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7β-Hydroperoxycholest-5-en-3β-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low density lipoprotein

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ABSTRACT **Modification of low density lipoprotein (LDL) by free radical oxidation renders this molecular complex cytotoxic. Oxidized lipoproteins exist *in vivo* in atherosclerotic lesions and in the plasma of diabetic animals, suggesting that lipoprotein-induced tissue damage may occur in certain diseases. We undertook purification and identification of the major cytotoxin in oxidized LDL. The lipid extract from oxidized LDL was subjected to multiple HPLC separations, and the fractions were assayed for cytotoxicity. Mass spectrometry and nuclear magnetic resonance identified the purified toxin as 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH-Chol). This molecule accounted for approximately 90% of the cytotoxicity of the lipids of oxidized LDL. We also found 7β-OOH-Chol in human atherosclerotic lesions from endarterectomy specimens obtained immediately after excision. These results are consistent with the hypothesis that the oxidized LDL present in lesions has the capacity to induce cell and tissue injury, leading to progression of the disease and the generation of the necrotic core of the lesion.**

Vascular cells are susceptible to the toxic effects of low density lipoprotein (LDL) or very low density lipoprotein (VLDL) after modification of the lipoproteins by free radical oxidation (1–4). Oxidation of LDL leads to cytotoxin formation whether oxidation is mediated by lipoxygenases (5), metal ions (6), or ultraviolet irradiation (7) in cell-free systems or by the action of free radicals from cultured endothelial cells (8), vascular smooth muscle cells (8), neutrophils (9), or stimulated human monocytes (10). The cytotoxic moiety of oxidized LDL (oxLDL) is extractable with organic solvents (4), and numerous candidate substances could be proposed to explain its toxic action (11).

Oxidized lipoproteins occur *in vivo* in vascular lesions (12–14) and in plasma of certain diabetic subjects (15). OxLDL has been proposed to play a causal role in atherosclerosis (11, 16–18). The oxidized lipoproteins found *in vivo* exhibit similar cytotoxic properties to lipoproteins oxidized *in vitro* (15, 19); therefore, toxic, oxidized lipoproteins could theoretically participate in cell damage associated with diabetes or atherosclerosis. In this study, we sought to identify the principal toxin(s) in oxLDL. Identification of the toxin(s) is an important step toward testing evolving theories of atherogenesis that include lipoprotein oxidation (11, 16–18) and vascular injury (20, 21) as putative early events.

METHODS

Human LDL and lipoprotein-deficient serum were isolated by sequential ultracentrifugation (6, 22). LDL was oxidized by dialysis against 2–6 μM cupric sulfate for various times (23). OxLDL preparations were dialyzed against 0.15 M NaCl/0.5 mM EDTA, pH 8.5, to remove cupric ions. Relative oxidation, measured as thiobarbituric acid reactivity (6, 24), was equivalent to 4–6 nmol of malondialdehyde (MDA) per mg of LDL cholesterol.

Human foreskin fibroblasts were plated in a 1:1 (vol/vol) mixture of Dulbecco's minimal essential medium and Ham's F-12 medium (DME/F-12) supplemented with 5% (vol/vol) fetal bovine serum (6) and 0.2 μCi (7.4 kBq) of [^{14}C]adenine per ml. Cytotoxicity was measured as the specific ^{14}C released into the culture medium (25). LDL lipid fractions in acetone/ethanol, 1:1 (vol/vol), were added to culture media at desired concentrations. The final organic solvent concentration was 1.0%. Media containing lipoprotein-deficient serum (6) and LDL lipid fractions were added to cells for 66–72 hr. Lipid fractions were specified in terms of total cholesterol equivalents of the original lipoprotein.

Aliquots of 5 mg of native LDL or oxLDL were lyophilized overnight, and the lipid was extracted with 5 ml of acetone. The mixture was sonicated for 10–20 sec, mixed for 1 min, and allowed to stand for 20 min. After centrifugation at 1000 × *g* for 10–20 min, the residue was reextracted twice, as above. Preliminary experiments revealed that the recovery of the cytotoxic activity by this extraction procedure was equivalent or superior to other means, including chloroform/methanol extraction of aqueous lipoprotein solutions. Pooled extracts were dried under nitrogen and redissolved in isopropanol/acetonitrile, 1:1 (vol/vol). After centrifugation for 5 min at 1000 × *g*, the supernatant was analyzed by reverse-phase HPLC (Waters μBondapak C_{18} preparative column). The solvent gradient for elution consisted of water/acetonitrile, 1:1 (vol/vol), which was increased over 5 min to 100% acetonitrile and then was changed to 100% isopropanol over 45 min. The flow rate was 5 ml/min. The dominant toxic fraction from reverse-phase HPLC was dried under nitrogen, resuspended in 0.5 ml of elution solvent (3% isopropanol in hexane), and applied to a μBondapak CN column (normal phase; Waters); the flow rate was 1 ml/min.

Proton and carbon NMR were carried out in monodeuterated chloroform or hexadeuterated benzene (MSD Isotopes) on a Bruker AM-300 WB NMR spectrometer. Chemical shifts are given in ppm referenced to tetramethylsilane (0 ppm). Coupling constants, *J*, are in Hertz (Hz). Mass spectrometric

Abbreviations: LDL, low density lipoprotein; oxLDL, oxidized LDL; 7β-OOH-Chol, 7β-hydroperoxycholest-5-en-3β-ol or 7β-hydroperoxycholesterol; TMS, trimethylsilane; NOE, nuclear Overhauser enhancement; PCI, positive chemical ionization.

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analyses were performed on a TSQ-70 mass spectrometer (Finnigan-MAT, San Jose, CA). Methane was the reagent gas for positive chemical ionization (PCI). Gas chromatography (GC) was performed on a Varian 3400 gas chromatograph equipped with a 30 m × 0.32 mm i.d. DB-17 fused silica capillary column (J & W Scientific, Rancho Cordova, CA) having a 0.15-μm film thickness. Typical GC parameters were 15 psi (psi = 6.89 kPa) of hydrogen carrier gas and splitless injection at an oven temperature of 180°C, which was increased to 230°C at 25°C/min, to 284°C at 6°C/min, and to 290°C at 1.2°C/min.

Trimethylsilyl (TMS) ethers were formed by incubating samples in Tri-Sil-TBT (Pierce) for 30 min at 60°C. The product was extracted with 150 μl of hexane and 50 μl of water, and the organic layer was dried under nitrogen. In some experiments, samples were reduced in 50 μl of methanol containing 0.1 mg of sodium borohydride overnight at room temperature. The reduced compound was extracted, dried, and derivatized as described above. 7α- and 7β-hydroxycholesterol were from Steraloids (Wilton, NH).

Freshly excised atherosclerotic lesions, obtained at carotid endarterectomy, were immediately placed into 0.15 M NaCl (pH 9.0) containing 1 mM EDTA, 0.1 mM butylated hydroxytoluene (BHT), 100 units of penicillin per ml, and 100 μg of streptomycin per ml; rinsed three times to remove residual blood; then frozen in fresh medium containing EDTA, BHT, penicillin, and streptomycin; and stored at −70°C under argon. After lyophilization samples were suspended in acetone containing 0.1 mM BHT and minced under a constant stream of nitrogen. The suspension was mixed in a Vortex mixer for 5 min and centrifuged at 750 × *g* at 5°C for 20 min. After three extractions, supernatants were evaporated under nitrogen. Extracts were stored at −70°C in chloroform containing 5 μM BHT until either HPLC (as above) or TLC on silica plates was performed under nitrogen with 1:1 (vol/vol) heptane/ethyl acetate as the developing solvent. Oxysterol bands from TLC were visualized with iodine vapors and compared with standards. Fractions from HPLC were subjected to TLC.

To determine if free cholesterol in lesions was oxidized during isolation, [^{14}C]cholesterol was injected into tissues immediately after excision (or was added to solvents without tissue) and subjected to our procedures. Final and initial amounts of labeled oxysterols could thus be compared.

RESULTS

Lipids extracted from native LDL and oxLDL were subjected to reverse-phase HPLC. Multiple qualitative and quantitative changes take place in LDL lipids upon oxidation (Fig. 1 *A* and *B*). Of particular interest was a fraction, "peak A" (retention time, 23.1 min), which appeared in oxLDL lipids, but not in native LDL lipids, and which contained more cytotoxic activity than other fractions (Fig. 1*C*). We subjected peak A to normal-phase HPLC (Fig. 2*A*) and tested fractions for cytotoxicity. The largest peak, "A2," contained most of the toxic activity (data not shown). The two dominant toxins, fractions A1 and A2, were individually rechromatographed. Fig. 2*B* displays relative toxicities of these subfractions relative to the total lipid extract. The extent to which A2 accounted for the total toxicity of the oxLDL lipid extract could be estimated by comparing the concentrations required

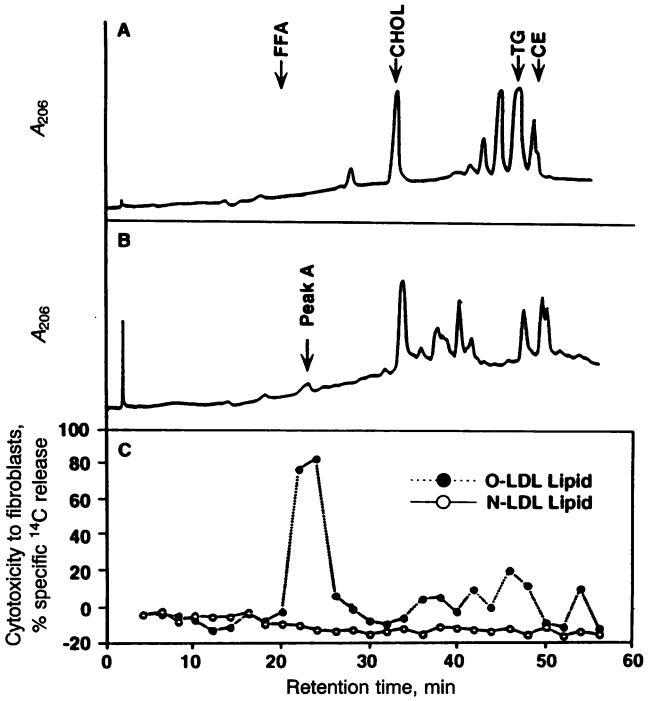

FIG. 1. Reverse-phase HPLC and relative cytotoxicity of native LDL and oxLDL lipids. (*A* and *B*) Extracts from native LDL (*A*) or oxLDL (*B*) were eluted from HPLC. Standards eluted in separate runs are shown by arrows for oleic acid (FFA), cholesterol (CHOL), triolein (TG), and cholesteryl docosahexaenoic acid (CE). (*C*) Relative toxicities of fractions from native LDL (N-LDL) or oxLDL (O-LDL) lipids added to fibroblast cultures at a concentration corresponding to 0.4 mg of cholesterol equivalents/ml of original native LDL or oxLDL (i.e., one ml of medium added to cells contained the designated elution fraction from 0.4 mg of cholesterol equivalents of total lipoprotein lipid). "Peak A" in *B* contained the dominant toxic fractions in *C* of oxLDL lipids.

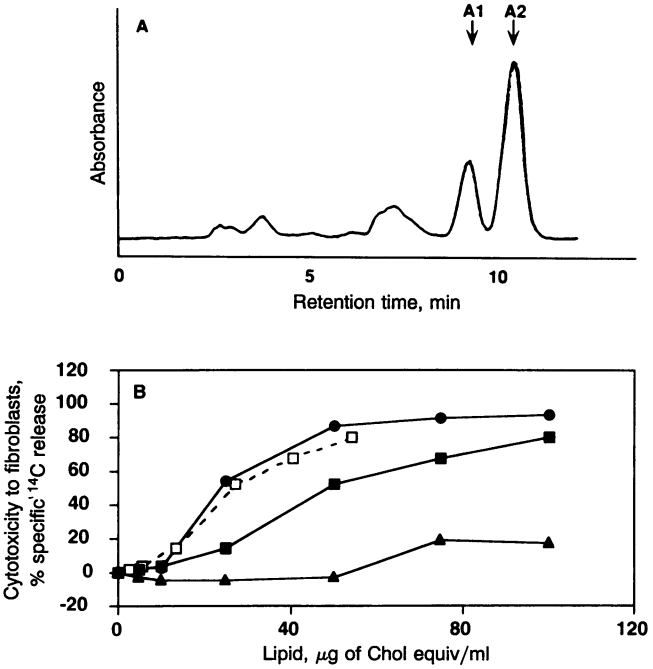

FIG. 2. Normal phase HPLC and toxicities of lipids from "peak A." (*A*) The dominant toxic fraction from reverse-phase HPLC of oxLDL lipids (Fig. 1) was resolved further by normal-phase HPLC (absorbance at 206 nm). (*B*) Toxicities (see Fig. 1 legend and *Methods*) are shown for the two most toxic fractions in *A*, designated A1 (▲) and A2 (■), relative to the toxicity of the total oxLDL lipids (●). Concentration is expressed as cholesterol equivalents of the original oxLDL sample (see Fig. 1 legend and *Methods*). The lipid concentrations indicated by ■ and ▲ were maxima based on a hypothetical 100% recovery; concentrations indicated by ■ were multiplied by 0.55 (recovery was 55%; see *Results*) to correct the equivalent concentration of A2 for preparative losses. □ and —— show corrected values.

to yield half-maximal cell death. When 100% recovery from HPLC was assumed, the toxicity of fraction A2 relative to the total lipids in the experiment shown was 47% and averaged 52% in four experiments performed with different preparations of oxLDL. When corrected for losses during isolation, which averaged 45% (see below), fraction A2 accounted for about 90% of the cytotoxic activity of the total lipid extract as illustrated in Fig. 2*B* (dashed line).

Proton and carbon NMR (Fig. 3) indicated that the structure of A2 was similar to that of cholesterol. Decoupling experiments and proton-difference NOE spectra (see the legend of Fig. 3) suggested a change at carbon-7 (C-7), which was supported by carbon NMR data. Indeed, loss of 6-Hz coupling to the hydrogen on carbon-6 (H-6) indicated the substitution of a C-7 hydrogen with a heteroatom, presumably oxygen because of chemical shifts of the "new" proton (4.13 ppm) and "new" carbon (86.61 ppm). The NOE established a spatial connection between H-6 and the proton at δ4.13, and since the positions of the only other possible NOE enhancements are known (H-4 and H-14), these data strongly suggested that the "new" proton was at position 7. Decoupling experiments (see the legend of Fig. 3) showed that the altered H-7 proton was axial (β position). Because of the absence of any additional protons or carbons as compared with cholesterol, structural possibilities included an epoxide, alcohol, or hydroperoxide [the proton NMR spectrum of 7α-hydroperoxycholest-5-en-3α-ol has been reported (26)]. Neither infrared spectra (not shown) nor NMR data supported an epoxide, and analysis of the TMS derivative of the compound by PCI mass spectrometry yielded spectra that differed from that of the di-TMS ether of 7-hydroxycholesterol, indicating that the unknown was not a diol (data not shown). The presence of a hydroperoxy group at C-7 was verified by reduction of the compound with sodium borohydride to form a diol. Trimethylsilylation of the diol produced a di-TMS ether that had a PCI mass spectrum and GC retention time identical to that of 7β-hydroxycholesterol (Fig. 4). Thus, the unreduced compound was 7β-hydroperoxycholest-5-en-3β-ol (7-hydroperoxycholesterol or 7β-OOH-Chol). Having identified the cytotoxin as 7β-OOH-Chol, we were then able to adopt HPLC or TLC schemes to determine the presence of the compound in sample extracts.

The amount of 7β-OOH-Chol in oxLDL was determined by adding [^{14}C]cholesterol to native LDL, oxidizing the LDL, and isolating 7β-OOH-[^{14}C]Chol by HPLC. This indicated that approximately 5% of LDL free cholesterol was oxidized to 7β-OOH-Chol (correcting for losses during isolation as given below). This varied with the degree of oxidation of the LDL (data not shown). The 7β-OOH-[^{14}C]Chol was added to the lipid extract of oxLDL and was used to assess recovery during HPLC isolation; 67%, 58%, and 55% of the labeled 7β-OOH-Chol were recovered after the C_{18} step and the first and second normal-phase runs, respectively (average of two experiments). This recovery was used to correct the degree to which cholesterol was converted to 7β-OOH-Chol and the degree to which the cytotoxicity of the oxLDL lipids was attributable to 7β-OOH-Chol (see above). The concentration of 7β-OOH-Chol resulting in half-maximal toxicity was approximately 0.6 μg/ml.

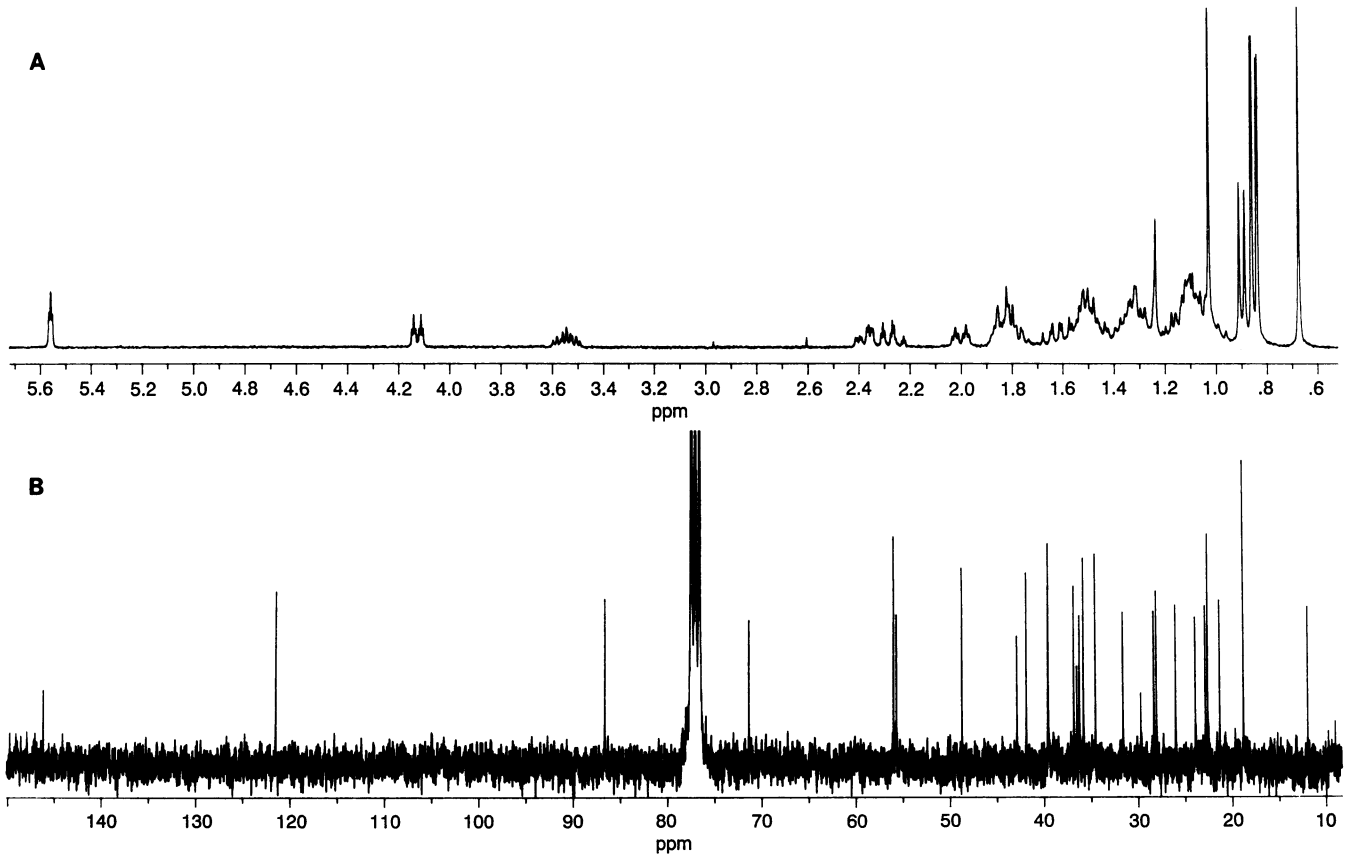

FIG. 3. The proton NMR spectrum. Fraction A2 in monodeuterated chloroform resembled cholesterol; however, H-6 (δ 5.56, 1H, dd, J = 2.09, 1.71) was shifted downfield by 0.24 ppm and lost a 6-Hz coupling. A "new" proton observed at δ4.13 (1H, ddd, J = 8.72, 2.09, and 1.99) was shown by a proton-difference Nuclear Overhauser Enhancement (NOE) experiment (not shown) to be spatially close (within 3–5 Å) to H-6. A homonuclear decoupling experiment (not shown) with irradiation at δ4.13 verified a scalar coupling with H-6 of 2.09 Hz and with the axial H-8 (δ 1.63) of 8.72 Hz, indicating an axial orientation. (*B*) The proton-decoupled carbon NMR spectrum exhibited 27 carbon resonances, the majority of which matched well with cholesterol. The major difference was the appearance of a methine carbon at 86.61 ppm, which coincided with the disappearance of the methylene C-7 ($\delta_{cholesterol}$ = 31.6).

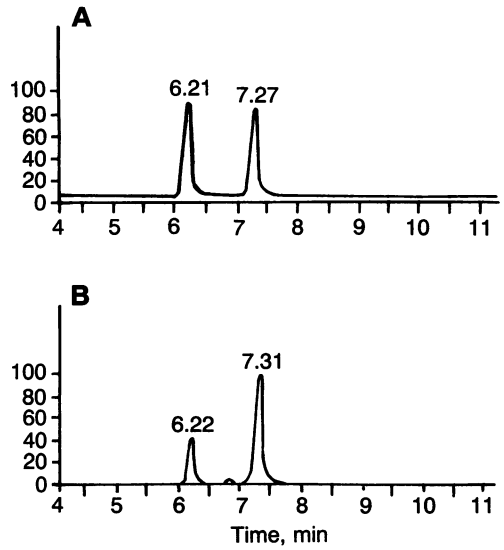

FIG. 4. GC of the TMS ether of the reduced cytotoxin. Fraction A2 was reduced with sodium borohydride, derivatized with Tri-Sil-TBT (Pierce), separated by GC, and evaluated by PCI mass spectrometry. The TMS derivative had a spectrum identical to that of the di-TMS ether of 7-hydroxycholesterol. (*A*) A chromatogram of a sample containing TMS ethers of authentic 7α- and 7β-hydroxycholesterol (retention times of 6:21 and 7:27 min, respectively). (*B*) When the TMS ether of the reduced unknown was added to this sample, the peak containing authentic 7β-hydroxycholesterol was enlarged, indicating that the structure of fraction A2 prior to reduction and derivitization was 7β-OOH-Chol.

We asked if 7β-OOH-Chol is present *in vivo* in human atherosclerotic lesions. Endarterectomy specimens were collected in antioxidants immediately upon excision from human carotid arteries. These were minced and then lyophilized and extracted, and the lipoproteins were separated as before. 7β-OOH-Chol was readily detected by TLC in five of five specimens for which total cholesterol of the total lesion extract exceeded 0.32 mg and in five of five specimens subjected to HPLC isolation for which total cholesterol of the lesion extract exceeded 2.23 mg (see Fig. 5).

To determine if postexcision procedures artifactually produced 7β-OOH-Chol or its degradation products from free cholesterol residing in the lesion, we injected [^{14}C]cholesterol into the lesion at the time of acquisition. We quantified the labeled HPLC fractions appearing (by TLC) as 7α- or β-OOH-Chol, 7α- or 7β-hydroxycholesterol, or 7-ketocholesterol in the original injectate and final extract. The initial labeled injectate contained 0.26% (15 fmol) of labeled cholesterol as the sum of the hydroperoxide, alcohol, and keto forms. After lyophilization, extraction, and separation, 0.20% of the labeled cholesterol recovered (11.8 fmol) was detected in these oxidized forms. Thus, no detectable formation of these oxysterols occurred during processing. The decrease in the labeled oxysterol recovered likely represented losses of oxysterols analogous to loss of the labeled 7β-OOH-Chol during HPLC alluded to above. No additional formation of the hydroperoxides or their degradation products could be detected in the solvents in the absence of tissue when [^{14}C]cholesterol was carried through the isolation procedure.

DISCUSSION

Findings demonstrating that oxidized forms of LDL exist *in vivo* in atherosclerotic lesions (14, 27) highlight the potential for their effects on surrounding cells in the intima. We have hypothesized that the capacity of oxLDL to injure cells could

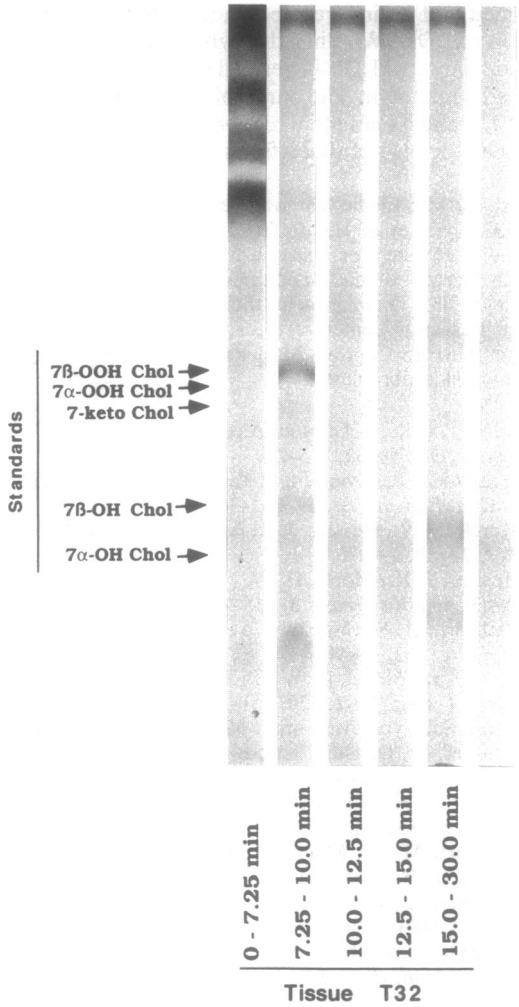

FIG. 5. TLC of extracts from human atherosclerotic lesions. TLC was performed on five normal-phase HPLC fractions containing various oxysterol components extracted from a human atherosclerotic lesion obtained during endarterectomy. The presence of 7β-OOH-Chol was noted and was distinct from 7α-hydroxycholesterol (7α-OH Chol), 7β-hydroxycholesterol, and 7-ketocholesterol as shown and from 25-hydroxycholesterol, epoxycholesterol, and cholestane triol (not shown).

potentially worsen atherosclerosis by two means (11, 21)—injury to endothelium leading to cellular dysfunction (20) and cell injury leading to dead cell debris in the necrotic core of advanced lesions.

We have demonstrated that 7β-OOH-Chol exists in human atherosclerotic lesions; it has been identified previously (28) *in vivo* in rat skin and was observed to increase with age. It has been shown to be an intermediate in the nonenzymatic oxidation of cholesterol that leads to the formation of 7β-hydroxycholesterol, 7α-hydroxycholesterol, and 7-ketocholesterol (29, 30), all of which have been observed *in vivo* (31). 7β-OOH-Chol is known to form if cholesterol is exposed to soybean lipoxygenase acting on its substrate (32, 33). The presence of 15-lipoxygenase activity has been inferred in atherosclerotic lesions (34, 35), offering a possible mechanism for the formation of 7β-OOH-Chol in lesions.

Numerous toxins are formed during LDL oxidation. 7α-Hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, epoxycholesterols (36, 37), malondialdehyde (38) 4-hydroxynonenal (38), and lysophosphatidylcholine (39) are formed upon the oxidation of LDL, and each has been shown to be cytotoxic (11, 21, 31, 40, 41). However, our results suggest that, based on the relative level of 7β-OOH-Chol

produced and its inherent potency, it is the dominant cytotoxin in oxLDL. Whether the cytotoxicity of 7β-OOH-Chol in particular or oxLDL in general plays a role in cell injury in lesions is unknown. The study of the mechanism of toxicity of oxLDL and its potential role *in vivo* can be examined more precisely because of these findings.

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