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# Increased apolipoprotein E and c-*fms* gene expression without elevated interleukin 1 or 6 mRNA levels indicates selective activation of macrophage functions in advanced human atheroma

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**ABSTRACT** **Cells found within atherosclerotic lesions can produce in culture protein mediators that may participate in atherogenesis. To test whether human atheromata actually contain transcripts for certain of these genes, we compared levels of mRNAs in carotid or coronary atheromata and in nonatherosclerotic human vessels by polymerase chain reaction (PCR) amplification of cDNAs reverse-transcribed from RNA. We measured PCR products (generated during exponential amplification) by incorporation of $^{32}$P-labeled primers. Levels of interleukin 1$\alpha$, 1$\beta$, or 6 mRNAs in plaques and controls did not differ. Compared to uninvolved vessels, plaques did contain higher levels of mRNA encoding platelet-derived growth factor A chain (42 ± 24 vs. 12 ± 10 fmol of product; mean ± SD; $n$ = 8 and 8, respectively; $P$ = 0.007) and B chain (41 ± 36 vs. 4 ± 3 fmol of product, $n$ = 14 and 6, respectively; $P$ = 0.024). Atherosclerotic lesions consistently had much higher levels of apolipoprotein E (apoE) mRNA than did control vessels (131 ± 71 vs. 5 ± 3 fmol of product; $n$ = 12 and 10, respectively; $P$ < 0.001). Direct RNA blot analyses confirmed elevated levels of apoE mRNA in plaque extracts. To test whether mononuclear phagocytes might be a source of the apoE mRNA, we studied a selective marker for cells of the monocytic lineage, the c-*fms* protooncogene, which encodes the receptor for macrophage colony-stimulating factor. Plaques also contained elevated levels of c-*fms* mRNA (30 ± 17 vs. 5 ± 3 fmol of product; $n$ = 10 and 7, respectively; $P$ = 0.002). Immunohistochemical colocalization demonstrated apoE protein in association with macrophages in plaques, whereas nonatherosclerotic vessels showed no immunoreactive apoE. ApoE produced locally in atheroma might modulate the functions of lesional T cells or promote "reverse cholesterol transport" by associating with high density lipoprotein particles, thus targeting them for peripheral uptake. Macrophages within the advanced human atheroma appear to exhibit a selective program of activation as they express high levels of apoE, whereas overall levels of interleukin 1 or 6 mRNAs in plaques are not elevated.**

It is now well accepted that blood lipoproteins and platelet products may influence the behavior of cells within human atherosclerotic plaques. Over the last few years it has become apparent that blood vessel wall cells and infiltrating leukocytes can themselves produce mediators that may act locally to modulate vascular function. Cell culture studies have established the ability of vascular endothelial and smooth muscle cells to express genes for growth factors (1–4), cytokines (5–13), and other biologically active proteins. Macrophages, such as those found within human atheroma, can produce a multitude of mediators of potential pathogenic significance.

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Studies of cultured cells permit the definition of functional capacities of well-characterized cell preparations under well-controlled conditions. However, the challenge remains to determine if properties of cells displayed *in vitro* are actually expressed *in vivo* and may play a role in pathology. Animal experiments are useful in this regard as they permit longitudinal and carefully controlled studies. However, no experimental model replicates human atherosclerosis exactly. Verification of the relevance of results of cell culture and animal experiments to human atherogenesis will require the analysis of gene expression in the human lesion itself. Proper application of immunohistochemistry can localize specific proteins within plaques. However, this approach is qualitative and cannot prove local production of the protein. Studies of mRNA by *in situ* hybridization combined with immunohistochemical identification of cells provides a powerful tool for analysis of local gene expression by specific cell types but also provides mainly qualitative information (14, 15). Conventional RNA blot analysis can identify specific mRNA in extracts of human atherosclerotic lesions (16, 17). However, this approach requires relatively large amounts of RNA, which restricts its application in view of the limited availability of fresh human atherosclerotic tissue.

RNA phenotyping by reverse transcription of mRNA with amplification by the polymerase chain reaction (PCR) is a convenient and very sensitive technique that requires little RNA (18, 19). Previous applications to a small number of human atherosclerotic lesions have established the potential utility of this method (20). We report here application of this approach to study the expression of a number of genes often invoked in discussions of atherogenesis but for which limited data from human lesions exist.

## MATERIALS AND METHODS

**Sources and Characterization of Specimens.** Human atheromata were obtained in a fresh state at carotid ($n$ = 10), coronary ($n$ = 3), or femoral ($n$ = 3) endarterectomy or from an aortic plaque ($n$ = 1). Intimal plaque was prepared from endarterectomy specimens by careful removal of the portion of tunica media usually removed by the surgeon along with the plaque. Nonatherosclerotic vessels studied included excess portions of internal mammary artery ($n$ = 7) or saphenous vein ($n$ = 5), obtained at a time of coronary bypass operations, and vessels from transplant donors ($n$ = 2). Immunohistochemical examination also used vascular specimens obtained at autopsy. Each specimen was carefully examined grossly and representative portions were prepared for routine light microscopic examination. One grossly nor-

Abbreviations: apoE, apolipoprotein E; IL, interleukin; PDGF, platelet-derived growth factor.

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mal artery was excluded from the group of control vessels because histologic examination revealed early atherosclerotic changes.

**Preparation and Characterization of RNA.** The portion of these specimens not reserved for morphologic examination was minced and homogenized in 4 M guanidinium isothiocyanate/10 mM Tris/0.43 M 2-mercaptoethanol/68.2 mM Sarkosyl, pH 7.4, with a Polytron device. The extracted RNA was isolated by centrifugation (116,000 × *g*, 18 h) through a $CsCl_2$ cushion. Integrity of the isolated RNA was evaluated by visualizing the ethidium bromide-stained nucleic acids after electrophoresis through agarose gels. RNA concentrations were calculated from the absorbance at 260 nm and the purity was determined by $A_{260}/A_{280}$.

**Reverse Transcription and PCR Amplification.** Cellular RNA (1 μg) was reverse-transcribed in a 10-μl reaction mixture containing 100 units of Moloney murine leukemia virus reverse transcriptase and 0.1 μg of oligo(dT) (12- to 18-mer) (20). We obtained equivalent results with random hexanucleotide primers or with oligo(dT) primers in the reverse transcriptase reaction (data not shown). Longer incubation times and higher concentrations of enzyme and deoxyribonucleotides did not increase the efficiency of PCR amplification of the products of the reverse transcriptase reaction. Thus, the conditions used for reverse transcription appeared maximally efficient.

The strategy employed for measurement of PCR products involved measuring the incorporation of $^{32}P$-end-labeled primers of known specific radioactivity as a function of cycle number. This method is generally applicable, requires no specially constructed standards, and is, therefore, well suited to initial surveys of large numbers of targets. The total amount of radioactivity incorporated from end-labeled primers is proportional to product copy number and is independent of product size. Sense and antisense primers for interleukin (IL) 1α, IL-1β, IL-6, and c-*fms* were labeled with $^{32}P$ using T4 polynucleotide kinase. The sequences used for the IL-1, PDGF, and apoE primers have been published (20). Sequences of the other primers used were for c-*fms* 5′-TCTGAGCAAGACCTGGACAA-3′ and 5′-GGATTCAGCCCAAGTGAGAA-3′ and for IL-6 5′-CTTTTGGAGTTTGAGGTATACCTAG-3′ and 5′-GCTGCGCAGAATGAGATGAGTTATC-3′. Unincorporated isotope was removed using NENsorb 20 columns (NEN), and percent incorporation was determined, permitting calculation of the specific radioactivity of the radiolabeled primers.

For PCR expansion, each 50-μl reaction mixture contained 1 μl of cDNA derived from 0.1 μg of total RNA, $1 \times 10^6$ cpm of labeled primers, 5 pmol of each unlabeled primer, 1 unit of Taq DNA polymerase, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and bovine serum albumin at 100 μg/ml. The PCR cycle conditions were melting for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 1.5 min at 72°C. A 250-μl reaction mixture was prepared and then divided into five 50-μl samples, assuring homogeneous initial concentrations of enzyme and substrates. One of these five tubes was removed at the completion of 20, 25, 30, 35, and 40 cycles. Preliminary experiments with each primer pair determined directly the range of cycles of expansion that yielded exponential increase in product and disclosed wide variation in the amplification efficiency of different primer pairs. Therefore, for each sample and each primer pair, we chose a cycle number for comparison of values that fell within the exponential amplification range, determined experimentally under identical conditions and verified directly during each experiment.

The PCR product was separated from unincorporated primer by electrophoresis through 3% agarose gels. The primer pairs and reaction conditions were chosen to amplify one principle size of product and to minimize "primer-dimer" formation. The band migrating at the predicted size was excised, and the radioactivity was determined by liquid scintillation spectroscopy (efficiency, >90%). The point in the exponential amplification range for comparison was selected by inspection of semi-logarithmic plots of cpm vs. cycle number. Control experiments demonstrated that the amount of PCR product was proportional to the input levels of target sequences, in the reverse transcriptase reaction and in the PCR amplification.

Southern blot analysis using end-labeled synthetic oligodeoxyribonucleotides complementary to internal sequences within the amplified products, was performed to investigate the identity of the products of amplification. After electrophoresis, Southern transfer to Hybond nylon membranes (Amersham), and UV fixation, the blots were prehybridized for 1 h at 37°C and hybridized at 40°C for 15–20 h with end-labeled oligodeoxynucleotides at $1 \times 10^6$ cpm/ml complementary to a 20-base-pair (bp) region between the sense and antisense primers. The membranes were washed for two 20-min periods in 6× standard sodium citrate at room temperature and analyzed by autoradiography. In addition, sequence analysis of PCR products generated with the primer pairs for IL-1α, IL-1β, and apoE by dideoxynucleotide chain-termination explored whether the DNA amplified by PCR corresponded to the published cDNA sequences of the intended targets.

**RNA Blotting Analysis.** RNA (1 μg) was applied to a nylon membrane by using a slot-blot apparatus, prehybridized, hybridized, and autoradiographed. A 1-kilobase *Ava* I–*Hin*fI fragment of apoE cDNA (21) and a 320-bp *Eco*RI–*Hin*dIII fragment of chicken β-actin cDNA labeled by random hexanucleotide priming with [$^{32}P$]dCTP were used as probes. Laser densitometry of the autoradiogram was used to calculate the ratio of apoE to the β-actin signal for each sample.

**Immunohistochemical Study.** Normal or atherosclerotic vessels were cut into 4-mm-thick transverse pieces, frozen in OCT embedding medium (Miles), and stored at −70°C until cryosectioning into 6-μm sections on chromalum-coated slides. Tissue sections were fixed in acetone at 4°C for 2 min, air-dried briefly, and baked for 15 h at 42°C. Slides were stored at −20°C until immunostaining, at which time they were brought to room temperature and rehydrated in phosphate-buffered saline (PBS, pH 7.4) for 15 min. Standard ABC staining techniques were employed using commercially available streptavidin reagents (Vector Laboratories). Antibodies used included the rabbit polyclonal anti-human apoE antibody GHE3-9 (22) (diluted 1:1000), a gift of Karl Weisgraber (Gladstone Foundation Laboratories, San Francisco); anti-macrophage antibody EBM11 (Dako, diluted 1:300); anti-smooth muscle α-actin (23) (diluted 1:300), a gift of Giulio Gabbiani (University of Geneva, Switzerland); and nonimmune rabbit serum (Vector Laboratories; diluted 1:1000). Controls included the omission of the primary antibody and the use of nonimmune rabbit serum diluted 1:1000 in PBS containing 10% (vol/vol) horse serum.

## RESULTS

Gross and histological examination of the lesions sampled in this study showed the considerable heterogeneity typical of advanced human atherosclerosis. Some lesions contained substantial amounts of connective tissue and were relatively lipid-poor. Others contained greater amounts of fat deposition. Highly calcified lesions were unsuitable for RNA extraction and were not entered into this study. Attempts to correlate the morphology of the lesions with the levels of expression of various genes proved inconclusive. The heterogeneity of the lesions sampled reflects the usual spectrum of human atherosclerosis and contributes to the variability

Table 1. Level of mRNA expression in extracts of human atherosclerotic plaques and uninvolved vessels

| PCR product | Product, fmol | | P | PCR cycles, no. |
|---|---|---|---|---|
| | Plaque | Normal | | |
| PDGF-A | 42 ± 24 (8) | 12 ± 10 (8) | 0.007 | 25 |
| PDGF-B | 41 ± 37 (9) | 4 ± 3 (6) | 0.024 | 30 |
| IL-1$\beta$ | 9 ± 6 (11) | 6 ± 6 (9) | NS | 35 |
| IL-1$\alpha$ | 26 ± 12 (9) | 23 ± 11 (5) | NS | 35 |
| IL-6 | 11 ± 10 (8) | 7 ± 6 (4) | NS | 35 |

Results are PCR product levels (fmol, mean ± SD; numbers in parentheses are *n*). NS, not significant. Statistical significance of plaque vs. normal tissue was evaluated by the unpaired Student's *t* test.

reflected by the generally large standard deviation in the levels of gene expression (Table 1).

Plaques contained more PDGF-A and -B (c-sis) mRNA than normal vessels, consistent with previous results (refs. 14, 17, 20, and 24 and Table 1). The lesions sampled did not contain elevated levels of IL-1 $\alpha$, IL-1 $\beta$, or IL-6 mRNA. Likewise, the estimates of levels of mRNA encoding interferon $\gamma$ showed no difference among the various samples studied (data not shown). ApoE mRNA in the plaque exceeded by far that in normal vessels. This difference was evident from inspection of the ethidium bromide-stained electrophoretic gels of the PCR products (Fig. 1). Quantitative analysis of the incorporation of $^{32}$P-end-labeled primers into the electrophoretically isolated apoE PCR products showed exponential expansion over the range of 20–30 cycles, as indicated by the linearity of the semi-logarithmic plot of cpm incorporated vs. cycle number (Fig. 2). By choosing the midportion of this exponential phase of amplification as a point for comparison, apoE PCR product derived from cDNA from atheromatous tissue exceeded that in uninvolved vessels by 25-fold (Fig. 3*A*). RNA blot analysis also showed an increase in steady-state levels of apoE mRNA in atheromata compared to normal vessels relative to the level of the RNA encoding the ubiquitous cytoskeletal protein $\beta$-actin. The average ratio of apoE to $\beta$-actin in four nonatherosclerotic vessels was 2 (range 1.0–4.5), whereas this ratio was 63 (range 38–120) in RNA from four atheromata.

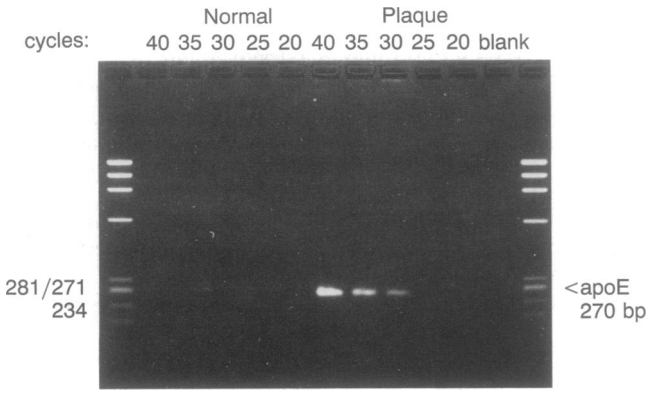


FIG. 1. Ethidium bromide visualization of PCR products obtained by analysis of RNA from a normal vessel and from an atherosclerotic plaque. RNA was extracted, and cDNA was synthesized and amplified. Samples of the PCR products were electrophoresed on a 3% agarose minigel and stained with ethidium bromide. *Hae* III fragments of phage ΦX174 were used as size markers (outer lanes); the size of the standard fragments in the region of interest are shown to the left. Products obtained at the cycle indicated were applied to different lanes. The position of migration of the apoE product (predicted size, 270 bp) is indicated to the right.

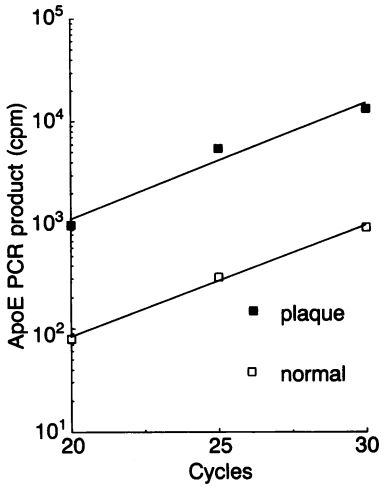


FIG. 2. Exponential amplification of apoE cDNA by PCR. The PCR products obtained at 20, 25, and 30 cycles of expansion were isolated by electrophoresis. The region of the gel containing the band migrating at the position of the expected PCR product was excised and the radioactivity was measured by liquid scintillation spectroscopy. The graph plots the logarithm of PCR product measured by the radioactivity in the excised portions of the gel as a function of cycle number. Solid squares represent PCR product derived from RNA from a human atherosclerotic plaque; open squares represent product derived from RNA from an uninvolved human vessel. Note the logarithmic increase in the products as a function of cycle number. The two lines are parallel indicating that the efficiency of amplification was similar during expansion of the PCR product from both specimens.

This accumulation of apoE mRNA corresponded to increased expression of apoE protein. All plaques studied by immunohistochemistry ($n = 4$) showed specific and localized staining for apoE (Fig. 4). Nonimmune rabbit serum and PBS controls showed no staining of plaques (data not shown). Sections of two normal iliac arteries did not stain with anti-apoE antibody, nonimmune serum, or PBS (data not shown). Plaques contained immunoreactive apoE in areas rich in cells identified as macrophages by specific antibody staining of serial sections (Fig. 4). Some plaques exhibited

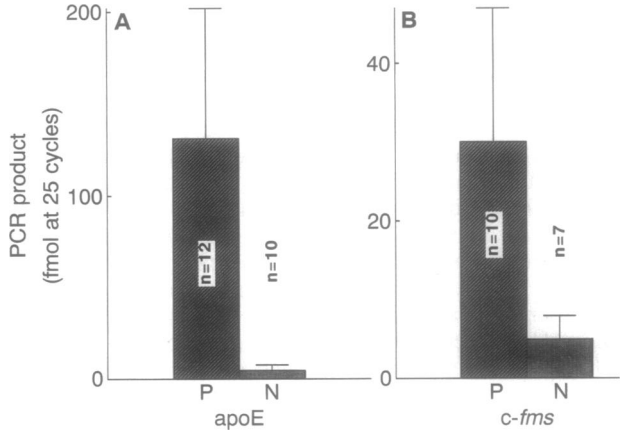


FIG. 3. Comparison of the levels of apoE and the c-*fms* protooncogene PCR products generated from cDNAs derived from RNA extracted from human atherosclerotic plaque (bars P) and nonatherosclerotic human blood vessels (bars N). RNA extraction, cDNA preparation, and amplification by PCR were performed. Level of PCR products for apoE (*A*) or for c-*fms* (*B*) (mean ± SD; the *n* within or over each bar denotes the number of specimens studied) are shown. The differences between plaque and normal tissue differed significantly as determined by the unpaired Student's *t* test (apoE, $P < 0.001$; c-*fms*, $P = 0.002$).

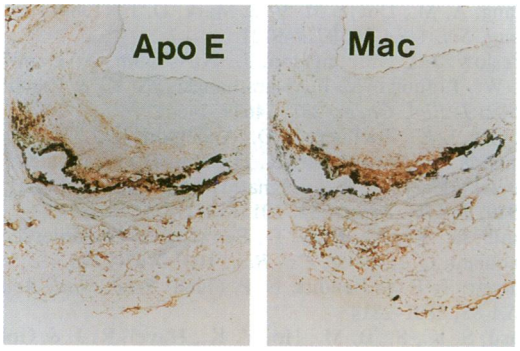


FIG. 4. Immunohistochemical analysis shows apoE protein in regions of an atherosclerotic human coronary artery (*Left*) infiltrated by macrophages, as shown by staining of a serial section of the same lesion with a specific monoclonal antibody EBM-11 (*Right*). Analysis of three other human atheromata showed similar results. Normal vessels did not stain for apoE (data not shown).

apoE immunostaining in areas containing cells bearing markers of smooth muscle origin as well (data not shown). Both macrophages and smooth muscle cells can synthesize apoE *in vitro* (25–27).

The c-*fms* protooncogene encodes the receptor for macrophage colony-stimulating factor. Barrett and Benditt (24) used c-fms mRNA as a marker for macrophages in their Northern blot analysis of gene expression in human atheromata (24). Neither endothelial cells nor smooth muscle cells appear to express this receptor (28). The elevated levels of c-fms mRNA in plaques compared to controls (Fig. 3*B*) confirm the substantial macrophage infiltration observed by histologic analysis of the specimens studied here and widely described (29–31).

Southern blot analysis, using end-labeled synthetic oligodeoxyribonucleotides complementary to internal sequences within the amplified products, verified the identity of the products of amplification (data not shown). In addition, the nucleotide sequences of PCR products obtained with the primers used here IL-1$\alpha$, IL-1$\beta$, and apoE, using human plaque RNA-derived cDNA as starting material, agreed with published sequences of the human target cDNAs (data not shown).

## DISCUSSION

Application of the PCR RNA phenotyping approach to advanced human atherosclerotic plaques showed elevations in PDGF-A and -B mRNA compared to uninvolved vessels (Table 1). These findings agree well with the results obtained by Northern blot analysis (17, 24), *in situ* hybridization (14), and PCR analysis of mRNA from a single plaque (20). Advanced human atherosclerotic plaques contain immunoreactive PDGF-B spatially associated with macrophages (32). Thus these findings indicate that locally produced PDGF may function as an autocrine or paracrine mediator even during the late phases of human atherogenesis (4, 24, 33).

The lack of increased IL-1 or IL-6 mRNAs in the advanced lesions sampled here does not imply that these genes do not contribute to earlier phases of the atherogenesis or that cells within complex atheromata do not express these genes inducibly. The level of cytokine gene expression by vascular cells and leukocytes depends on the presence of stimuli. When the integrity of a plaque is disrupted by rupture, ulceration, or hemorrhage, products of thrombosis and coagulation may elicit increased levels of growth factor and cytokine gene expression locally (2, 34). Likewise, bacterial lipopolysaccharide and endogenous pyrogens, such as IL-1 and tumor necrosis factor, circulating levels of which may increase during systemic illness or sepsis, can elicit cytokine or PDGF gene expression in vascular wall cells and leukocytes alike (5, 6, 35–38). We have found that aortas of rabbits rendered atherosclerotic by diets enriched in cholesterol and fat contained low basal levels of IL-1$\alpha$ or -$\beta$ mRNA. However, intravenous administration of lipopolysaccharide, a classical IL-1-inducing stimulus, elevated these mRNAs more in atheromatous than in normal aortas (39). Thus, interpretation of the present results requires recognition that they represent static observations on late-stage atheromata obtained in most cases at elective surgery on stable patients. Nonatherosclerotic control vessels were also obtained in most cases from patients undergoing elective surgery. Practical considerations make it difficult to obtain normal human carotid arteries in a fresh enough state for RNA extraction, which is why we used internal mammary arteries for comparison in this study.

The magnitude and consistency of the accumulation of apoE mRNA documented here is striking. Experimental studies have previously documented apoE expression in atherosclerotic lesions of rabbits (40–42). Interestingly, our rabbit experiments that showed no elevation in IL-1$\alpha$ and -$\beta$ mRNA levels in atheromatous aortas did disclose increased apoE mRNA, consistent with the present observations on advanced human lesions (39). The accumulation of c-fms mRNA is consistent with the presence of macrophages within lesions, and colocalization of immunoreactive apoE protein with lesional macrophages (Fig. 4) indicates that phagocytic leukocytes are one likely source of the apoE mRNA. However, our results do not exclude that smooth muscle cells or foam cells derived from smooth muscle cells contribute to increased apoE mRNA in plaque extracts. J. N. Wilcox (personal communication) has found high levels of apoE mRNA in advanced human atheromata by *in situ* hybridization analysis, mostly in association with macrophages. Immunoreactive apoE was previously found in human atheromata (43, 44) and a recent report showed apoE mRNA in two human lesions studied on a Northern blot (42), in accord with the present comparative analysis of RNA and local apoE protein expression in atheromatous and uninvolved human vascular tissue.

The elevated levels of apoE mRNA documented here have several implications for human atherogenesis. ApoE produced locally within the atherosclerotic plaque may associate with high density lipoprotein particles that contact and remove cholesterol from foam cells. Association of apoE with these cholesterol-enriched high density lipoprotein particles might aid their clearance by promoting recognition by the hepatic low density lipoprotein receptors (the "apoB,E receptor"). Thus, this apoE might aid targeting of high density lipoprotein particles involved in "reverse cholesterol transport" for peripheral catabolism (45, 46). In addition fractions of plasma lipoproteins that contain apoE can inhibit T-cell activation *in vitro* (47, 48). There is considerable evidence for T-cell activation in complicated human atherosclerotic plaques (49). Thus, locally produced apoE may also play a role in regulation of this local immune reaction by attenuating T-cell activation.

Morphologic demonstration of the presence of macrophages within atheroma discloses little about the functional state of these cells. The present observations go beyond merely marking macrophage accumulation in the complicated human atheroma by providing insight into the activation program(s) displayed by these cells *in vivo*. Our findings argue against "all-or-none" activation of macrophage functions within atheroma but suggest instead that these cells can display interesting subsets of inducible functions. Culture experiments have established that lipopolysaccharide, tumor necrosis factor, or IL-1 itself augment IL-1 expression in monocytes and intrinsic vascular cells (6, 36, 38, 50). Expo-

sure to oxidized low density lipoprotein can actually inhibit cytokine gene expression by cultured macrophages (51). In contrast, modified low density lipoprotein increases apoE expression in these cells (25, 45). Our finding that advanced human atherosclerotic plaques have elevated levels of apoE and c-fms mRNA, without overall changes in IL-1 expression, suggests that the distinct pathways of activation, exemplified by the responses elicited *in vitro* by cytokines vs. modified low density lipoprotein, also pertain to human atherogenesis. Our results support the view that macrophages within lesions can display graded expression of diverse inducible functions that may vary considerably depending on local exposure to different stimuli at various times during the evolution of an atherosclerotic plaque.

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