Radiolabeled Native Low-Density Lipoprotein Injected Into Patients With Carotid Stenosis Accumulates in Macrophages of Atherosclerotic Plaque

Effect of Vitamin E Supplementation

L. Iuliano, MD; A. Mauriello, MD; E. Sbarigia, MD; L.G. Spagnoli, MD; F. Violi, MD

Background—Accumulation of LDL within the arterial wall appears to play a crucial role in the initiation and progression of atherosclerotic plaque. The dynamic sequence of this event has not been fully elucidated in humans.

Methods and Results—In 7 patients with previous transient ischemic attack or stroke and critical (>70%) carotid stenosis, autologous native [^{125}I]-labeled LDL or [^{125}I]-labeled human serum albumin were injected 24 to 72 hours before endarterectomy. Carotid specimens obtained at endarterectomy were analyzed by autoradiography and immunohistochemistry. Autoradiographic study showed that LDL was localized prevalently in the foam cells of atherosclerotic plaques, whereas the accumulation in the lipid core was negligible. Immunohistochemistry revealed that foam cells that had accumulated radiolabeled LDL were mostly CD68 positive, whereas a small number were α-actin positive. No accumulation of the radiotracer was detected in atherosclerotic plaques after injection of radiolabeled human serum albumin. In 3 patients treated for 4 weeks with vitamin E (900 mg/d), an almost complete suppression of radiolabeled LDL uptake by macrophages was observed.

Conclusions—This study shows that circulating LDL rapidly accumulates in human atherosclerotic plaque. The prevalent accumulation of LDL by macrophages provides strong support to the hypothesis that these cells play a crucial role in the pathogenesis of atherosclerosis. (***Circulation***. 2000;101:1249-1254.)

Key Words: atherosclerosis ■ lipoproteins ■ stenosis ■ plaque ■ oxidant stress

The oxidative modification hypothesis of atherosclerosis is based on the assumption that the formation of the initial lesion, which is characterized by the presence of macrophage-derived foam cell, is dependent on the uptake of oxidized LDL by macrophages through a specific receptor.[1,2] Several lines of experimental evidence support this hypothesis. For instance, it has been shown that LDL extracted from animal and human atherosclerotic lesions are oxidatively modified.[3,4] Antibodies against oxidized LDL react with antigen of atherosclerotic plaque.[5] In addition, patients with clinically proven atherosclerosis have circulating antibodies that react against oxidized LDL.[6]

According to the oxidative-modification hypothesis, LDL would initially accumulate in the subendothelial space and, after oxidative changes induced by resident cells such as endothelial cells, macrophages, or smooth muscle cells, would be taken up by macrophages through the scavenger receptor. However, the temporal sequence of these events has never been demonstrated in human beings. To test this hypothesis in human atherosclerosis, we analyzed in patients with critical (>70%) carotid stenosis whether injected radio-labeled autologous native LDL accumulated in the carotid atherosclerotic plaque. To this purpose, 24 to 72 hours after injection of radiolabeled LDL, atherosclerotic specimens were obtained at endarterectomy and analyzed by autoradiography and immunohistochemical autoradiography. In the present study, we demonstrated that circulating LDL accumulates prevalently in the monocyte-macrophages of human atherosclerotic plaque and that vitamin E supplementation prevents it.

Methods

Materials

$CuSO_4$, hydrogen peroxide, ICl, and tetraethoxypropane were purchased from Aldrich (Sigma-Aldrich); Paragon agarose gel–precoated plates were from Beckman; α-tocopherol, α-tocopherol acetate, poly-L-lysine, bicinchoninic acid, and Sephadex G25 were from Sigma Chemical Co (Sigma-Aldrich); and carrier-free Na ^{125}I was from Amersham International Ltd. Human serum albumin, 20% solution, was from Centeon (Centeon SpA). All other reagents were of the highest grade available from Merck.

Preparation of LDL

LDL (density 1.025 to 1.050 g/mL) obtained from human plasma was processed under sterile conditions to minimize exposure to endotoxin. To minimize oxidation, LDL was prepared in the presence of EDTA and by fast sequential flotation ultracentrifugation at 100 000 rpm in an Optima TLX Ultracentrifuge (Beckman) with a TLA 100.4 rotor. LDL was collected by upward fractionation to minimize albumin contamination.[7] The purity of LDL evaluated by agarose gel electrophoresis was >98%. Before labeling, the purified lipoproteins were desalted by Sephadex G25 chromatography, at 4°C, with saline used as the exchanging solution. Protein concentration was determined by the bicinchoninic acid method.[8] LDL and human serum albumin were radiolabeled with ^{125}I by use of the iodine monochloride method.[9] After labeling, the proteins were passed through a Sephadex G25 column to remove free ^{125}I, with 0.1 mol/L sodium bicarbonate in 0.9% NaCl (pH 8) used as the exchanging buffer. Before injection, radiolabeled proteins were sterilized by passage through a 0.22-μm Millipore filter. LDL samples were assayed for oxidation by measuring the levels of thiobarbituric acid–reactive substances, lipid hydroperoxides, and electrophoretic mobility as previously described.[10] Before injection, both LDL and human serum albumin were always tested for endotoxin contamination by the *Limulus amebocytes* lysate assay, as previously described.[11] Final endotoxin contamination was always <0.02 U/mg LDL cholesterol.

In Vivo Studies

We studied 10 patients (4 women and 6 men, ages 68 to 74 years) who were eligible for endarterectomy (previous transient ischemic attack or stroke and carotid stenosis >70%). Informed consent was obtained from all patients before the study. All procedures were approved by the local human ethics committee. Blood was withdrawn from each patient after overnight fasting and immediately processed for LDL separation and radiolabeling with ^{125}I as described above. Among 7 patients injected with ^{125}I-LDL, 3 were treated with 900 mg/d vitamin E for 4 weeks before endarterectomy. Radiolabeled autologous LDL were bolus-injected within 24 hours from blood withdrawal. Commercial human albumin was adopted as control with the same LDL protocol used for radiolabeling and injection. The amount of radiolabeled protein injected was 5 μCi of [^{125}I]-LDL or 10 μCi of [^{125}I]-albumin. No side effects were observed in patients after injection of radiolabeled proteins. Patients underwent surgery 24 to 72 hours after radiolabeled protein injection. Immediately after surgery, endarterectomy specimens were rinsed twice with cold PBS containing EDTA to remove contaminated blood.

Autoradiography

Light microscopy autoradiographic studies were carried out on 4 carotid endarterectomy specimens obtained from patients injected with autologous [^{125}I]-native LDL and 3 carotid specimens obtained from patients injected with [^{125}I]-albumin as control. Patients injected with native LDL (4 men and 3 women, ages 70 to 74 years) or human serum albumin (2 men and 1 woman, ages 68 to 70 years) had comparable prevalence of risk factors such as diabetes, hypercholesterolemia, hypertension, or smoking. Total cholesterol, LDL cholesterol, and HDL cholesterol plasma levels in patients treated with vitamin E were comparable to those of control patients.

After surgical procedures, samples were immediately fixed in 0.1 mol/L phosphate buffer, pH 7.4, containing 10% (wt/vol) formaldehyde and 0.5% (wt/vol) glutaraldehyde. After decalcification, if necessary, the specimens were sliced transversally every 3 mm and embedded in paraffin.

Five sections per slice (3 μm thick, mean 50 sections per plaque) were processed for autoradiographic studies. Before autoradiographic procedures, 2 sections per slice were processed immunohistochemically to characterize the cell types present within the plaque.[12] All samples were processed for autoradiography within 72 hours of the surgical procedure.

For autoradiography, sections were coated with Ilford K2 emulsion (Ilford Ltd) diluted 1:1 with 1% glycerol. After 6 weeks of exposure at 4°C in light-proof boxes containing silica gel, sections were developed in Kodak D-19 (Kodak-Pathé) and then cleared in Ilford Hypam, washed in water, and stained with hematoxylin and eosin.

In each section, silver grain density over plaque cells and interstitial tissue was obtained by determining the number of silver grains per surface area by an oil-immersion objective. Surface area was measured with the aid of Scion Image software. To quantify the intracellular radiolabeled LDL content, we randomly analyzed 20 macrophages corresponding to $\geq$62 000 μm^2, which were present in the cap region of the plaque of each subject. Values were expressed as number of silver grains (mean$\pm$SE)/ 100 μm^2 cytoplasm. Grain density over plaque structures was corrected for background radiation, which was measured in a similar manner over areas of the slides devoid of vascular tissue. Background radiation had a mean value of 0.95 grains/100 μm^2.

Immunohistochemical Study

Two serial sections cut from paraffin blocks were mounted on slides previously treated with poly-L-lysine solution. To characterize the phenotype of the cells that were positive in the autoradiography analysis, sections were incubated with the monoclonal primary antibody anti-CD68 (anti-human monocyte/macrophages) (Dakopatts)[13] and the anti–α-smooth muscle actin antibody (Dakopatts), which recognizes vascular smooth muscle cells.[14] These antibodies were used as previously described.[12]

Plasma Vitamin E Assay

After overnight fasting, blood samples were taken into tubes containing heparin and centrifuged at 500g for 10 minutes to obtain plasma. The plasma was stored at -20°C until assay. Plasma vitamin E was measured by high-performance liquid chromatography with UV detection, and vitamin E acetate was used as the internal standard.[15]

Statistical Analysis

Data analysis was carried out by ANOVA and by Student's t test. Significance was accepted at the level of $P<0.05$.

Results

Patients with carotid stenosis injected with radiolabeled autologous native LDL or human serum albumin underwent endarterectomy after 24 to 72 hours. The carotid plaques of patients injected with native [^{125}I]-labeled LDL or [^{125}I]-labeled human serum albumin were all advanced lesions belonging to type VI according to Stary's classification.[16] The degree of plaque calcification was comparable in the subjects injected with LDL or albumin. Evidence of plaque fissuring and thrombosis was present in 3 out of 4 carotid samples of patients injected with LDL, in 2 out of 3 carotid samples of patients in the vitamin E group, and in 2 out of 3 carotid samples of patients injected with albumin.

The plaques were composed of a great number of inflammatory cells, including monocyte/macrophages, CD68-positive cells, and lymphocytes, principally localized in the shoulder and cap of the plaque. Cell density in the cap and shoulder of the plaque was similar among the 3 groups of patients. The CD68-positive cells were mainly composed of foam cells with a round, often eccentric nucleus, and the cytoplasm was filled entirely with lipid droplets. A small number of CD68-positive cells were monocytes. A few muscular foam cells, preferentially

localized in the cap region, also were present. These foam cells were α-smooth muscle actin positive and were featured as elongated cells with a fusiform nucleus; the cytoplasm was largely or entirely filled by lipid droplets.

After intravenous injection, autologous radiolabeled native LDL was detected in atherosclerotic plaques by light microscopy autoradiography. Deposition of radioactivity was detected in specimens obtained 24 hours after LDL injection (Figure 1) and was seen in samples obtained 72 hours after LDL injection (not shown).

Developed silver grains in number**s** exceeding that of the background were always present over the foam cells of the cap and shoulder. The grain density over the foam cells ranged from 5.88 to 9.05/100 μm^2, compared with 0.95/100 μm^2 of background. Radiolabeled LDL was observed within the cytoplasm, whereas extracellular deposition was negligible (Figure 1, A and B). Most of the labeled foam cells were CD68 positive (Figure 1C and Figure 2), whereas few cells were α-actin positive (Figure 1D and 2).

No difference in radiolabeling was observed in the lipid core, compared with the grain density of background (Figure 1E).

Localization of radioactivity within cells but not in the lipid core suggested that LDL distribution within the atherosclerotic plaque was specific. This suggestion was corroborated by demonstrating that very low radioactivity was accumulated within the atherosclerotic plaque after intravenous injection of radiolabeled human serum albumin (Figure 1F). However, albumin radioactivity was comparable to the background radiation seen with LDL infusion, and the overall radioactivity accumulated with albumin infusion was equally distributed in cells and extracellular areas.

Because native LDL generally does not accumulate in macrophage-derived foam cells,[17] our findings would imply that native LDL has probably undergone oxidative modification and was then taken up by macrophages. We could exclude that LDL underwent oxidative modification in vitro because after the radiolabeling, no formation of malondialdehyde equivalents and lipid hydroperoxides was detected. Furthermore, no electrophoretic mobility modification was observed in radiolabeled LDL compared with freshly separated LDL (not shown).

To further explore this hypothesis, we measured macrophage LDL uptake in carotid plaques taken from patients given vitamin E (900 mg/d) for 4 weeks before procedure. We found that vitamin E–treated patients had significantly lower radiolabeled LDL content than did untreated patients (Figure 3). Serum vitamin E was 28±6.1 μmol/L at baseline and increased to 54±8.6 after vitamin E supplementation.

Discussion

The injection of radiolabeled autologous LDL has been used as a tool to study in vivo distribution and metabolism of LDL and for noninvasive imaging of atherosclerotic plaque in humans.[18,19] These studies have demonstrated uptake by atherosclerotic plaque of native and oxidized radiolabeled LDL with the use of in vivo tomographic scintigraphy.

However, to the best of our knowledge, no autoradiographic studies at the cellular level have been done to investigate the traffic of LDL in the atherosclerotic plaque. The investigation of this specific issue is of particular relevance to analyze the validity of the hypothesis that suggests that circulating LDL crosses the arterial wall and after oxidative modification is taken up by macrophages. As recently shown by Napoli et al,[20] LDL accumulation and oxidation within the vessel wall appears to precede monocyte recruitment and foam cell formation. Oxidation of LDL would play an important role in this event because a coexistence of oxidized LDL and macrophages was detected in the vast majority of atherogenic sites. The perpetuation of this sequence of events is likely to play a crucial role in the progression of the fatty streak to a more advanced lesion. This also could be deduced by interventional studies with lipid-lowering drugs, which demonstrated regression of atherosclerotic lesions in humans after reduction of serum LDL cholesterol.[21] On the basis of these assumptions, the progression of atherosclerotic plaque would occur as a consequence of continuous accumulation of LDL, but the dynamic sequence of this event has never been demonstrated in humans. We demonstrated that after intravenous injection, native LDL accumulated minimally in the extracellular compartment, whereas the vast majority was detected within foam cells. The uptake of LDL by macrophages was relatively rapid because LDL accumulated intracellularly 24 hours after injection. This localization was not casual for 2 reasons. First, atherosclerotic areas depleted of foam cells showed negligible radioactivity. Second, the intravenous injection of radiolabeled human albumin was not followed by specific accumulation of radioactivity in the plaque.

On the basis of current knowledge, it is believed that macrophages express abundant scavenger receptors that recognize only modified LDL, such as oxidized LDL but not native LDL.[1,2,17] Thus it could be suggested that intracellular deposition of radiolabeled LDL after intravenous injection occurred as a consequence of oxidative modification of LDL.

Although it seems logical that accumulation of LDL within the foam cells may be explained only by assuming the oxidative change of LDL, it remains to be established where and how LDL is oxidized, that is, before or after crossing the vessel wall. As far as the first point is concerned, we can reasonably exclude that circulating LDL was already oxidized before crossing the arterial wall. We used a fast procedure to separate LDL from plasma, and LDL did not show any sign of oxidative modification before injection. The possibility of oxidation after injection, during circulation of LDL in the blood compartment and before crossing the vessel wall, could be excluded considering the abundance of antioxidants present in plasma.[22] From these data, it can be deduced that LDL was not oxidized in the circulation system but very likely after crossing the arterial wall. Further support for the hypothesis that LDL uptake by macrophages occurred as a consequence of oxidation was provided by the interventional study with antioxidants. Thus we observed that after treatment with vitamin E, uptake of radiolabeled
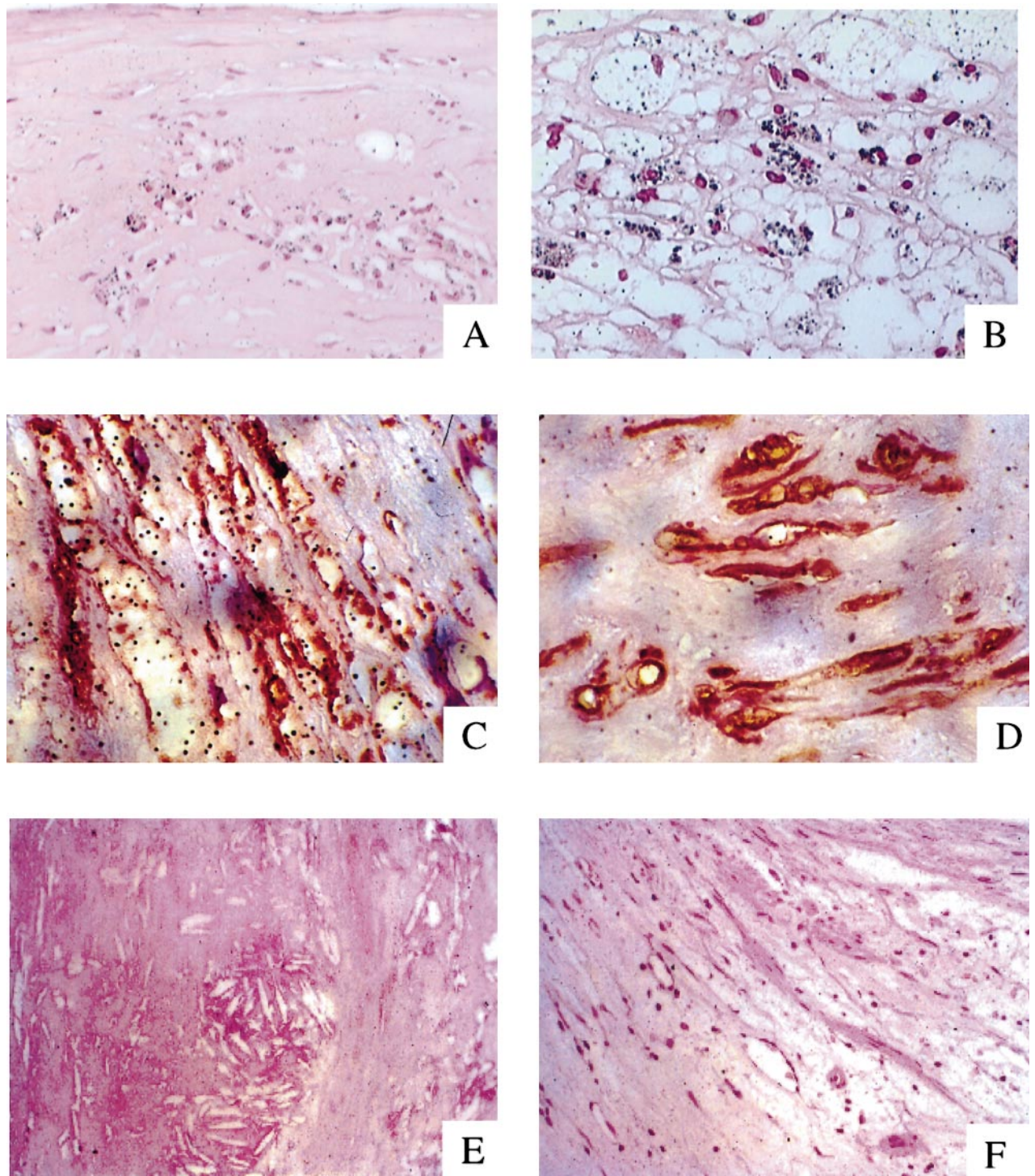
Figure 1. Autoradiography and immunohistochemical autoradiography of atherosclerotic plaques of patients injected with radiolabeled LDL or radiolabeled human serum albumin. A, Autoradiography. Foam cells infiltrating fibrous cap of an atherosclerotic plaque of patient injected 24 hours before endarterectomy with native [125I]-LDL. Foam cells are strongly labeled by silver grains (×20). B, Autoradiography (higher magnification). Silver grains specifically label cytoplasm of foam cells. Very low background is present (×50). C, Immunohistochemical autoradiography. Silver-labeled foam cells show clear-cut positivity for CD68 monoclonal antibody (×50). D, Immunohistochemical autoradiography. α-Smooth muscle–positive cells show very faint label like that of background (×50). E, Autoradiography. Atheromatous core of plaque of patient injected 24 hours before endarterectomy with native [125I]-LDL shows negative label at autoradiography (×50). F, Autoradiography. No label is detected in foam cells infiltrating fibrous cap of patient injected with [125I]-albumin (×50).

LDL by plaque-resident macrophages was almost completely suppressed.

This suggestion is strongly corroborated by an experimental study in which Calara et al[23] evaluated the localization of native radiolabeled LDL after intravenous or intraperitoneal injection in Sprague-Dawley rats. With the use of antibodies against Apolipoprotein B and epitopes present on oxidized LDL, these investigators localized
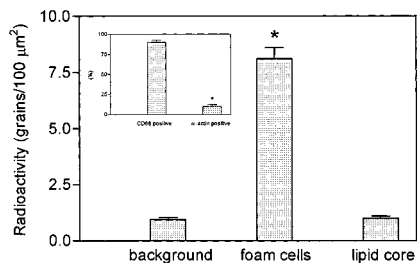
Figure 2. Quantitative evaluation of radiolabeled LDL accumulation in various regions of atherosclerotic plaque. Silver grain density was determined by counting the number of silver grains per surface area with an image analyzer (see Methods section for details). Inset shows immunohistochemical evaluation with anti-CD68 and anti–α-actin antibodies, which revealed that foam cells that accumulated the radiotracer were mostly derived from the monocyte-macrophage line (y-axis represents percentage of cells; filled bar, CD68 positive cells; and open bar, α-actin–positive cells). Each bar represents mean±SE. *P<0.0001, foam cells vs background and vs lipid core.

LDL and oxidized LDL in the arterial endothelium and media within 6 hours of injection; accumulation peaked at 12 hours for native LDL and 24 hours for oxidized LDL. These investigators also demonstrated a reduced number of epitopes present on oxidized LDL in the case of injection of LDL enriched by the antioxidant probucol. Our findings showing that vitamin E inhibits LDL uptake by macrophages in atherosclerotic plaque might have potential clinical implications because they represent an important approach to the prevention of progression of atherosclerosis. Consistent with this suggestion is the demonstration
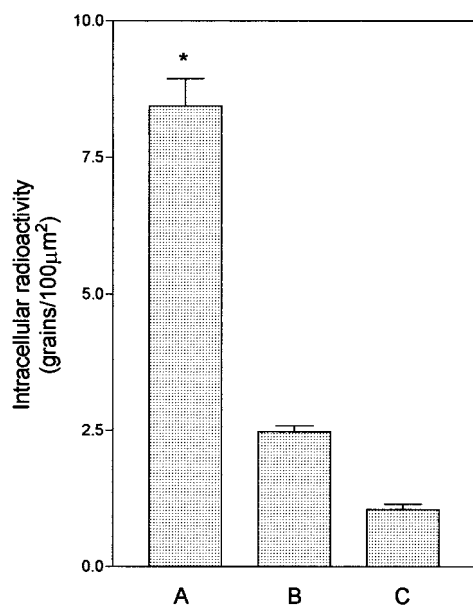
that vitamin E supplementation reduces atherosclerotic lesions in an experimental model of Apolipoprotein E–deficient mice. Praticò et al[24] found that this atherosclerotic model is associated with enhanced oxidative stress and that vitamin E supplementation reduces aortic lesions coincidentally with inhibition of oxidative stress.

In conclusion, we showed that circulating LDL accumulates within the foam cells of human atherosclerotic plaque. This process is likely to occur after oxidative modification of LDL within the vessel wall, but further study is necessary to fully elucidate the exact sequence of events leading to intracellular LDL accumulation.

Acknowledgments

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Figure 3. Effect of vitamin E treatment on uptake of radiolabeled LDL in atherosclerotic plaque. Radioactivity was determined by counting the number of silver grains (mean±SE) in cytoplasm with an image analyzer (see Methods section for details). A, Plaque macrophages (n=80) of patients injected with [^{125}I]-LDL; B, plaque macrophages (n=60) of patients injected with [^{125}I]-LDL and treated with vitamin E (900 mg/d) in previous 4 weeks; and C, plaque macrophages (n=60) of patients injected with [^{125}I]–human serum albumin. *P<0.0001, B vs A and vs C.

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