Presence of Hypochlorite-modified Proteins in Human Atherosclerotic Lesions

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Abstract

Oxidation of LDL may contribute to atherogenesis, though the nature of the in vivo oxidant(s) remains obscure. Myeloperoxidase, the enzyme responsible for hypochlorous acid/hypochlorite (HOCl) production in vivo, is present in active form in human atherosclerotic lesions, and HOCl aggregates and transforms LDL into a high-uptake form for macrophages in vitro. Here we demonstrate HOCl-modified proteins in human lesions using an mAb raised against HOCl-modified LDL that recognizes HOCl-oxidized proteins but does not cross-react with Cu^{2+}-, malondialdehyde-, or 4-hydroxynonenal–modified LDL. This antibody detected significantly more material in advanced atherosclerotic lesions than normal arteries, even though azide and methionine were included during sample work-up to inhibit myeloperoxidase and to scavenge HOCl. The epitope(s) recognized was predominantly cell associated and present in monocyte/macrophages, smooth muscle, and endothelial cells. The intima and cholesterol clefts stained more heavily than the center of the thickened vessels; adventitial staining was apparent in some cases. Immunostaining was also detected in a very early lesion from an accident victim, beside healthy areas that were unreactive. LDL oxidized by HOCl in vitro, but not native LDL, effectively competed with the epitopes in lesions for antibody binding. Density centrifugation of plaque homogenates and Western blot analysis showed that, in the apo B–containing lipoprotein fraction, the mAb recognized protein(s) of molecular mass greater than apo B, similar to those produced during oxidation of LDL with HOCl in vitro. Three major proteins were recognized by the anti-HOCl–modified protein antibody but not by an anti–apo B antibody in the apo B–free fraction. Together, these results demonstrate HOCl-oxidized proteins in human atherosclerotic lesions, implicating this oxidant in LDL modification in vivo. (*J. Clin. Invest.* 1996. 97:1535–1544.) Key words: antioxidants • atherogenesis • oxidation • low density lipoprotein • myeloperoxidase

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Introduction

Oxidation of LDL may contribute to the early stages of atherogenesis (1–3). Evidence for the occurrence of oxidized LDL in vivo includes localization of material recognized by antibodies directed against various types of oxidatively modified LDL in situ. Previous studies describe antibodies raised against in vitro copper-oxidized LDL (Cu-ox-LDL)[1] or LDL modified by end products of lipid peroxidation, such as malondialdehyde (MDA) or 4-hydroxynonenal (HNE), as these reactive compounds can convert native LDL into an atherogenic form. Atherosclerotic lesions of varying severity from Watanabe Heritable Hyperlipidemic rabbits, and foam cells and LDL isolated from these lesions, all contain material recognized by mAbs raised against MDA-, HNE-, and Cu-ox-LDL (4–7). Diseased aortas from human autopsy specimens also react with a polyclonal antibody recognizing Cu-ox- and HNE-LDL (8), and plasma of humans with established cardiovascular disease contains epitopes recognized by polyclonal antibodies raised against MDA-modified LDL (MDA-LDL) (9), although there is no evidence for MDA-LDL in plasma of Watanabe rabbits (4). Autoantibodies against MDA-LDL were found in human and rabbit sera, although there is controversy concerning the relationship between these autoantibodies and atherosclerosis (10, 11).

Despite much interest in the oxidation theory of atherosclerosis, little progress has been made in the identification of the putative in vivo oxidant of LDL. Although it is commonly assumed that lipid peroxidation precedes, and to some extent causes, oxidative modification of apolipoprotein B-100, hypochlorite (HOCl) can transform the lipoprotein into a high uptake form without significant lipid (per)oxidation. Apo B-100, the single major protein associated with LDL, is the main target for this oxidant (12, 13) and becomes aggregated and cross-linked (13, 14). HOCl is an oxidant produced from H_2O_2 and chloride by myeloperoxidase (MPO, E.C. 1.11.1.7) present in neutrophils and monocytes. The latter infiltrate the intima from the earliest stages of atherosclerosis (15). Importantly, human lesions contain large amounts of active MPO (16), making HOCl a candidate oxidant for in vivo modification of LDL.

To investigate the potential presence of HOCl-modified

1. *Abbreviations used in this paper:* Cu-ox-LDL, copper oxidized LDL; HNE, 4-hydroxynonenal; HNE-LDL, HNE-modified LDL; HOCl, hypochlorite or hypochlorous acid, since both forms are present at pH 7.4; HOP-1, a murine mAb specific for hypochlorite-oxidized protein; MDA, malondialdehyde; MDA-LDL, MDA-modified LDL; MPO, myeloperoxidase; PTAH, phosphotungstic acid/hematoxylin; SMC, smooth muscle cells; TPBS, Tween PBS.

LDL in human atherosclerotic lesions, we used an mAb (defined as HOP-1) that specifically recognizes HOCl-modified proteins including LDL but does not cross-react with native LDL or other forms of oxidatively modified LDL, including Cu^{2+}-, MDA-, or HNE-LDL (17). We report here that this antibody recognizes several proteins in human atherosclerotic arteries, including material present in the apo B-100–containing lipoprotein fraction of plaque homogenate. Our results indicate that oxidation of LDL and other proteins by HOCl may indeed contribute to atherogenesis.

Methods

Materials. Nanopure or Milli-Q water was used for all buffers and aqueous solutions. Bicinchoninic acid was obtained from Sigma Chemical Co. (St. Louis, MO), and reagent HOCl (5% available chlorine minimum) was from Aldrich Chemical Co. (Milwaukee, WI). PBS1 (50 mM phosphate, 150 mM Cl^-, pH 7.4) was used with EDTA (0.1% wt/vol) for LDL isolation and without EDTA for gel filtration, resuspension, and oxidation of the lipoprotein. Before its use, PBS1 was treated with Chelex-100 (Bio-Rad Laboratories, Richmond, CA) to remove contaminating transition metals. PBS2 (PBS1 including 1 mM EDTA, 200 mM methionine, 0.1 mM sodium azide, pH 7.4) or carbonate buffer (100 mM Na_2CO_3, 1 mM EDTA, 200 mM methionine, 0.1 mM azide, pH 11.5) was used for normal artery and plaque homogenization. Tween PBS (TPBS, 9.5 mM PO_4^{-3}, 140 mM Cl^-, 0.5 ml/liter Tween-20 (Merck, Darmstadt, Germany), 10 g/liter BSA (Sigma Chemical Co.; pH 7.4) was used for antibody dilutions and washes during probing of nitrocellulose. Antibody dilutions for immunohistochemistry and washes were carried out in Tris-HCl buffer (0.1 M, pH 7.4). The primary mouse mAb (HOP-1, IgG_{2b}, referred to previously (17) as mAb B: clone 2D10G9), was raised against HOCl-modified LDL (800 molecules HOCl per LDL particle), and the myeloma supernatants were concentrated 20-fold by ammonium sulphate precipitation before use. The concentrate contained 22 mg protein/ml, of which 18.5% was IgG. Characterization of HOP-1 revealed it to be highly specific for HOCl-modified LDL and other HOCl-modified proteins (BSA, HSA [17], and HDL [Malle, E., and G. Waeg, unpublished observations]), and not cross-react with native LDL, LDL modified by Cu^{2+} oxidation, MDA, HNE (17), or peroxynitrite (Schwarz, W., L.J. Hazell, and R. Stocker, unpublished observations).

LDL preparation and oxidation. Blood was obtained from nonfasted, healthy male or female subjects (23–35 yr). LDL was rapidly isolated (2 h, 15°C) from fresh plasma by ultracentrifugation (18) and gel filtered (PD-10 column; Pharmacia LKB Biotechnology, Uppsala, Sweden), and its protein concentration was determined using bicinchoninic acid (19, 20) and BSA as a standard.

The concentration of the commercial HOCl solution was determined and oxidation of LDL was carried out as described previously (12), by addition of freshly diluted reagent HOCl to an LDL solution (0.5–2.0 mg protein/ml) at 4°C, followed by brief mixing (< 1 s). HOCl was used at a ratio of 400 or 800 molecules per LDL particle, and the HOCl-modified LDL (HOCl-LDL) was used within 3–4 mo. HOCl-LDL can be stored for this length of time at 4°C in transition metal–free PBS1 without loss of recognition by HOP-1 (Hazell, L.J., and G. Waeg, unpublished observations).

Sources of human tissue. Normal iliac arteries were obtained from liver transplant donors (accident victims) as control samples and human plaques from patients undergoing carotid endarterectomy. For quantitation of HOCl-oxidized protein(s) by slot-blot analysis, fresh samples from seven patients aged 45–71 yr (64±9 mean±SD, five males) and four normals aged 13–37 yr (24±11, all males) were used.

Immunohistochemistry was carried out on nine diseased and five control arterial samples, of which six diseased and three controls were fresh samples. The remainder was paraffin-embedded archival material, derived largely from postmortem samples. The results shown in Figs. 1–6 were derived from three fresh (from males aged 23, 72, and 74 yr) and one archival samples (from a female aged 59 yr); the severity of the lesions is described in the appropriate figure legend. Fresh samples of diseased and normal arteries were obtained and processed within ≤ 2 and ≤ 36 h, respectively. The postmortem sample (type IV atheroma) was taken 17 h after death from a coronary artery disease victim in 1976.

The fresh diseased samples were from patients ($n = 9$, seven males) of whom seven showed clinical signs of atherosclerosis in at least two major vascular beds. All patients were past or current smokers, six were hypercholesterolemic (one normocholesterolemic, two unknown) and seven were hypertensive (two normotensive). As expected for endarterectomy specimens, all nine samples contained advanced fibro-fatty lesions, although microscopic examination also revealed areas with less severe atherosclerosis. All samples were obtained by qualified hospital staff, and all procedures were approved by the local human ethics committee.

Lesion nomenclature. The nomenclature used in this manuscript for describing the severity of atheromatous lesions is based on that of Stary et al. (21). Type I: very early lesions; small isolated groups of macrophages containing lipid droplets, adaptive intimal thickening (often eccentric). Type II: early lesions; first grossly visible fatty streaks, macrophage foam cells in adjacent layers, intimal smooth muscle cells (SMC) also containing lipid droplets. Type III: intermediate lesions (also known as transitional lesions and preatheroma); visible extracellular lipid pools (multiple but separate, no lipid core) which may disrupt SMC layers. Type IV: advanced lesions; lipid core, intimal disorganization, arterial deformity. (Each increase in severity incorporates the features of the previous type, with new features mentioned only.)

Quantitation of HOCl-oxidized protein in homogenates. Arteries (30 or 100 mg wet tissue/ml PBS2 or carbonate buffer) were homogenized using a teflon piston (Fisons Scientific Equipment, Homebush, New South Wales, Australia) and glass homogenizer (Wheaton, Millville, NJ) for up to 4 h at 4°C, and were then stored at this temperature. To quantify HOCl-oxidized protein in lesions and normal arteries, homogenized tissues (50–150 μg protein/well) were bound to Hybond-C nitrocellulose (0.45 μm, Amersham International, Little Chalfont, UK) using a Bio-dot slot format apparatus (Bio-Rad Laboratories). Because of the particulate nature of plaque homogenates, the solutions often caused blockage of the pores of the nitrocellulose, and this was not overcome by a variety of denaturants. We therefore incubated plaque homogenates on the nitrocellulose for 2 h without flow through the membrane, and then rinsed the solution off the top of the membrane with PBS3 (9.5 mM phosphate, 140 mM Cl^-, pH 7.4). The apparatus was then dismantled, protein binding sites on the membrane were blocked by incubation with TPBS for 30 min, and the membrane was subsequently incubated with HOP-1 followed by the secondary antibody (peroxidase-conjugated goat anti–mouse IgG; Bio-Rad Laboratories) for 1 h each. Before use, HOP-1 and the secondary antibody were diluted in TPBS to 1:5,000 and 1:80,000, respectively. The membrane was washed thoroughly with TPBS after each incubation and given a final wash in PBS4 (2 mM NaH_2PO_4, 16 mM Na_2HPO_4, 150 mM NaCl). To visualize bound antibody, ECL-Western blotting detection reagents (Amersham International) and Hyperfilm-ECL (Amersham International) were used according to the manufacturer's instructions, and films were developed in an automatic processor. The films obtained were scanned (OmniMedia Scanner, XRS, Torrance, CA), and the integral of each band was measured using NIH-Image software (National Institutes of Health, Bethesda, MD) and standardized using dilutions of in vitro HOCl-oxidized LDL (800 molecules of HOCl per LDL particle) from 1–50 pM with a logarithmic line of best fit (R value $= > 0.95$). Results were expressed as mean±SD of different tissue samples, each analyzed in quadruplicate. For statistical analysis, the Wilcoxon two-sample rank sum test was used.

Partial characterization of HOCl-oxidized plaque proteins. Ultra-

centrifugation was used to separate an apo B–rich lipoprotein fraction from other proteins present in plaque. For this purpose, plaques (100 mg wet tissue/ml carbonate buffer) were homogenized as described above, the density was adjusted with KBr to $\rho = 1.35$ mg/ml, the homogenate layered under PBS2 and then centrifuged as described for the isolation of LDL (18). The top 2/3 and lower 1/3 fractions (excluding the pellet) of the gradient were collected, lyophilized, delipidated with chloroform/methanol (3:1, vol/vol), and washed with methanol followed by water before the remaining protein pellet was redissolved in 8% SDS. The resulting protein solutions ($\sim$ 3 mg/ml) were then separated by SDS-PAGE (2 h, 150 V) using Tris-glycine-SDS buffer (25 mM Tris, 190 mM glycine, 3.5 mM SDS, pH 8.3) on precast 4–15% gradient gels (Tris-HCl mini-protean II ready gels; Bio-Rad Laboratories). After separation, proteins were blotted onto nitrocellulose (1 h, 30 V) using a Mini-Blot module (Novex, San Diego, CA) and Tris-glycine buffer (12 mM Tris, 96 mM glycine). The membranes were blocked and probed with antibodies as described in the previous paragraph. Some nitrocellulose blots were probed with polyclonal sheep anti–human apo B antibody (1:4,000 dilution, Serotec, Oxford, England) and peroxidase-conjugated rabbit anti–sheep immunoglobulins (3:40,000 dilution, Dakopatts, Glostrup, Denmark) to detect apo B.

Tissue preparation and immunohistochemistry. Fresh human arteries were fixed overnight in 10% phosphate-buffered formaldehyde, and then transferred to 70% ethanol until use. Subsequently, they were dehydrated, cleared, and embedded in paraffin, and 5-μm sections were cut. To examine whether HOP-1–reactive material was produced during tissue fixation and embedding, 180 mM methionine, 0.9 mM EDTA, and 0.9 mM azide were added to the fixative used for some arteries.

Some sections were stained with Mallory's phosphotungstic acid/hematoxylin stain (PTAH) using a shortened, weaker (acidified 0.05% $KMnO_4$ for 15 s) rather than the full-strength bleaching procedure described in reference 22, to reduce the intensity of elastin staining while maintaining the majority of myofibril staining. For immunohistochemistry, tissue sections were deparaffinized, rehydrated, and incubated with 1% H_2O_2 in methanol (30 min) to block endogenous peroxidase activity and then incubated with 10% horse serum (CSL Diagnostics, Parkville, Australia) in Tris-HCl buffer (30 min) to block nonspecific binding before staining. HOP-1 was used at a 1:500 dilution of the concentrated IgG solution. For competition experiments, the competitor specified was added to the sections together with the primary antibody, at the concentrations indicated. Biotinylated anti–mouse IgG (Immunodiagnostic, Camperdown, New South Wales, Australia) was used as secondary antibody at a 1:200 dilution. A Vecta stain avidin-biotin kit (Immunodiagnostic) was used for amplification, and the antibody was visualized with conjugated horseradish peroxidase, hydrogen peroxide, and 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.), which gave a red-brown color. For the isotype control experiment, purified mouse myeloma IgG_{2b} (ICN ImmunoBiologicals, Lisle, IL) was used at the same IgG concentration as HOP-1. For identification of macrophages, sections were treated with a protease solution before incubation with horse serum, and then DAKO-CD68 (clone KP1, Dakopatts) was used as the primary antibody at 1:200 dilution. According to the specifications supplied by the manufacturer, this antibody recognizes macrophages in a wide variety of human tissues, also staining cases of chronic and acute myeloid leukemia and some B cell neoplasms. All sections stained immunologically were counterstained lightly with Harris' hematoxylin. To visualize hemosiderin, sections were stained with Perls' stain before hematoxylin. The reagent for Perls' stain consisted of potassium hexacyanoferrate(II) trihydrate (47 mM) and HCl acid (0.64 M) (1:1, vol/vol), and was mixed immediately before use. The slides were incubated in this reagent for 15 min before being washed extensively with tap water. The immunological staining was not altered by this process, and the aquamarine color due to Perls' stain was distinct from that of the hematoxylin staining. Cell types were identified on the basis of their morphology as assessed by hematoxylin and eosin staining, as well as by serial sections stained with either HOP-1, PTAH (for SMC), or DAKO-CD68 (for macrophages).

Results

Using the slot-blot protocol, HOP-1 recognized material in each homogenate of eight different advanced diseased arteries, prepared using azide and methionine-free buffer. The amount of reactive material in these homogenates corresponded to 3–30 ng HOCl-modified protein/mg total protein, calculated from a standard curve of in vitro HOCl-oxidized LDL (not shown). As human atherosclerotic lesions contain active MPO (16), homogenates were also prepared using an azide- and methionine-containing buffer, to inhibit this enzyme and scavenge any HOCl potentially produced during the work-up of the samples. Under these conditions, the amount of material detected by HOP-1 in seven different plaque samples from endarterectomies was lower than that detected in the absence of azide and methionine, though it was significantly greater than that in four normal arteries (2.04 ± 2.2 vs 0.15 ± 0.07 ng HOCl-modified protein/mg total protein, $P = 0.025$). There was large variation in the content of material recognized by HOP-1 in the seven plaque samples (range 0.14–5.28) but not in normal arteries (0.07–0.25). Only one plaque had a value less than the mean of the control samples. Together, these findings strongly support the presence of active MPO in atherosclerotic lesions (16) and are consistent with a role for HOCl in atherosclerosis.

We next used HOP-1 for immunohistochemical studies. Consistent with the slot-blot results, both advanced lesions (type IV), intermediate lesions (type III), and foam cells contained large amounts of material recognized by HOP-1 (Fig. 1). The results in Fig. 1 are representative of nine different lesions examined. Inclusion of methionine and azide during tissue fixation did not result in any apparent differences in the staining (not shown) when compared with tissues fixed using the standard procedure, indicating that the material recognized by HOP-1 was not generated during sample preparation. Among the general features of the staining pattern, we noted that the area around cholesterol clefts stained most strongly, thereby overshadowing other areas of staining in the advanced lesion (Fig. 1 *a*). Intermediate lesions also stained clearly, with the epitope detected primarily inside or associated with cells, and sometimes associated with connective tissue (Fig. 1 *c*). Foam cells, located beneath intact endothelial cell layer, also stained strongly with a grainy appearance (Fig. 1 *e*). In each case, omission of the primary antibody resulted in a lack of detectable stain (Fig. 1, *b, d,* and *f*). In areas of vessel that did not contain a necrotic core, the intima and the adventitia were often more heavily stained than the center of the thickened arteries (not shown).

Omission of the primary mAb (Fig. 2 *a*) or replacement of HOP-1 with an IgG_{2b} isotype control (not shown) eliminated all staining, demonstrating that the staining observed was due to HOP-1 rather than nonspecific effects. Also, competition with authentic HOCl-LDL (40 μg/ml), but not native LDL even at a relatively higher concentration (i.e., 80 μg/ml), prevented antibody binding in intermediate (Fig. 2, *c* and *d*) and advanced atheroma (not shown), demonstrating that the staining was specific for HOCl-oxidized protein.

To identify the cell types that contained material recognized by HOP-1, serial sections prepared from fresh advanced

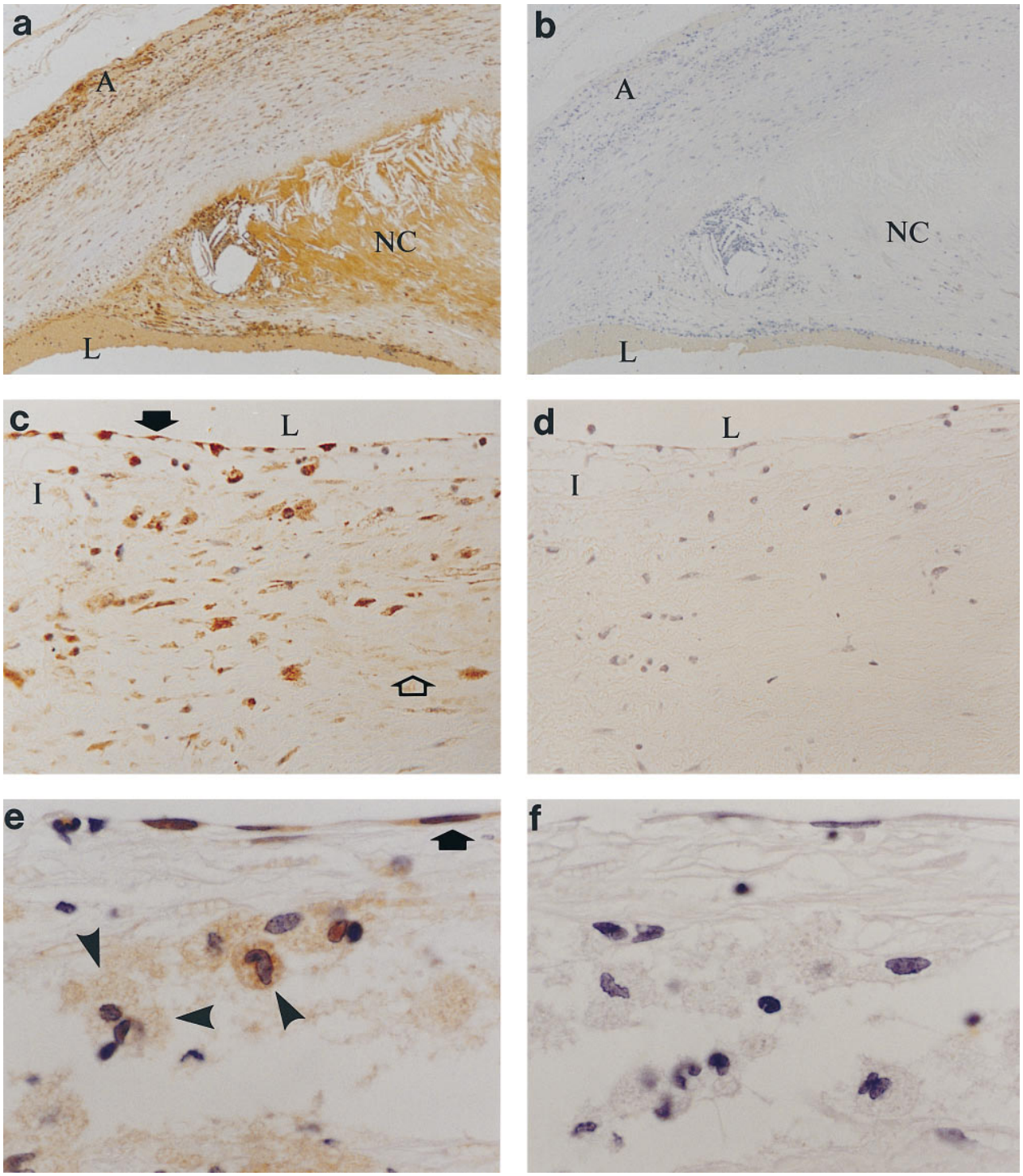

Figure 1. The HOCl-modified protein-specific mAb, HOP-1, recognizes material in advanced lesions with cholesterol clefts, intermediate lesions with no large lipid deposits, and foam cells. (*a*) Heavy staining of acellular cholesterol clefts and necrotic core in advanced (type IV) lesion (×40). At higher magnification, cell-associated staining was also seen in the intima, media, and adventitia. The sample was from archival material, prepared from a 59-yr-old female who died of cardiac arrest. (*b*) Field corresponding to that shown in *A*, derived from a serial section with primary antibody omitted. (*c*) Cell-associated staining in intermediate (type III) atheroma from a vessel also containing advanced lesion. Some endothelial cells (*solid arrow*) and extracellular matrix (*open arrow*) also stained with HOP-1 (×200). The sample was obtained freshly from a 74-yr-old male patient undergoing endarterectomy. (*d*) Field corresponding to that shown in *c* with primary antibody omitted. (*e*) Grainy staining of a cluster of foam cells (*arrowheads*) beneath the endothelial layer in the same artery as that shown in *c* and *d* (×400). Note also the staining of endothelial cells (*solid arrow*) with HOP-1. (*f*) Field corresponding to that shown in *e* with primary antibody omitted. HOP-1 was used at a 1:500 dilution. Avidin-biotin amplification with conjugated horseradish peroxidase was used to visualize the antibody, with 3,3′-diaminobenzidine tetrahydrochloride giving rise to a red-brown color. The section was counterstained lightly with Harris' hematoxylin (blue) to visualize cell nuclei. Three archival and six fresh lesions from different donors were examined and gave qualitatively similar results, although not all features were present in each section. *A,* adventitia; *I,* intima; *L,* lumen; *NC,* necrotic core.

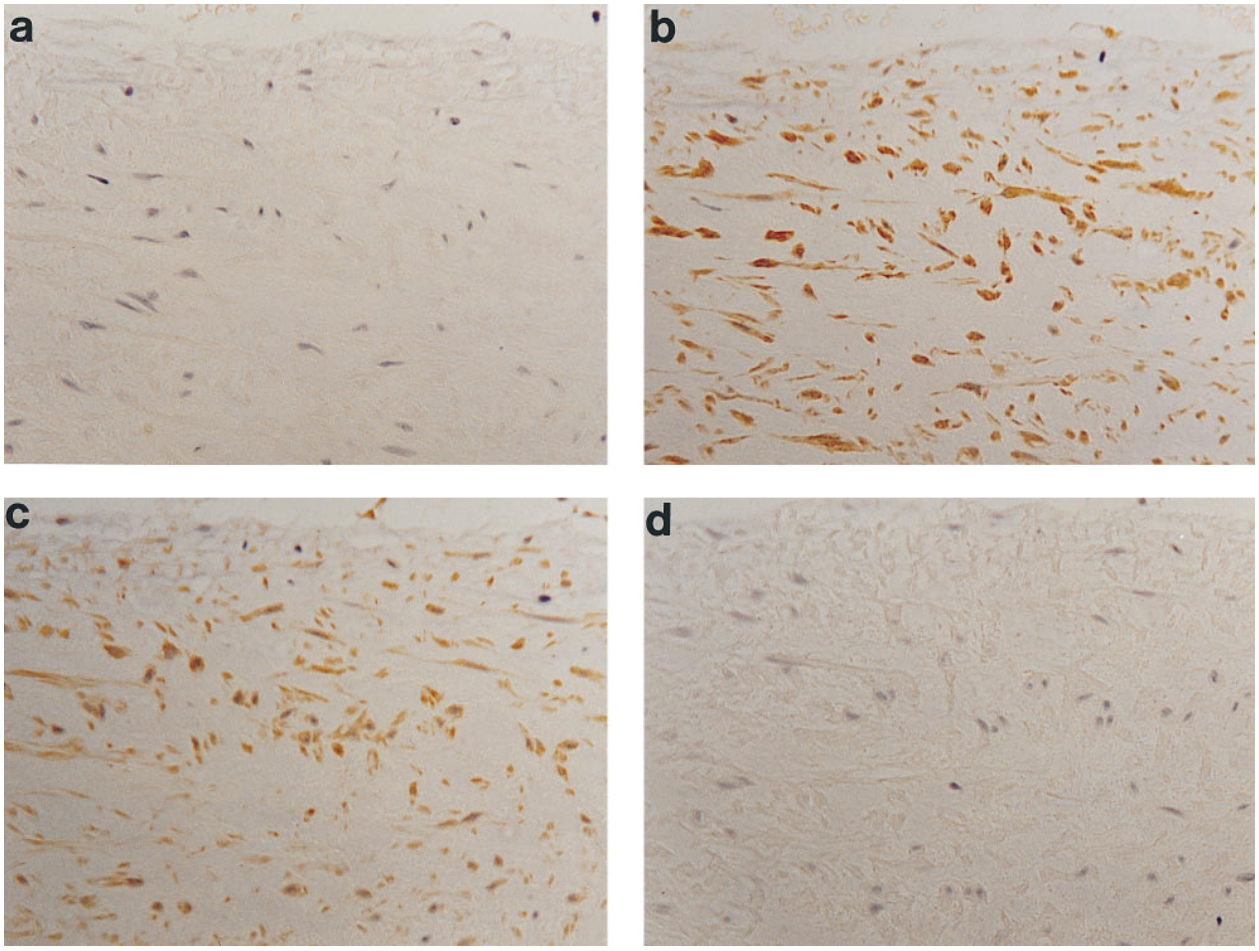

Figure 2. HOCl-LDL but not native LDL inhibits binding of HOP-1 to atherosclerotic tissue. The sample was the same as that shown in Fig. 1, *c–f* (×200). (*a*) Primary antibody omitted. (*b*) HOP-1 with no competitor. (*c*) HOP-1 with native LDL at 80 μg/ml. (*d*) HOP-1 with HOCl-LDL at 40 μg/ml. Immunohistochemistry was carried out as described in the legend of Fig. 1. Competitor was added to the section at the same time as primary antibody. Similar results were obtained with the sample used for Fig. 1, *a* and *b*.

lesions were examined. As shown in Fig. 3, anti-CD68 antibody and HOP-1 detected material that colocalized to the same cells, identifying them as macrophages containing HOCl-modified proteins. The aquamarine staining in some macrophages was due to the presence of hemosiderin treated with Perls' stain, as seen clearly when the primary antibody was omitted (Fig. 3 *c*). Foam cells maintained their foamy appearance, despite removal of lipid during tissue preparation, probably because fixed proteins outlined the area where lipid droplets were located (Figs. 3 *b* and 1 *e*). Long, thin cells also stained with HOP-1 (Fig. 3 *b*) but not the macrophage marker (Fig. 3 *a*), suggestive of SMC or fibroblasts. In support of this, serial sections from areas of the vessel rich in SMC stained heavily with PTAH, which stains myofilaments blue (Fig. 4 *a*), as well as HOP-1 (Fig. 4 *b*). Omission of the primary antibody (Fig. 4 *c*) again eliminated staining. Endothelial cells lining the blood vessel also clearly stained with HOP-1 (Figs. 1, *c* and *e*, and 3 *b*).

An artery from an accident victim, chosen initially as a control tissue, showed signs of an initial lesion (type I atheroma [21]) with thickening of the intima and early infiltration of monocyte/macrophages (Fig. 5 *a*). Clearly, the thickened area contained epitopes recognized by HOP-1 (Fig. 5 *b*), whereas the adjacent, healthy area that had little thickening (Fig. 5 *a*) was unreactive (shown in part in Fig. 5 *b*). As with more advanced lesions, the omission of the primary antibody resulted in a loss of staining (Fig. 5 *c*).

Consistent with previous reports (13, 14), in vitro treatment of LDL with HOCl (400 molecules of oxidant per particle) resulted in non-disulfide cross-linked apo B with little if any apo B monomer remaining (Fig. 6 *a*, lanes *1* and *2*). The HOCl-oxidized, cross-linked apo B was also recognized by the commercial anti–apo B antibody used, albeit to a somewhat lesser extent than apo B derived from the unoxidized LDL. In contrast, HOP-1 did not recognize monomeric apo B of native LDL, whereas it produced a strong signal with HOCl-LDL–derived cross-linked apo B (Fig. 6 *b*, lanes *1* and *2*).

To obtain more information about the nature of the material recognized by HOP-1, homogenates obtained from advanced atherosclerotic lesions were subjected to density centrifugation, and the resulting buoyant lipoproteins were separated from the more dense protein fraction. Both fractions were lyophilized, lipid extracted, and water washed before separation by SDS-PAGE and Western blotting. Lanes *3* and *4* in Fig. 6 *a* show that density centrifugation of plaque homogenate largely separated an apo B–rich fraction from one

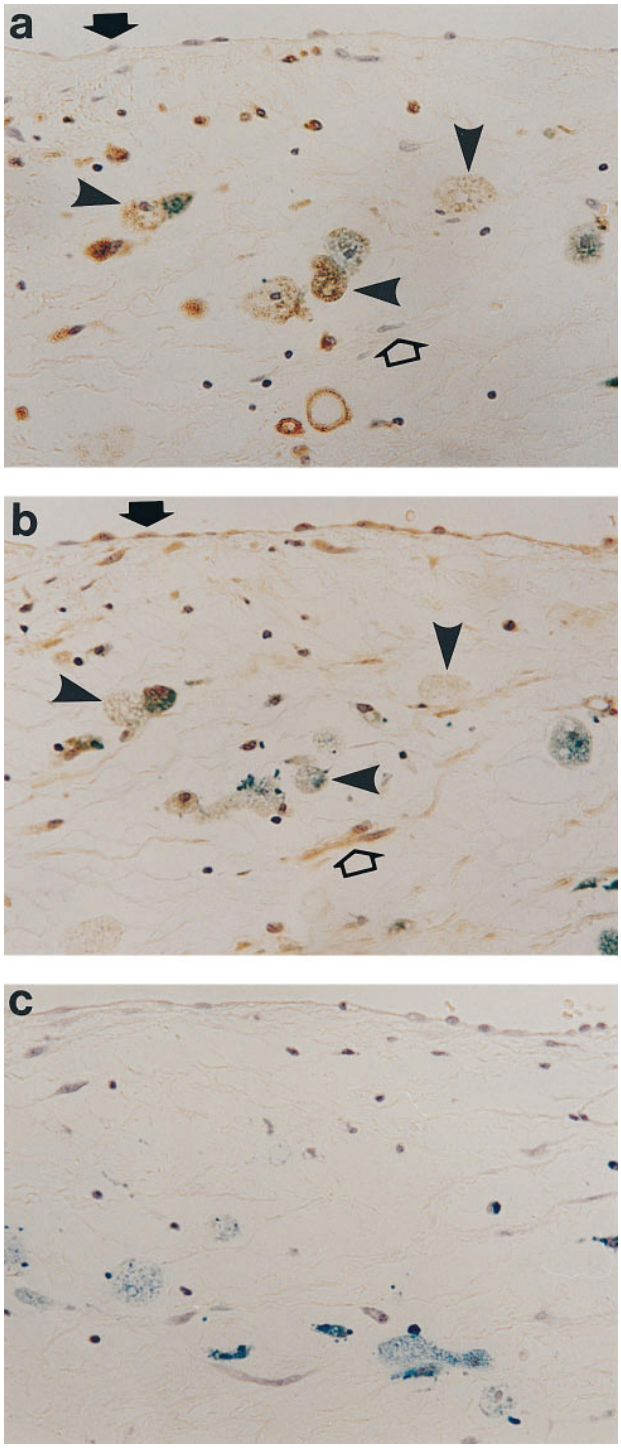

Figure 3. Cellular localization of antigen(s) recognized by HOP-1. The sample was obtained freshly from a 72-yr-old male patient undergoing endarterectomy. An area of vessel adjacent to a neovascular site in the advanced lesion is shown (×200). Serial sections stained with (*a*) an antimacrophage antibody (DAKO-CD68); (*b*) HOP-1; (*c*) primary antibody omitted. Immunohistochemistry was carried out as described in the legend of Fig. 1, except that all sections were stained with Perls' stain after immunohistochemistry and before hematoxylin staining. Perls' stain indicates the presence of hemosiderin by an aquamarine color. HOP-1 staining is localized to macrophages (*arrowheads*), long thin cells (*open arrow*), which are likely SMC or fibroblasts, and endothelial cells (*solid arrow*). A large number of regions containing foam cells that stained with HOP-1 and DAKO-CD68 were noted in four different fresh samples. Colocalization of staining from both antibodies to particular cells was readily possible in two of the four samples, both of which also contained hemosiderin.

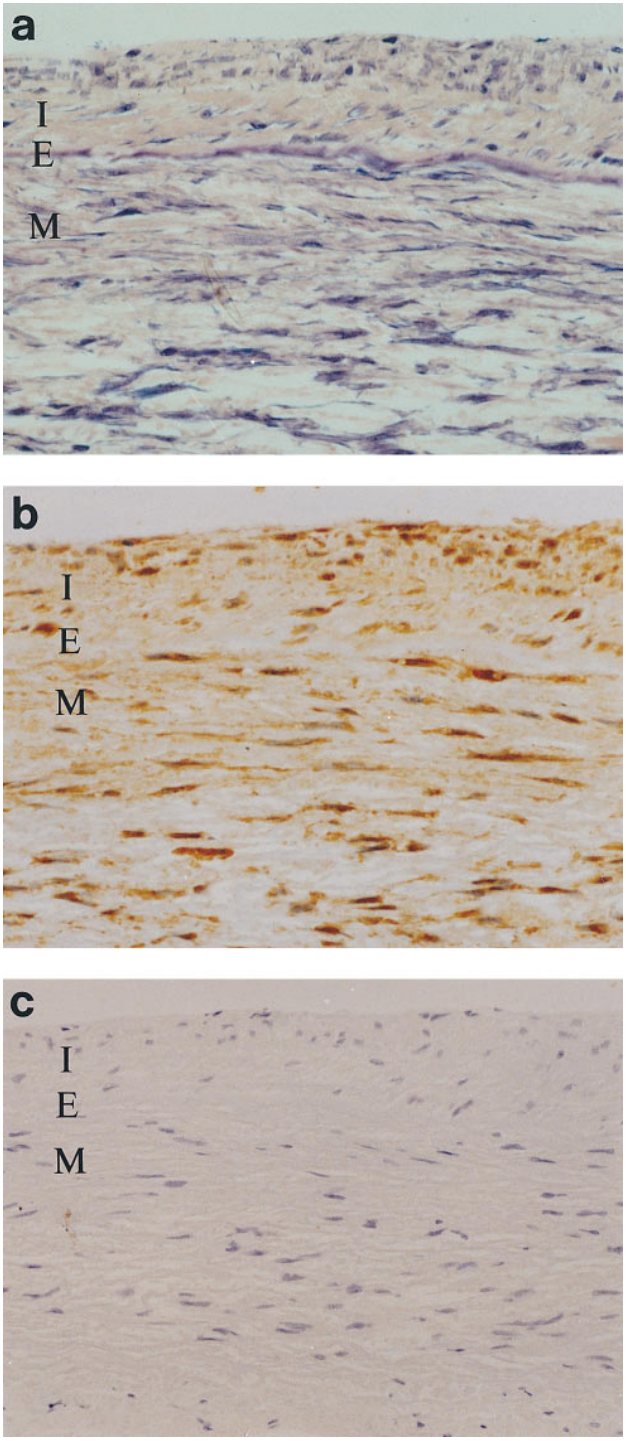

Figure 4. HOP-1 stains areas rich in SMC. The sample was the same as that shown in Fig. 1, *a* and *b*. An area of vessel adjacent to the core of the lesion is shown (×200). (*a*) PTAH-stained section visualizing different areas of the artery. The intima (*I*) appears as the upper pink layer, with the internal elastic lamina (dark pink to violet, *E*) immediately beneath, followed by the media (*M*) containing SMC (dark blue myofibrils). Cell nuclei also stain blue. (*b*) Serial section to that shown in *a* stained with HOP-1 rather than PTAH. (*c*) Primary antibody omitted. Immunohistochemistry was carried out as described in the legend of Fig. 1. Long thin cells (probably SMC or fibroblasts) that stained with HOP-1 were seen in two archival and six fresh samples obtained from different donors. These cells were more frequently seen in disordered areas of tissue than in ordered areas such as that shown in the figure.

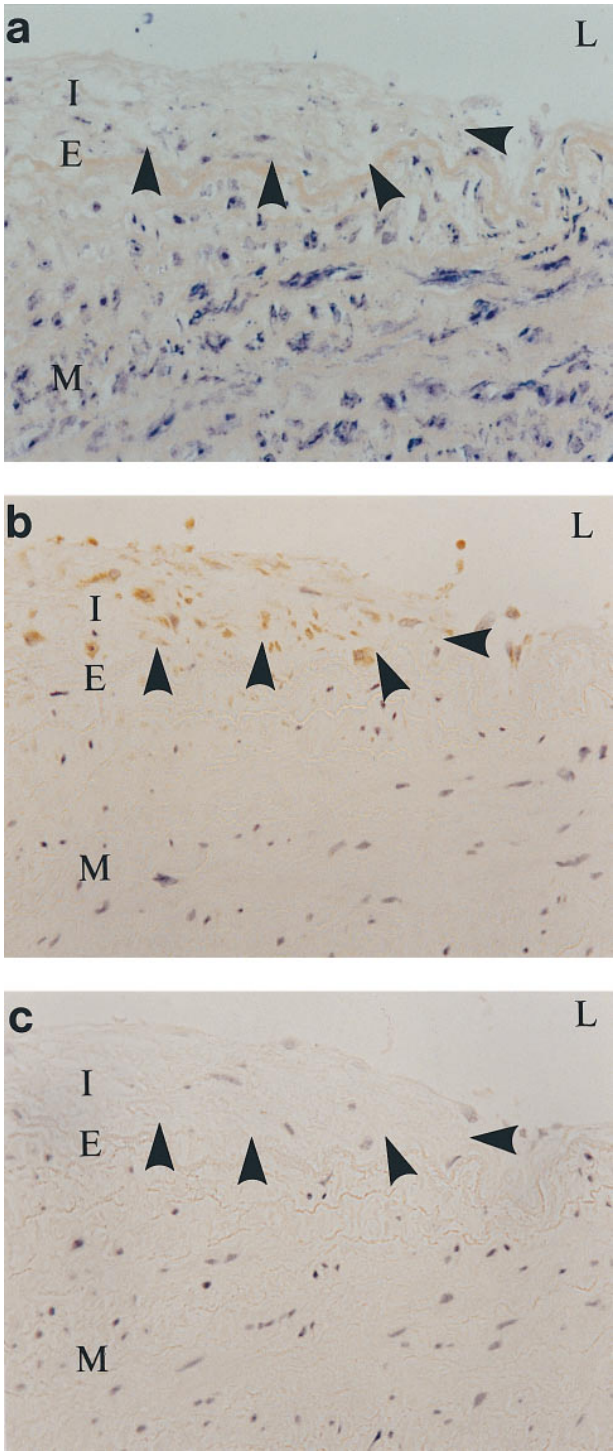

Figure 5. Very early lesion also contains material recognized by HOP-1. The sample was obtained from the iliac artery of a 24-yr-old male accident victim (×200). (*a*) Section stained with PTAH showing a small, localized intimal thickening (*arrowheads*). The normal, very narrow intima can be seen next to the thickening, at the right edge of the photograph. (*b*) Serial section to that shown in *a* stained with HOP-1 rather than PTAH, showing cells containing HOCl-oxidized protein in the thickened intima but not the adjacent healthy intima. (*c*) Serial section with primary antibody omitted. Immunohistochemistry was carried out as described in the legend of Fig. 1. *e,* internal elastic lamina; *I,* intima; *L,* lumen; *M,* media.

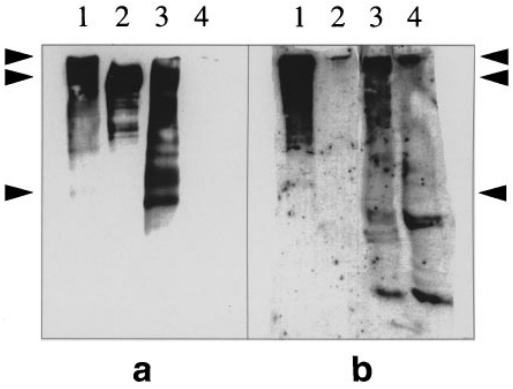

Figure 6. Partial characterization of proteins recognized by HOP-1 in advanced human atherosclerotic lesions. Plaque homogenates were separated into an apo B–rich lipoprotein and more dense protein fraction, their proteins were concentrated and subjected to SDS-PAGE and Western blotting, and the proteins were detected as described in Methods. The primary and secondary antibodies used in *a* were polyclonal sheep anti–human apo B antibody (1:4,000 dilution) and peroxidase-conjugated rabbit anti–sheep immunoglobulins (3:40,000 dilution), respectively. In *b*, HOP-1 (1:5,000 dilution) and peroxidase-conjugated goat anti–mouse IgG (1:80,000 dilution) were used. The film was exposed for 30 s in *a,* lanes *1* and *2*, 2 min in *a*, lanes *3* and *4,* or overnight in *b.* Samples loaded onto gel were as follows: (*a*) lane *1,* 2 μg in vitro HOCl-oxidized LDL; lane *2,* 2 μg native LDL; lane *3,* 13.2 μg apo B–rich lipoprotein fraction from density gradient centrifugation of plaque homogenate; and lane *4,* 18.7 μg protein from apo B–poor fraction from density gradient centrifugation. (*b*) Lane *1,* 50 ng in vitro HOCl-oxidized LDL; lane *2,* 50 ng native LDL; lane *3,* 26.4 μg apo B–rich lipoprotein fraction from density gradient centrifugation of plaque homogenate; and lane *4,* 18.7 μg protein from apo B–poor fraction from density gradient centrifugation. The upper, central, and lower arrowheads indicate the locations of the top of the gel and apparent molecular weights of 500 and 68 kD.

that did not contain substantial amounts of apo B. In the apo B–rich fraction, the anti–apo B antibody recognized a protein with an apparent molecular mass of 500,000 D, several lower molecular mass proteins, and comparatively smaller amounts of material with molecular mass greater than that of apo B (Fig. 6 *a*, lane *3*). These results support several previous reports (23–25) showing a significant portion of apo B in advanced lesions to be present as fragments, and suggest that only a minor portion of lesion apo B is aggregated/cross-linked.

HOP-1 recognized high molecular weight aggregates primarily in the apo B–rich lipoprotein fraction of human plaque homogenate (Fig. 6 *b*, lane *3*), which migrated poorly during PAGE, as did cross-linked apo B derived from in vitro HOCl oxidized LDL (Fig. 6 *b*, lane *1*). Apo B monomer and its fragments (detected by the anti–apo B antibody in the apo B–rich fraction of plaque homogenate) were not recognized strongly by HOP-1, as clear bands were not seen at the corresponding positions (*b*), although antibody binding above background was noted (Fig. 6 *b*, lane *3*). However, HOP-1 also recognized several additional, unidentified proteins that were present in larger amounts in the apo B–poor than the apo B–rich fraction of plaque homogenate (Fig. 6 *b*, lanes *3* and *4*). These proteins were not recognized by the anti–apo B antibody (Fig. 6 *a*, lanes *4*).

Discussion

The results presented here show for the first time that HOCl-modified proteins recognized by HOP-1, a specific mAb raised against HOCl-modified LDL, are present in vivo. Epitopes were detected in both early and advanced human atherosclerotic lesions, and inside and outside different cell types. HOCl-modified but not native LDL effectively competed for antibody binding to the lesion. HOP-1 recognized significantly more material in advanced atherosclerotic lesions than in normal arteries, and some of the reactive plaque material was present in the apo B–containing lipoprotein fraction as high molecular weight aggregates, similar to that formed from apo B during in vitro oxidation of LDL with HOCl. Together, these results demonstrate HOCl-oxidized proteins in atherosclerotic lesions and suggest that this type of oxidative modification of lipoproteins, including LDL, does occur during atherogenesis.

The majority of epitopes recognized in the lesions was localized to cells with macrophage characteristics, suggesting that the antibody recognized phagocytosed material. This is consistent with HOCl production inside the phagolysosomes of activated monocyte/macrophages. However, the presence of immunoreactive material in SMC and endothelial cells not previously shown to contain MPO argues against restriction of HOCl-mediated damage to the phagolysosome. Furthermore, acellular areas of connective tissue (Fig. 1 *c*) and, in particular, cholesterol clefts (Fig. 1 *a*) stained clearly with the antibody, demonstrating HOCl-modified protein outside cells.

The relative intensity of staining observed in different lesion areas in this study was similar but not identical to that reported using an antibody specific for MPO (16), the enzyme known to produce HOCl in vivo. One difference was the granularity of staining due to MPO, which contrasted with the more widespread HOCl staining. The HOP-1 also stained the entire necrotic core around the cholesterol clefts in a relatively even, intense red, whereas the anti-MPO antibody was localized in clusters close to the clefts. If the antioxidant defences were unable to contain the putative HOCl formed within a localized area, the oxidant could diffuse away from its source and cause the diffuse staining observed. In less advanced areas of the lesion, the material recognized by HOP-1 was more closely cell localized, although not exclusively so. Inclusion of the MPO inhibitor azide and HOCl scavenger methionine in the buffer during sample work-up substantially reduced the amounts of material recognized by HOP-1, strongly implicating active MPO in human lesions. This fully supports a previous report that MPO can be isolated from atherosclerotic lesions in its active form (16). Together, these findings directly indicate HOCl production in vivo and are in line with HOCl-mediated oxidation of LDL/proteins contributing to atherogenesis.

The overall staining patterns observed here with HOP-1 and those with the anti-MPO antibody reported by Daugherty et al. (16) were strikingly similar to those reported previously using mono- and polyclonal antibodies raised against Cu-ox-LDL or LDL modified by products of lipid peroxidation, such as HNE and MDA (7, 10, 26). This may be due to LDL lipid peroxidation and HOCl-mediated oxidation occurring in similar locations. Indeed, tyrosyl radicals, if generated by MPO in vivo, may initiate LDL lipid peroxidation (27). Alternatively, epitopes formed during Cu^{2+}-, MDA-, and HNE-mediated LDL modification may be similar to those formed by HOCl. Indeed, there is evidence that HOCl-mediated LDL oxidation involves formation of aldehydes from chloramines and cross-linking reactions of these aldehydes with apo B's lysine residues (13), similar to those of aldehydes derived from lipid peroxidation (28). Furthermore, many polyclonal antibodies and mAbs raised against Cu-ox-LDL and HNE-modified LDL cross-react with HOCl-oxidized LDL and vice versa, although HOP-1 used in the present study was specific for HOCl-modified proteins only (17). In light of the possibility that some of the polyclonal antibodies and mAbs raised against Cu-ox-LDL and HNE- or MDA-modified LDL used in previous studies (7, 10, 26) are cross-reactive with HOCl-modified LDL, the conclusion of extensive lipid peroxidation in lesions drawn in those studies may no longer be justified.

The identification of MPO (16) and oxidized LDL/protein shown here and previously (6) in the adventitia of advanced lesions is interesting, as the adventitia is supplied with blood via the vasa vasorum. This suggests that chemotactic factors for monocytes (as a source of MPO) may also be present in the adventitia, whereas current theories of atherogenesis focus primarily on intimal location and activities of these factors.

HOCl-mediated LDL/protein oxidation appears to occur during the very earliest stages of atherogenesis, as shown by positive staining in type I atheroma (Fig. 5). Other reports support the view that (LDL) oxidation is an early event in atherogenesis. Thus, arteries from normolipidemic rabbits contain low levels of epitopes recognized by an antibody raised against MDA-LDL (4). Also, Jürgens et al. (8) noted the absence of HNE adducts in "normal" areas of tissue adjacent to stained lesions, as well as heavy reactivity in an early human atherosclerotic lesion. With increasing severity of lesions in rabbits, a progression of staining from cell associated to non–cell associated and from the intima to the media was reported using a variety of antibodies (6). In fatty streaks, all five of the antibodies used in the above study recognized material intracellularly, suggesting that (LDL) oxidation does not only occur as a result of atherosclerosis, but may at least in part also cause the disease. It is also conceivable that the iron in the hemosiderin detected in this study is redox active and contributes to LDL oxidation.

Obviously, the reliability of conclusions drawn from immunohistochemical studies depends upon the specificity of the antibodies used. As mentioned above, most antibodies used previously for the detection of LDL modified by different products of lipid peroxidation (4, 29) cross-reacted with at least two of these different antigens. They also recognized proteins or peptides other than LDL modified by these oxidants, although often with lower affinities. HOP-1 does not cross-react with native LDL, Cu-ox-LDL, peroxynitrite-oxidized LDL, HNE-LDL, or MDA-LDL, whereas it recognizes HOCl-modified LDL and other HOCl-modified proteins, including fatty acid–free human albumin (17) and high density lipoprotein (Malle, E., and G. Waeg, unpublished observations). This strongly suggests that HOP-1 recognizes HOCl-modified protein rather than protein–lipid adduct(s) or oxidized lipids (e.g., chlorohydrins) that can be formed from HOCl (30, 31).

The use of HOP-1 offers the advantage that HOCl modification of different proteins can be detected. Our preliminary data (Fig. 6) indicate that lipoproteins are among these proteins, as indicated by the presence of HOP-1–recognized material in the apo B–containing lipoprotein fraction prepared

from plaque homogenate. The material recognized was poorly mobile during PAGE, fully consistent with HOCl modification of apo B in human lesions, which results in increased cross-linking of LDL's apo B with increasing oxidant-to-lipoprotein ratios (13, 14). However, further experiments are required to demonstrate unambiguously such modification of LDL's apo B in atherosclerotic lesions. As a result of the high reactivity of HOCl, we would not expect this oxidant to oxidize apo B (or any other protein) specifically; rather, oxidation of a range of proteins seems more likely, a view supported by the various proteins recognized by HOP-1 in the apo B–poor fraction of plaque homogenate.

HOCl-mediated modification to proteins other than LDL could contribute to atherogenesis. For example, HOCl produced by activated neutrophils is critically involved in the detachment of endothelial cells from an anchoring matrix in vitro (32). This and other destructive activities may involve HOCl-mediated inactivation of inhibitors of proteases such as elastase (32), which is present in macrophages (33). HOCl could also directly damage (endothelial) cells as well as various components of extracellular matrix (see reference 34) and thereby contribute to atherogenesis directly and/or via enhancement of inflammatory processes.

HOCl differs in its oxidation characteristics, and in the antioxidant defences that are effective against it, from the various free radical oxidants studied more extensively (35, 36). Its differences raise interesting questions concerning the targeting and design of antiatherosclerotic drugs that are based on (LDL) antioxidation, as well as appropriate in vitro model(s) for the assessment of LDL "oxidizability" and method(s) for measuring the extent of LDL oxidation. None of the commonly used in vitro tests consider HOCl a relevant oxidant, and at least some oxidation parameters commonly used would not detect HOCl-inflicted damage to LDL.

The results presented here show that HOCl, a natural, non–metal-dependent and powerful oxidant, causes modification to proteins in human atherosclerotic lesions in vivo. They suggest that this oxidant may contribute significantly to atherogenesis.

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