium without fetal calf serum (with or without 10 ng/mL lipopolysaccharide [LPS]) was added, and the cells were incubated for an additional 2 hours. The cells were then washed again, re-fed with fresh medium (without LPS), and incubated for 24 hours. The resulting 24-hour conditioned media were collected and probed for the presence of secreted apoE using Western blot analysis with the monoclonal antibody RE-4 (generated against apoE purified from rabbit β-VLDL). For the Western blot analysis, 0.2 mL conditioned medium was electrophoresed on 3% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels in the

presence of 1% sodium dodecyl sulfate as previously described.[25] The bands were visualized by using the Imun-Lite Chemiluminescence Assay Kit (Bio-Rad).

Results

When human aortic lesions were examined, the majority of macrophages in each lesion (regardless of the severity of the lesion) hybridized with the apoE probe. Fig 1 shows a representative example of simultaneous in situ hybridization with the human apoE riboprobe and immunocytochemistry with the human macrophage–specific antibody HAM-56. Note that although most macrophages appeared to express mRNA for apoE, there were also pockets of macrophages that did not (Fig 1D). Fig 2 is a composite of serial sections from a human aortic lesion demonstrating the distribution of macrophages (panel C), smooth muscle cells (panel D), and those cells in the lesion that expressed apoE (panels A and B). Note that in these sections the majority of the cells expressing apoE were stained with the macrophage-specific antibody HAM-56. In addition, Fig 2 shows the patterns of immunostaining with antibodies specific for apoE (panel E), apoB (panel F), and MDA-lysine, an oxidation-specific lipid-protein adduct characteristic of oxidized low-density lipoprotein (LDL) (panel G). In this lesion we observed extensive staining for apoE, predominantly in the area containing the
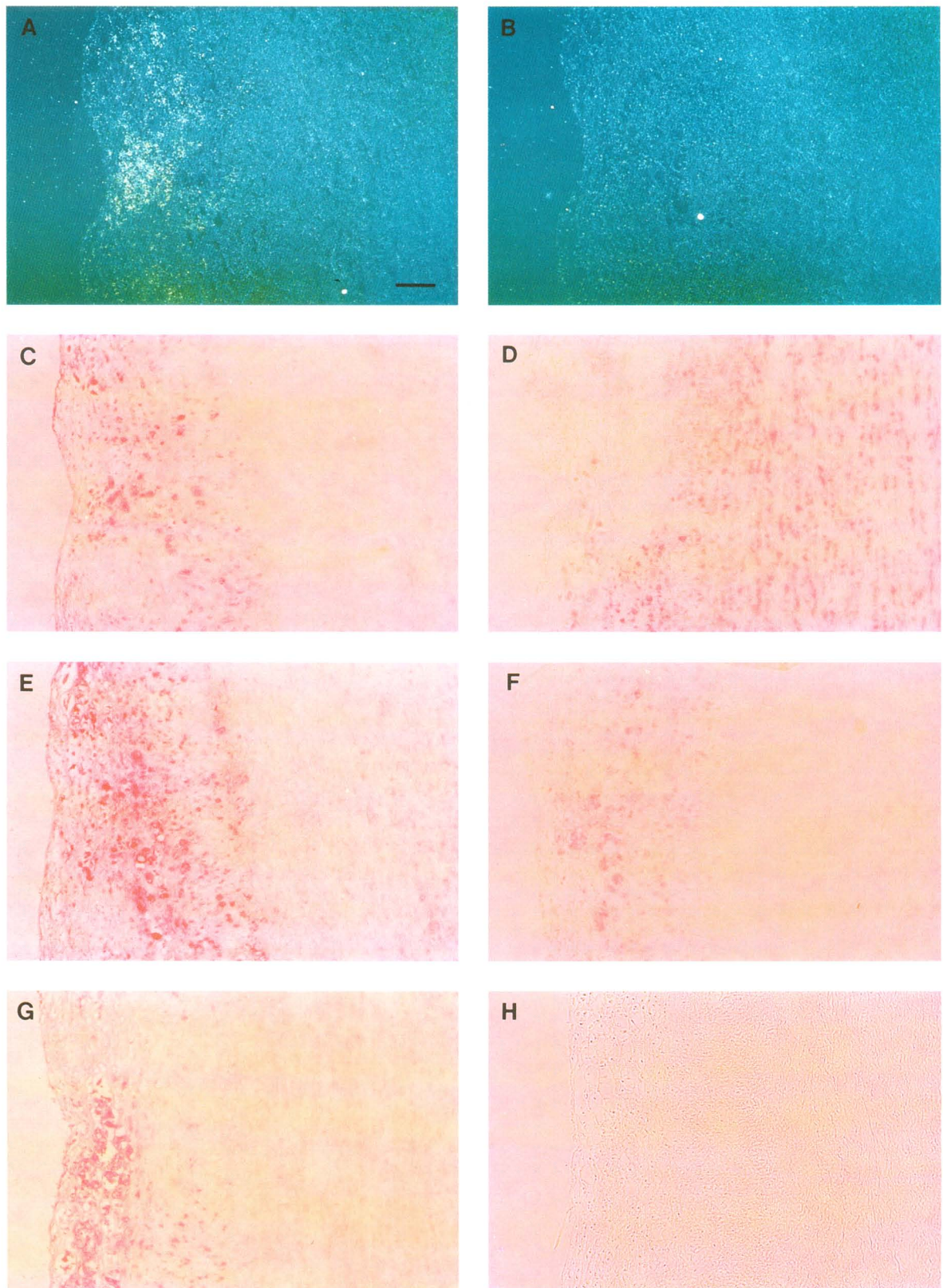
FIG 2. *Photomicrographs showing serial sections of an atherosclerotic lesion from a human thoracic aorta. A, Epiluminescence image of hybridization with an apoprotein (apo) E–specific antisense probe. B, Epiluminescence image of hybridization with corresponding sense probe for apoE. C through H, Bright-field images of immunocytochemical staining. C, Macrophages (HAM-56); D, smooth muscle cells (HHF-35); E, apoE (1E); F, apoB (MB47); G, malondialdehyde-lysine (MDA-2); and H, nonimmune control (phase contrast) (original magnification ×66 [all panels]; bar=100 μm).*
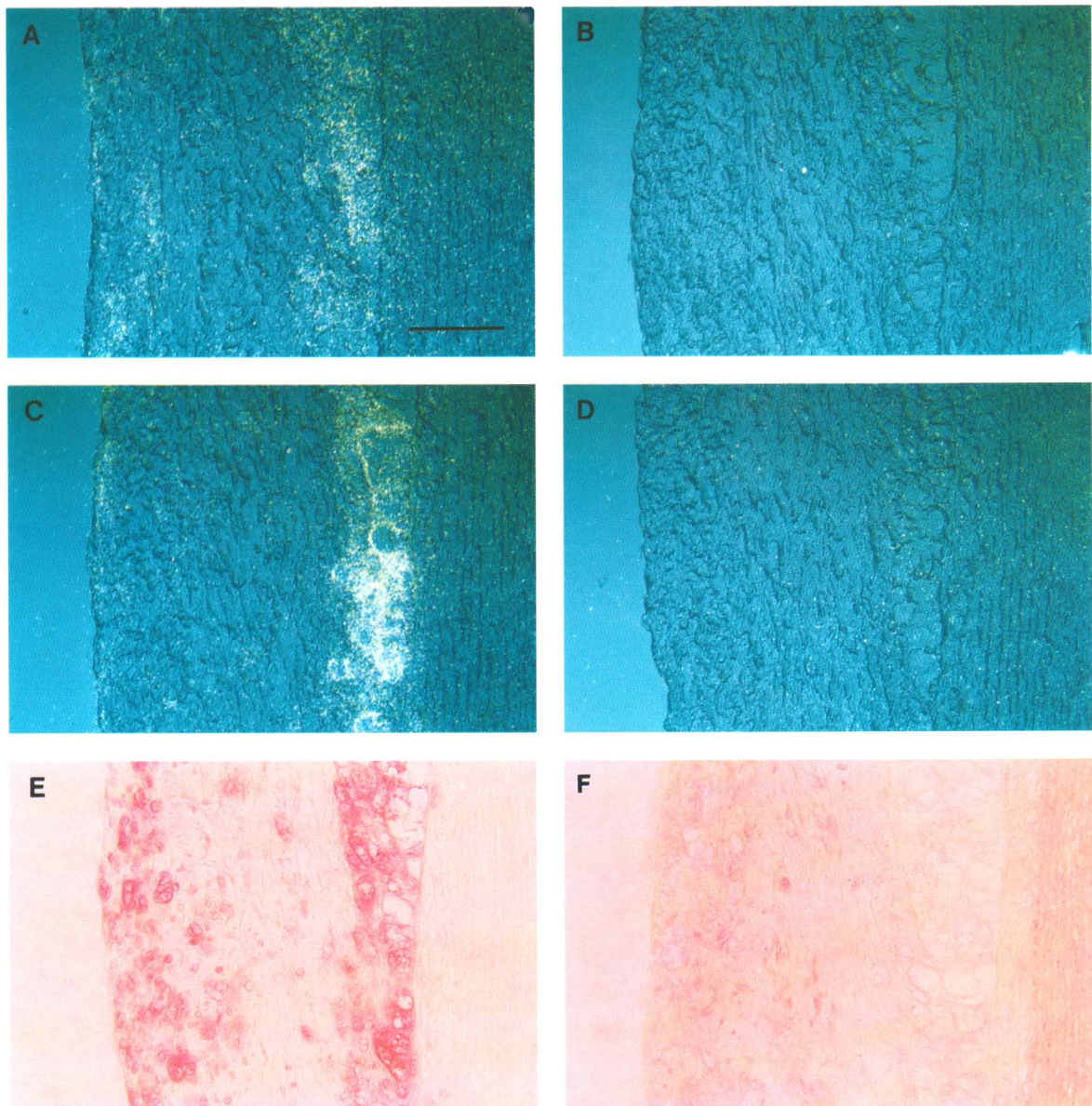
FIG 3.   *Photomicrographs showing serial sections of an atherosclerotic lesion from the thoracic aorta of a hypercholesterolemic New Zealand White rabbit. A through D, Epiluminescence images of in situ hybridization studies with the following probes: A, human apoprotein (apo) E antisense probe; B, human apoE sense probe; C, rabbit apoE antisense probe; and D, rabbit apoE sense probe. E and F, Bright-field images of immunocytochemical staining. E, Macrophages (RAM-11) and F, smooth muscle cells (HHF-35) (original magnification ×133 [all panels]; bar=100 μm).*

macrophages, whereas there was limited staining for epitopes of native or oxidized LDL.

Fig 3 is a similar composite showing serial sections of aortic lesions from a cholesterol-fed rabbit. Panels A through E demonstrate that both the human- (panel A) and rabbit- (panel C) specific antisense probes positively hybridized with the rabbit tissue. Immunostaining of the serial sections with RAM-11, a rabbit macrophage–specific monoclonal antibody (panel E), and HHF-35, the muscle actin–specific antibody (panel F), again revealed that the majority of cells expressing apoE in the rabbit lesions were immunoreactive for RAM-11. In most of the lesions studied by in situ hybridization we saw a very strong signal with the apoE riboprobe, which always collocalized with a macro-

phage-rich region of lesions, whether they were fatty streaks or more advanced complicated lesions. Among the various gene products we have examined, the apparent abundance of apoE message was most impressive. This was most prominent in the early lesions examined from hypercholesterolemic rabbits. Fig 4 displays examples of macrophage-rich regions of early lesions in the cholesterol-fed rabbit as well as lesions taken from the spontaneously hypercholesterolemic Watanabe heritable hyperlipidemic rabbit.

To further verify that macrophage-derived foam cells express and secrete apoE, studies were done using macrophage-derived foam cells that were freshly isolated from rabbit aortic lesions. As shown in Fig 5, there was positive hybridization of the rabbit apoE probe with
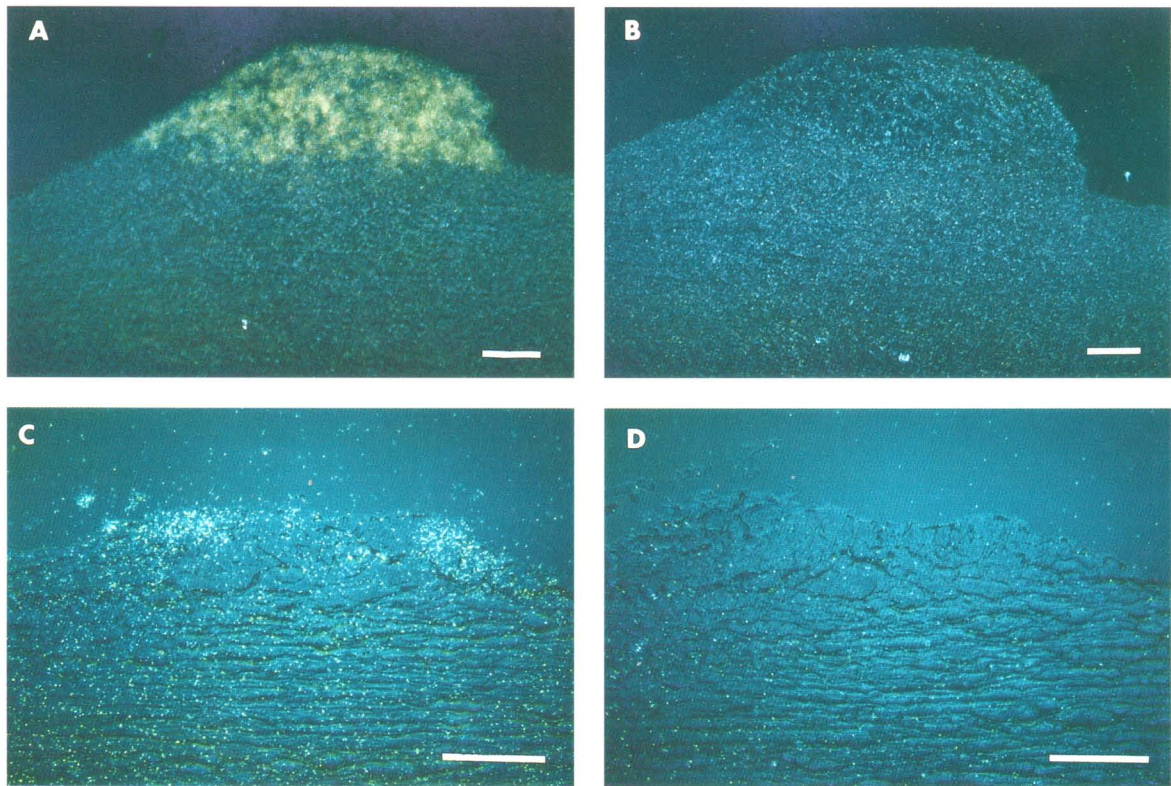
FIG 4. *Epiluminescence images of in situ hybridization with rabbit apoprotein (apo) E probes in aortic lesions taken from a cholesterol-fed New Zealand White rabbit (A and B) and a Watanabe heritable hyperlipidemic rabbit (C and D). A and C, Rabbit apoE antisense probe. B and D, Rabbit apoE sense control (original magnification ×66 [A, B], ×133 [C, D]; bars=100 μm).*

total mRNA extracted from the foam cells (panel b, lane 2). In contrast, there was no hybridization with the mRNA extracted from alveolar macrophages obtained from the same animals (panel b, lane 1). Furthermore, the 24-hour conditioned medium from the foam cells contained secreted apoE, as shown by using Western blot analysis with antibody RE-4 (Fig 6, lane 2). LPS treatment of these foam cells, however, did not appear to significantly alter the level of apoE secreted.

Discussion

The data presented in this article demonstrate that macrophages in atherosclerotic lesions of both human and
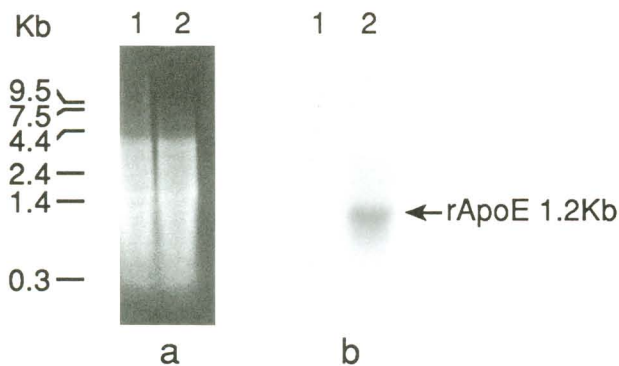

FIG 5. *Northern blot analysis of total mRNA with rabbit apoprotein (apo) E–specific probe. Expression of apoE mRNA by isolated rabbit arterial macrophage-derived foam cells and alveolar macrophages. a, Ethidium bromide staining. b, Alveolar macrophages (lane 1) and arterial foam cells (lane 2).*

FIG 6. *Secretion of apoprotein (apo) E by isolated arterial macrophage-derived foam cells. Western blot analysis of 24-hour conditioned media from isolated arterial macrophage-derived foam cells. Lane 1, Purified rabbit apoE from β-very-low-density lipoprotein; lane 2, conditioned medium from nonactivated foam cells; lane 3, conditioned medium from lipopolysaccharide-treated foam cells; and lane 4, nonconditioned medium.*

rabbit aortic tissue are the primary cell type responsible for the apoE synthesized in these lesions. By using techniques of in situ hybridization and immunocytochemistry, we demonstrated the marked abundance of apoE mRNA in macrophages as well as the secretion of apoE by isolated arterial macrophage/foam cells. The presence of apoE in atherosclerotic lesions of both humans and experimental animals has previously been demonstrated by using immunocytochemistry.[16-20] Because apoE is an important component of lipoproteins such as VLDL, intermediate-density lipoprotein, and high-density lipoprotein (HDL), all of which are found in the artery wall,[1,31,32] it is possible that some or all of the arterial wall apoE observed could come from the plasma. Recent studies by Crespo et al[3] and Salomon et al[4] have used Northern blot analysis and quantitative PCR to demonstrate the presence of apoE mRNA in rabbit and human atherosclerotic lesions, but the source of this mRNA was not defined. Thus, our data, and that of other investigators, now clearly establishes that there is an abundance of apoE in atherosclerotic lesions and that macrophages in the lesion make a significant amount of this apoE.

Previous studies have demonstrated that peritoneal macrophages in culture synthesize apoE,[15,33] a process that can be enhanced by an increased cellular content of free cholesterol.[34] Our observation that the isolated arterial macrophage/foam cells express and secrete apoE is consistent with these data. Although these foam cells contain 600 to 1000 μg total cholesterol/mg protein, between 10% and 15% of the cellular cholesterol is unesterified.[28] Thus, the large amount of free cholesterol in the arterial macrophage may be responsible for inducing the observed apoE expression. However, hypercholesterolemia per se is not sufficient to induce apoE expression by all macrophages, as a subset of alveolar macrophages obtained from the same animals did not contain large amounts of cholesterol, nor did they express message for apoE (Fig 5). Thus, in vivo, it is the accumulation of free cholesterol within the macrophages that appears to induce the expression of apoE. The accumulation of such large amounts of intracellular cholesterol by the arterial but not the alveolar macrophages suggests that the environment of the artery wall promotes the conversion of macrophages to foam cells.

We have observed that the isolated foam cells contain a basal level of interleukin-1 even without exposure to stimuli for interleukin-1 expression (data not shown), suggesting that the isolated foam cells are at least partially activated. Previous in vitro studies have demonstrated that differentiation stimuli, such as phorbol esters, enhance apoE expression, whereas activation of macrophages with agents such as LPS inhibits the expression of apoE.[33] The fact that LPS did not inhibit the secretion of apoE by the isolated foam cells (Fig 5) suggests that the foam cells may be fully activated (or refractory to the effects of LPS). Thus, the observed accumulation of apoE in the medium probably reflects the balance of a number of factors that influence apoE synthesis and release. Clearly, additional studies are required to determine whether there are changes in the threshold response to free cholesterol in activated macrophages with respect to apoE release. Another important consideration for future research is the relationship between the cellular uptake and content of oxidized lipids and the capacity of the cells to express apoE. We have previously shown that isolated foam cells

as well as arterial macrophages in vivo (Fig 2) are readily stained with antibodies generated against epitopes of oxidized LDL.[23,25] This would suggest that oxidized lipids within the cells do not prevent the cells from making or secreting apoE, although our studies do not address the issue of whether the presence within the cells of products of oxidized LDL might influence apoE synthesis or secretion.

It is interesting to speculate as to why arterial macrophages express apoE in atherosclerotic lesions and what role this expression may play in the atherogenic process. Because there is such a strong correlation between apoE expression and the cellular content of free cholesterol, and because too much free cholesterol is toxic to cells, the secretion of apoE may be a protective mechanism against the continued accumulation of more cholesterol. For example, the cellular content of free cholesterol would be reduced if more acceptor were present outside the cell to remove the excess cholesterol. The secreted apoE could rapidly associate with extracellular phospholipid[15] or with HDL[35] and facilitate the process of removal of cellular cholesterol.[1,2] If HDL is the ultimate acceptor for any cholesterol that the cell can unload, apoE secreted by macrophages can associate with intimal HDL. The extra apoE on the surface of HDL may expand its surface and thereby promote its ability to accept more free cholesterol as well as to facilitate its enhanced clearance by the liver.[2] Alternatively, it is possible that the secreted apoE might bind to the surface of intimal lipoproteins or lipid vesicles and help promote macrophage uptake,[36] possibly mediated by low-density lipoprotein receptor–related protein–mediated mechanisms.[37]

Finally, apoE secreted by the arterial macrophages may play an important role in regulating local immune responses occurring in the lesions. Recent immunocytochemical studies of both human and rabbit atherosclerotic lesions have shown that there are considerable numbers of T cells resident within the lesions.[38] Interactions between apoE and T lymphocytes could participate in the complex set of factors that influence lesion progression or regression. For example, previous in vitro studies have demonstrated that apoE can inhibit the activation of T lymphocytes.[39] Activated T cells elaborate factors that recruit and maintain monocytes within the lesion.[40] T-cell proliferation and activation are also required for the local expression and secretion of interferon gamma, a cytokine that both protects against the development of atherosclerosis in cholesterol-fed animals[41] and regulates macrophage production of apoE.[42]

Whatever role apoE plays in macrophage function, it is likely to be very important. The strength of the expression of apoE mRNA and protein in the lesions, especially in the markedly hypercholesterolemic rabbit models, is among the most dramatic of the various genes we have examined. It is tempting to speculate that the advanced atherosclerotic lesions seen in apoE-deficient mice are the result not only of the marked increase in plasma lipoprotein levels but also of an inability of the arterial macrophages to synthesize and secrete apoE. We suspect that this inability to secrete apoE contributes in a major way to the marked cholesterol accumulation and lesion progression that so rapidly occurs in the arteries of these mice.[8,9]

Acknowledgments

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