Role of Endogenous Ceruloplasmin in Low Density Lipoprotein Oxidation by Human U937 Monocytic Cells

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Abstract

Oxidation of lipids and lipoproteins by macrophages is an important event during atherogenesis. Activation of monocytic cells by zymosan and other agonists results in the release of multiple oxidant species and consequent oxidation of LDL. We now show evidence that ceruloplasmin, a copper-containing acute phase reactant, is secreted by zymosan-activated U937 monocytic cells, and that the protein has an important role in LDL oxidation by these cells. In one approach, ceruloplasmin has been shown to exhibit oxidant activity under the appropriate conditions. Exogenous addition of purified human ceruloplasmin stimulates U937 cell oxidation of LDL to nearly the same extent as activation by zymosan. In contrast to previous cell-free experiments (Ehrenwald, E., G.M. Chisom, and P.L. Fox. 1994. Intact human ceruloplasmin oxidatively modifies low density lipoprotein. *J. Clin. Invest.* 93:1493–1501.) in which ceruloplasmin by itself (in PBS) oxidizes LDL, under the conditions of the current experiments (in RPMI 1640 medium) ceruloplasmin only oxidizes LDL in the presence of cells; the mechanism by which cells overcome the inhibition by medium components has not been ascertained. As further evidence for a role of ceruloplasmin, activation of U937 cells with zymosan induces ceruloplasmin mRNA and ceruloplasmin protein synthesis after a 5–6 h lag that is consistent with that preceding LDL oxidation. Finally, neutralization by a highly specific polyclonal antibody to human ceruloplasmin inhibits LDL oxidation by at least 65%. Moreover, multiple antisense oligodeoxynucleotides targeted to different regions of the ceruloplasmin mRNA block LDL oxidation by up to 95%. The specific action of the antisense oligonucleotides has been verified by showing inhibition of ceruloplasmin synthesis and by the ability of exogenous ceruloplasmin to overcome the inhibition. In summary, these results are consistent with a mechanism in which cell-derived ceruloplasmin participates in oxidation of LDL by U937 monocytic cells. The data also show that cellular factors in addition to ceruloplasmin, possibly active oxygen species and/or lipoxygenases, are essential and act synergistically with ceruloplasmin to oxidize LDL. (*J. Clin. Invest.* 1996. 97:884–890.) Key words: ceruloplasmin • lipid peroxidation • low density lipoprotein • monocytic cells • zymosan

Introduction

Activated monocytes and macrophages are recognized for their remarkable diversity of secreted products, including multiple reactive oxygen and nitrogen intermediates, involved in the injury or killing of invasive organisms such as bacteria, parasites, and tumor cells (1). The same oxidation reactions may also cause secondary oxidative damage to host macromolecules and tissues during chronic inflammatory processes. For example, there is now abundant evidence that particles resembling oxidatively modified LDL are present in atherosclerotic lesions (2). Recent evidence suggests that oxidative damage is a critical factor in disease progression since antioxidants may reduce the onset or severity of cardiovascular disease (3) and inflammatory bowel diseases (4). The mechanisms of cellular oxidation processes have been subjected to intense scrutiny, but despite these efforts the physiological source(s) of transition metals required for metal ion–dependent oxidation reactions is not known (4, 5). A clue to the resolution of this enigma may be found in the observation by Cathcart et al. (6, 7) that activated human monocytes, or monocytic cells such as the U937 cell line, oxidize LDL in vitro even in iron and copper ion–free media. This finding distinguishes these cells from endothelial cells and smooth muscle cells that require exogenous copper or iron salts in the medium to achieve optimal oxidation rates (8, 9). One explanation is that monocytic cells may provide their own transition metal ions to maintain high oxidation rates and the inhibition of monocytic cell lipid oxidation by divalent cation chelators is consistent with this idea (10). Alternatively, macrophage-derived hypochlorous acid or nitric oxide may be precursors of highly reactive hydroxyl radicals by metal ion–independent mechanisms (11).

We have recently shown that physiological concentrations of purified human ceruloplasmin (200–400 μg/ml in healthy adults) increase the oxidation of LDL in vitro by up to 50-fold when measured as thiobarbituric acid–reactive substances (TBARS)[1] (12). Ceruloplasmin is a monomeric, 132-kD acute

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1. *Abbreviations used in this paper:* DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; TBARS, thiobarbituric acid-reactive substances.

phase protein that contains about seven atoms of copper per molecule and carries up to 95% of the total plasma copper (for review see references 13, 14). The newly identified oxidant property is contrary to earlier reports of ceruloplasmin antioxidant activity (15, 16). The apparent discrepancy may be explained by the fact that the oxidant activity is completely dependent on the structural integrity of the protein and on the presence of one specific copper atom (12). The oxidant activity of ceruloplasmin (in PBS) is inhibited by several proteins and by a mixture of amino acids raising uncertainty with respect to the physiological relevance of the observation (12). As will be shown in this study, cells act in concert with ceruloplasmin to overcome the inhibitory activity of amino acids (or other medium components). Other investigators have also reported oxidant activity of ceruloplasmin, but in contrast to our results using native protein, potentially damaging modifications of the protein were required for activation (17, 18).

Plasma ceruloplasmin concentration increases about 10-fold during development and further rises up to threefold during pregnancy and during multiple pathological processes including trauma and inflammation (for review see reference 19). Elevated serum ceruloplasmin has been found to be a risk factor for coronary heart disease (20, 21), and a strong correlation exists between serum ceruloplasmin and serum lipid oxidation (22). Ceruloplasmin in human atherosclerotic lesions has been detected (23, 24), but the cellular sources responsible for ceruloplasmin accumulation during this and other inflammatory processes are not known. While there is evidence for transcriptional regulation of ceruloplasmin in hepatic cells during inflammation (25, 26), Fleming et al. (27) have shown that cytokine-stimulated alveolar macrophages also express the ceruloplasmin gene and synthesize and secrete the protein. In contrast, ceruloplasmin production by vascular endothelial cells and smooth muscle cells has not been reported. The separate observations of synthesis of ceruloplasmin by activated macrophages, the oxidant activity of the purified protein, and the ability of macrophages to oxidize LDL even in the absence of exogenous metal ions led us to investigate the role of cell–derived ceruloplasmin in monocytic cell-mediated oxidation of LDL.

Methods

Materials. Rabbit polyclonal anti–human ceruloplasmin IgG, rabbit anti–human α_1-antitrypsin IgG, and rabbit anti–human serum albumin IgG were obtained from Accurate Chemical and Science Corp. (Westbury, NY). [L-3,4,5-^{3}H]leucine was obtained from Dupont NEN (Boston, MA). Chelex-100 was from Bio-Rad (Richmond, CA). Zymosan, cycloheximide, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), and all assay reagents were obtained from Sigma Chemical Co., St. Louis, MO. O-linked, sense and antisense oligodeoxynucleotide 15-mers targeted to ceruloplasmin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequences were synthesized and purified by Genosys Biotechnologies, The Woodlands, TX.

Preparation of human ceruloplasmin. Ceruloplasmin was purified from human plasma by a modification (28) of the method of Noyer et al. (29). In brief, plasma was applied sequentially to QAE-Sephadex A50 batch chromatography, ammonium sulfate precipitation, hydroxyapatite chromatography, Sephadex G50 chromatography, and Mono Q fast protein liquid chromatography. Only the major ceruloplasmin fraction from the hydroxyapatite column, containing three N-linked oligosaccharides (13), was used during subsequent purification. Ceruloplasmin was quantitated by absorbance ($OD^{1\%}_{1cm}$,610 nm = 0.66). The resulting preparation was > 99% pure as determined by amine oxidase specific activity (measured with *p*-phenylenediamine as substrate) (30), by an absorbance ratio (610 nm/280 nm) > 0.045 (13, 31), and by homogeneity according to PAGE and silver staining. Purified ceruloplasmin was primarily the intact 132-kD monomer, but also contained a small amount (< 15%) of the 115- and 19-kD proteolytic fragments of ceruloplasmin that are also present in serum (32). In some experiments partially purified ceruloplasmin that was not subjected to gel filtration was used; this preparation (> 95% pure) had higher specific oxidant activity than protein subjected to the entire purification protocol.

Lipoprotein methods. Human LDL was prepared from freshly drawn, citrated normolipemic plasma to which EDTA was added before ultracentrifugation. LDL (density = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation as described previously (8). Immediately before use, LDL was extensively dialyzed at 4°C against saline. Lipoprotein oxidation was measured as formation of TBARS by a modification (33) of the method of Schuh et al. (34). Lipoprotein samples were compared to a standard curve prepared with malondialdehyde, and TBARS quantitated by fluorescence at 515/553 nm emission.

U937 cell oxidation of LDL. For measurement of cellular oxidation of LDL, LDL (1 mg cholesterol/ml) was incubated with 10^5 U937 cells in the presence of zymosan (3–6 mg/ml) in 0.06 ml of iron- and copper-free RPMI 1640 medium (unless otherwise indicated) for 24 h at 37°C. In all experiments, zymosan was precoated with 10 mg/ml BSA instead of plasma, to prevent contamination by ceruloplasmin. Cells and zymosan were removed by low-speed centrifugation and LDL oxidation was measured as TBARS and results expressed as nmol of malondialdehyde-equivalents per mg of LDL cholesterol (mean±SEM, n = 3).

Immunoblot analysis of U937 cell-derived ceruloplasmin. Conditioned medium from 1.2×10^6 U937 cells in 1.5 ml was centrifuged, and the supernatant concentrated by ultrafiltration and subjected to 4–12% gradient SDS-PAGE electrophoresis. The proteins were transferred to a polyvinyldifluoride membrane and probed with rabbit anti–human ceruloplasmin IgG (1:20,000). Bound antibody was detected with a secondary horseradish peroxidase antibody and chemiluminescent development of XAR-5 x-ray film. The immunoblots were quantitated densitometrically using a charge-coupled device camera (Sony Corp., Park Ridge, NJ) and the N.I.H. Image program provided by Dr. Wayne Rasband, National Institutes of Mental Health. The specificity of the antibody was verified by its recognition of only ceruloplasmin in human serum.

Measurement of protein synthesis. Synthesis of total secreted protein by U937 cells was measured by addition of [^{3}H]leucine (1 μCi/ml) to cells during the last 5 h of a 24-h incubation. The conditioned medium was collected by low-speed centrifugation and TCA (10%)-precipitable material was determined by vacuum filtration using 0.22-μM GS filters (Millipore Corp., Bedford, MA).

RNA blot analysis of ceruloplasmin gene expression in U937 cells. Total RNA was extracted from zymosan-activated U937 cells, fractionated on a 1% agarose-formaldehyde gel (30 μg/lane), and transferred to nitrocellulose (35). The blot was hybridized with a [^{32}P]dCTP, random primer-labeled human ceruloplasmin cDNA probe (pCP3) kindly provided by Dr. Jonathan Gitlin, Washington University, St. Louis, MO (25). The blot was stripped and rehybridized using a GAPDH cDNA probe. The hybridization blots were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

Stimulation of U937 cell oxidation of LDL by exogenous, purified human ceruloplasmin. Addition of purified, intact human ceruloplasmin increased LDL oxidation (measured as TBARS [34]) by unstimulated U937 cells by at least 20-fold in metal ion-free medium (Fig. 1 *A*). The stimulation by ceruloplasmin

was at least as great as that by zymosan, yeast cell wall fragments that are classical activators of macrophage function and that stimulate LDL oxidation by U937 and other monocytic cells (36, 37). Exogenous ceruloplasmin did not greatly increase LDL oxidation by zymosan-activated cells indicating that a common pathway may be used. In the absence of cells, ceruloplasmin did not significantly enhance LDL oxidation indicating that a U937 cell product(s) or process(es) unrelated to ceruloplasmin was also required for ceruloplasmin-mediated LDL oxidation under these experimental conditions (see Discussion).

Zymosan increased U937 cell-mediated LDL oxidation, even in the absence of exogenous ceruloplasmin or other metal ions, but only after a lag of about 6–9 h (Fig. 1 *B*). This finding is consistent with a previous report (6) and also with a requirement for new synthesis of an activating agent. Inhibition of mRNA transcription by DRB or inhibition of protein synthesis by cycloheximide completely prevented zymosan-stimulated LDL oxidation, indicating that the observed lag is likely due to delayed synthesis of a requisite protein. The addition of ceruloplasmin to zymosan-stimulated cells eliminated the lag before initiation of oxidation (Fig. 1 *B*), a finding consistent with a requirement for ceruloplasmin for maximal cellular oxidation of LDL. In summary, these experiments show that ceruloplasmin has the requisite oxidant activity in the presence of U937 cells and is thus a viable candidate for a cell-derived source of divalent metal ions for LDL oxidation.

Ceruloplasmin production by zymosan-activated U937 cells. Ceruloplasmin production by U937 cells was measured by immunoblot analysis of conditioned medium using a rabbit anti–human ceruloplasmin IgG. The time course experiment in Fig. 2 *A*, C showed that zymosan-activated U937 cells released ceruloplasmin into the medium, but only after a 5–6 h delay, similar to that preceding LDL oxidation. Densitometric quantitation of immunoblots from multiple experiments showed that the amount of ceruloplasmin in 24-h conditioned medium was between 5 and 10 ng per 1.2×10^6 U937 cells. Ceruloplasmin was not detectable in medium from unstimulated cells (see Fig. 4 *A*, *inset*). Nearly all of the secreted ceruloplasmin was present as the intact, 132-kD form of the protein that has oxidant activity (12). In some experiments, up to 10%

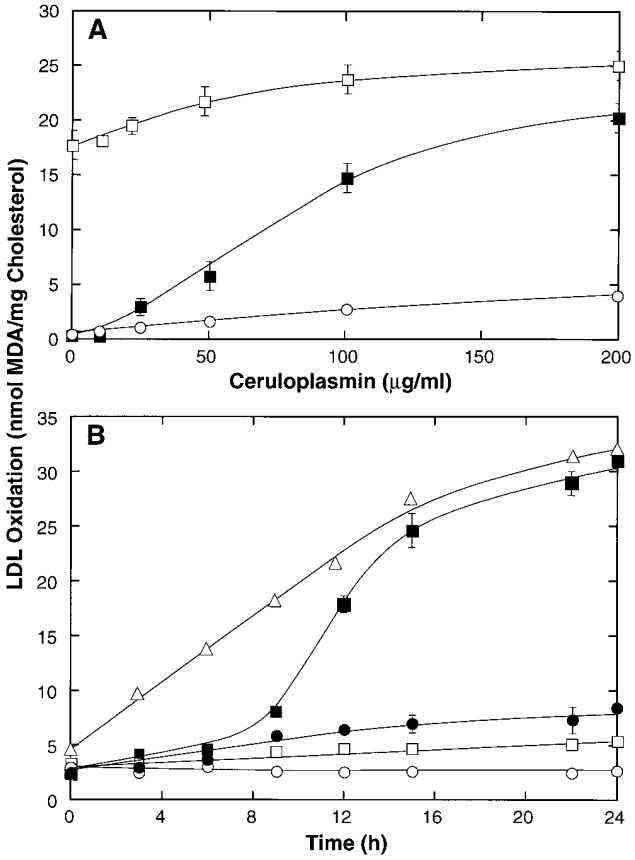

Figure 1. Oxidation of LDL by U937 cells. (*A*) LDL (1 mg cholesterol/ml) and purified human ceruloplasmin were incubated with nonactivated (■) or zymosan (4 mg/ml)-activated (□) U937 cells, or under cell-free conditions (○) for 24 h. LDL oxidation was measured as TBARS in conditioned medium. (*B*) Zymosan-activated (■) or untreated U937 cells (○) were incubated with LDL (1 mg cholesterol/ml) in the absence of exogenous ceruloplasmin or other added metal ions. In parallel wells, zymosan-stimulated cells were treated with the transcription inhibitor DRB (100 μM, □), with the protein synthesis inhibitor cycloheximide (10 μg/ml, ●), or with exogenous, purified human ceruloplasmin (100 μg/ml, △). After 24 h, LDL oxidation was measured as TBARS. Cells treated with either inhibitor were shown to be viable (≥ 90% trypan blue exclusion) at the end of the experiment.

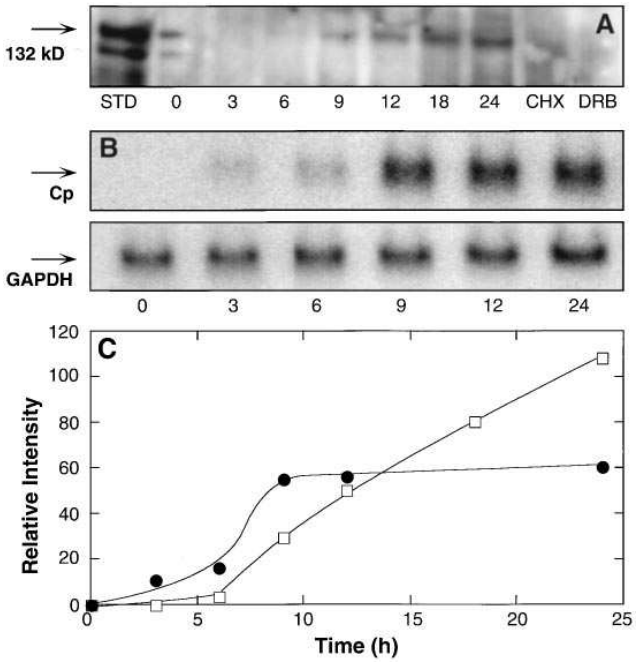

Figure 2. Ceruloplasmin production by zymosan-activated U937 cells. (*A*) U937 cells (1.2×10^6 cells in 1.5 ml) were activated with zymosan (4 mg/ml) for up to 24 h as indicated. Activated U937 cells were also treated with DRB (100 μM) or cycloheximide (10 μg/ml, *CHX*) for 24 h. The far left lane contained 20 ng of purified human ceruloplasmin as standard (*STD*) (*B*) RNA (30 μg) isolated from 10^7 U937 cells was subjected to electrophoresis and transferred to polyvinyldifluoride membranes. The blot was hybridized with radiolabeled human ceruloplasmin and GAPDH cDNA probes. *Cp*, ceruloplasmin. (*C*) The relative intensity of the ceruloplasmin bands in the immunoblot in *A* was determined by densitometry of the autoradiograph (□). The relative intensity of ceruloplasmin gene expression in the RNA blot in *B* was quantitated with a PhosphorImager and the signal normalized to that of GAPDH (●).

of the total ceruloplasmin in the conditioned medium was present as the 115-kD proteolytic cleavage product that lacks activity. The relative amount of intact protein (≥ 90%) was comparable to that in our purified preparations of human ceruloplasmin with high oxidant activity (12). Ceruloplasmin production by stimulated U937 cells was blocked by cycloheximide and DRB, thereby demonstrating the cellular requirement for transcription and translation. Furthermore, ceruloplasmin was not detectable in lysates of U937 cells, confirming the importance of de novo synthesis and secretion, rather than release from a preformed intracellular pool.

Total RNA isolated from zymosan-activated U937 cells was subjected to RNA blot hybridization using a radiolabeled human ceruloplasmin cDNA probe. Ceruloplasmin mRNA was strongly induced by zymosan (Fig. 2 *B*). The size of the mRNA was about 3.7 kb, corresponding to the single ceruloplasmin transcript expressed by rat alveolar macrophages (27) and to the smaller of the two transcripts expressed by multiple human cell lines (38). Ceruloplasmin mRNA was quantitated by PhosphorImage analysis and normalized to that of GAPDH mRNA, and showed a 5–6 h lag before significant increases of mRNA were observed (Fig. 2 *C*). The similar lag intervals of ceruloplasmin gene expression and LDL oxidation are consistent with a requirement for ceruloplasmin production for U937 cell-mediated oxidant activity.

Suppression of U937 cell oxidation of LDL by inhibition of ceruloplasmin activity or synthesis. To examine directly whether endogenous ceruloplasmin participates in U937 cell oxidation of LDL, specific inhibitors of ceruloplasmin activity were investigated. Polyclonal rabbit anti–human ceruloplasmin IgG added to zymosan-stimulated U937 cells blocked LDL oxidation by at least 65% while type- and species-matched irrelevant antibodies including anti–human α_1-antitrypsin IgG and anti-human serum albumin IgG had little inhibitory activity (Fig. 3). The antibodies were added 6 h after zymosan-stimulation since earlier addition resulted in lower inhibitory activity, perhaps due to free radical–mediated damage to the antibody by the zymosan-elicited respiratory burst (39). The incomplete inhibition may be due to the late addition of the antibody or, more likely, to the kinetic requirement for a high antibody titer to inactivate a continuously secreted protein. To show that the antibody treatment did not alter cell viability, total synthesis of proteins secreted by zymosan-stimulated cells was measured as incorporation of [^{3}H]leucine into TCA-precipitable material; the ceruloplasmin-specific antibody (and control antibody) was not inhibitory indicating specific suppression of ceruloplasmin production.

We have previously shown that treatment of purified human ceruloplasmin with Chelex-100 removes one of seven copper atoms from the protein and suppresses its oxidant activity (12). The addition of Chelex-100 to zymosan-activated U937 cells suppressed LDL oxidation in a concentration-dependent manner with a maximal inhibition of 80% found at 4 mg/ml of the solid-phase chelator (not shown). This result is consistent with an oxidant role for ceruloplasmin, and in fact for the specific oxidant ceruloplasmin copper, and is also consistent with studies by others showing that chelators of divalent cations block LDL oxidation by other monocytic and leukocytic cells (10, 40, 41).

In an independent approach designed to block ceruloplasmin synthesis rather than activity, antisense oligodeoxynucleotides targeted to human ceruloplasmin transcripts were used.

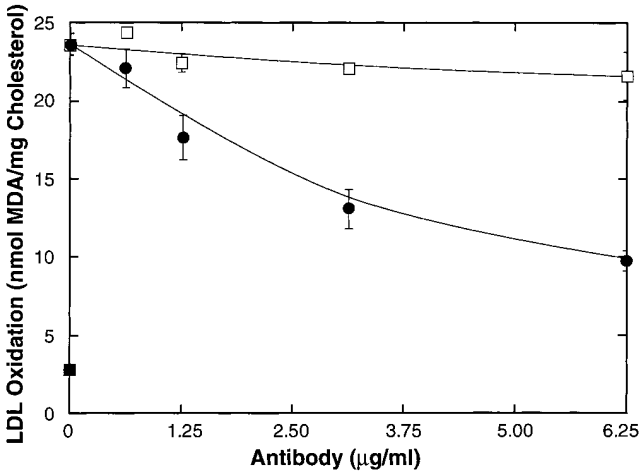

Figure 3. Effect of anticeruloplasmin IgG on U937 cell-mediated LDL oxidation. U937 cells were activated with zymosan in the presence of LDL (1 mg cholesterol/ml) for 24 h. Six hours after activation, anti–human ceruloplasmin IgG (●) or anti–human α_1-antitrypsin IgG (□) were added for 18 h. As a control, LDL was incubated for 24 h in the absence of cells (■). LDL oxidation was measured as TBARS of conditioned medium.

An O-linked oligodeoxynucleotide 15-mer complementary to the translation-initiation site of ceruloplasmin mRNA was used. At the maximal concentration of 150 μM, the antisense oligonucleotide suppressed cell-mediated LDL oxidation by 95% while the control sense oligonucleotide was only slightly inhibitory (Fig. 4 *A*). The effectiveness of the treatment was demonstrated by immunoblot analysis of conditioned medium followed by densitometric analysis (Fig. 4 *A*, *inset*). Antisense oligonucleotide (at 100 μM) inhibited ceruloplasmin production by 70% (up to 90% inhibition was seen in some experiments) compared to controls while inhibition by the sense oligonucleotide was about 15%. At this oligonucleotide concentration, the effects of sense and antisense treatments on ceruloplasmin secretion were comparable to their effects on LDL oxidation (inhibition of 85 and 17%, respectively). To confirm the specificity of action of the antisense oligonucleotide, the total synthesis of protein secreted by zymosan-stimulated cells was measured as incorporation of [^{3}H]leucine into TCA-precipitable material; the antisense (and sense) oligonucleotide did not significantly inhibit overall protein synthesis and secretion. Furthermore, conditioned medium from treated and untreated cells were subjected to SDS-PAGE and silver staining; significant differences were not found, confirming that the antisense treatment did not nonspecifically alter protein secretion rates (not shown).

The short antisense oligonucleotides used in these experiments are likely to be at least partially homologous to regions in genes unrelated to ceruloplasmin. To establish the specificity of the antisense treatment, two 15-mer oligonucleotides targeted to distinct regions of ceruloplasmin mRNA were also tested. The target sequences were chosen on the basis of their identification as putative "loop" regions in the secondary structure of the ceruloplasmin mRNA, as predicted by the energy minimization method of Jaeger et al. (42). These antisense oligonucleotides were as effective as those targeted to the translation-initiation site, confirming the specificity of the

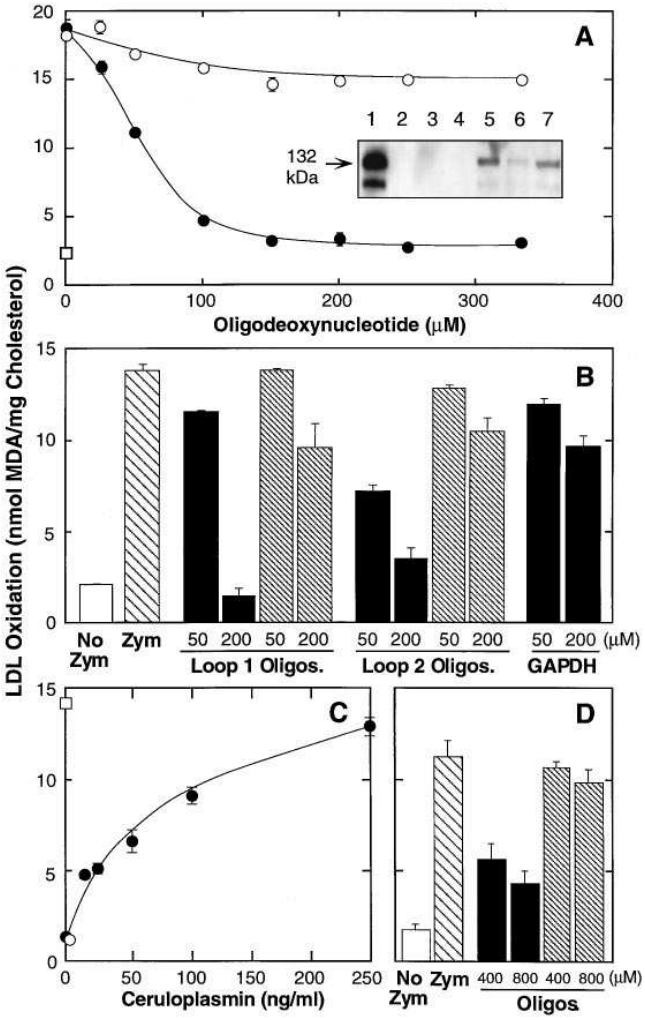

Figure 4. Inhibition of monocytic cell–mediated LDL oxidation by antisense deoxynucleotides targeted to ceruloplasmin mRNA. (*A*) An antisense oligodeoxynucleotide 15-mer targeted to the translation-initiation site of ceruloplasmin mRNA (5′-TATCAAAATCTTCAT-3′) and its sense control were used. U937 cells were preincubated with antisense (●) and sense (○) oligomers for 4 h before activation with zymosan and addition of LDL (1 mg cholesterol/ml). After 24 h, LDL oxidation was measured as TBARS. Baseline LDL oxidation was determined in the absence of cells (□). *Inset*: Inhibition of ceruloplasmin production by antisense oligodeoxynucleotides. Zymosan-activated U937 cells (6 x 10^5 cells) were incubated with 100 μM antisense 15-mer (and sense control) in the presence of 0.1 μM $CuSO_4$. Free copper was added to these cells (but not to cells used to measure LDL oxidation) since it increased U937 cell production of ceruloplasmin and minimized the volume and the amount of oligonucleotide required (similar results were seen in the absence of copper, not shown). The conditioned medium was concentrated and subjected to immunoblot analysis using anti–human ceruloplasmin IgG as described in Fig. 2 *A*. Applied to the gel were purified human ceruloplasmin (20 ng, lane *1*, *arrow*), medium alone (lane *2*), zymosan alone (lane *3*), conditioned medium from unstimulated cells (lane *4*), and conditioned medium from zymosan-activated cells treated with medium alone (lane *5*) or with antisense (lane *6*), and sense (lane *7*) oligonucleotides. (*B*) Theoretical secondary structure loops in ceruloplasmin mRNA were calculated by energy minimization (42). Antisense (*black bars*) oligonucleotide 15-mers targeted to putative loop segments at positions 66–80 in the ceruloplasmin cDNA (*Loop 1*, 5′-CCAATGTAATAATGC-3′) and at positions 614–628 (*Loop 2*, 5′-CTTTTTCTTTATCTA-3′), and their sense controls (*dense striped bars*), were prepared. These oligomers and one directed against the translation-initiation site of GAPDH (5′-CTTCACCTTCCCCAT-3′) were incubated with zymosan (*Zym*)-activated U937 cells as in *A* and LDL oxidation measured as TBARS. LDL oxidation by unstimulated (*open bar*) and zymosan-activated (*light striped bar*) cells are shown as controls. (*C*) U937 cells were pretreated for 4 h with antisense (100 μM) targeted to the ceruloplasmin mRNA translation-initiation site as in *A*. Zymosan (4 mg/ml) and LDL (1 mg cholesterol/ml) were then added, followed 1.5 h later by partially purified human ceruloplasmin at up to 250 ng/ml and LDL oxidation measured as TBARS (●). LDL oxidized by zymosan-activated cells in the absence of the oligonucleotide (□) and LDL incubated without cells (○) served as controls. (*D*) Freshly prepared human peripheral blood monocytes were preincubated for 4 h with antisense (*black bars*) and sense (*dense striped bars*) oligonucleotides targeted to the ceruloplasmin mRNA translation-initiation site. Zymosan (*Zym*) and LDL were added for 24 h and LDL oxidation measured as TBARS as in *A*. Oxidation by unstimulated (*open bar*) and zymosan-activated (*light striped bar*) cells are shown as controls.

inhibition (Fig. 4 *B*). Neither the sense 15-mer controls nor an oligonucleotide targeted to GAPDH (as a control for nonspecific inhibition by RNA/DNA heteroduplexes) was inhibitory. These data confirm the specificity of the inhibition by antisense oligomers and the specific role of ceruloplasmin synthesis in U937 cell-mediated oxidation of LDL. In a complementation experiment, the ability of exogenous ceruloplasmin to rescue the oxidant activity of antisense-blocked U937 cells was tested. At 250 ng/ml of ceruloplasmin, near complete restoration of LDL oxidation was observed (Fig. 4 *C*). At 10 ng/ml of exogenous ceruloplasmin (the approximate concentration in U937 cell–conditioned medium), significant oxidation of LDL occurred and half-maximal oxidation was observed at about 50 ng/ml. Thus, while more exogenous ceruloplasmin was required for maximal activity than was secreted by activated U937 cells (see Discussion for possible explanations), the results are consistent with suppression of LDL oxidation by specific antisense inhibition of ceruloplasmin synthesis, rather than a nonspecific effect of the oligonucleotide (e.g., chelation of ceruloplasmin copper by oligonucleotides, inactivation of nonceruloplasmin cellular factors, etc.).

The role of ceruloplasmin in LDL oxidation by normal human monocytes was examined. Peripheral monocytes were freshly prepared from human blood by a Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) gradient (37). Antisense oligonucleotide targeted to the translation-initiation site of ceruloplasmin mRNA blocked nearly 70% of zymosan-stimulated LDL oxidation while the sense oligonucleotide was inactive (Fig. 4 *D*).

Discussion

In this report we show that exogenously added ceruloplasmin stimulates the oxidative modification of LDL by U937 cells, that zymosan-activated U937 cells synthesize and secrete ceruloplasmin with a time course consistent with that of LDL oxidation, and that inhibition of ceruloplasmin activity or synthesis suppresses the oxidative activity of U937 cells. Together these results are consistent with a mechanism in which monocytic cell-derived ceruloplasmin, in conjunction with other cellular factors or processes, participates in the cellular reactions that cause LDL oxidation.

We have previously shown that ceruloplasmin itself is sufficient to oxidize LDL when coincubated in PBS (12). In contrast, under the current incubation conditions ceruloplasmin by itself does not oxidize LDL, but only expresses oxidant activity when added in the presence of U937 cells. Apparently, a component of RPMI 1640 medium (and other media tested) suppresses the oxidant activity of ceruloplasmin, and a U937 cell–derived product (distinct from ceruloplasmin) is also required for activity. The observed inhibition may be due to the supplemented amino acids since we have shown that free amino acids and proteins are highly inhibitory (12). We have not yet identified the cell-derived factor(s) (besides ceruloplasmin) required for LDL oxidation under these conditions. One candidate is the superoxide anion which is abundantly secreted by activated monocytic cells during an early "burst" period, and then continuously at a lower rate (6, 43). There is evidence for participation of superoxide ion in monocytic cell oxidation of LDL (6, 44), but its specific contribution is controversial (45). Many proteins nonspecifically scavenge superoxide (and other free radicals) (46) thus providing a mechanism for the suppressive activity of amino acids. The much higher potency of exogenous ceruloplasmin on activated cells (Fig. 4 *B*) than on nonactivated cells (Fig. 1 *A*) suggests that the factor is produced preferentially by activated cells, again consistent with a role for superoxide. There is evidence for the participation of multiple monocytic cell–secreted factors in LDL oxidation, including other reactive oxygen species, e.g., hydrogen peroxide and hydroxyl radical, reactive nitrogen species such as nitric oxide and peroxynitrite anion, and thiols (6, 44, 47, 48). Finally, enzymatic action of phospholipase A_2 and lipoxygenase on LDL mimics in many respects cell modification of LDL (49), however, the role of these enzymes in monocytic cell oxidative processes remains controversial (50–52).

The rescue of oxidant activity by ceruloplasmin in zymosan-activated, antisense-blocked U937 cells presents two apparent paradoxes: (*a*) more exogenous ceruloplasmin was required for maximal LDL oxidation than was produced by activated U937 cells and (*b*) much less exogenous ceruloplasmin was required for LDL oxidation by zymosan-activated cells than for nonactivated cells. We have not yet completely resolved the first issue but several explanations may be considered. The time of addition of exogenous ceruloplasmin may not be optimal for oxidant activity. Consistent with this mechanism we have found that delaying ceruloplasmin addition until 6 h after cell activation results in much more oxidation, most likely by avoiding ceruloplasmin damage by the initial superoxide burst (53). Alternatively, cell-derived ceruloplasmin may be preferentially directed to a compartment, e.g., the cell surface, where oxidative processes may be enhanced. The description of putative ceruloplasmin receptors on human blood monocytes (54) suggests a mechanism in which membrane-bound ceruloplasmin may interact with LDL. LDL receptors may also regulate macrophage LDL oxidation (55), but this requirement is controversial (56). Lastly, U937 cell–derived ceruloplasmin may have higher oxidant activity than purified plasma ceruloplasmin due to a specific modification by the cells or to a loss of activity of the plasma protein during the purification procedure. The variability in specific oxidant activity of different ceruloplasmin preparations (most commercial preparations are completely inactive) suggests that the purification procedure itself is a critical factor. The second apparent discrepancy, i.e., the lower requirement for ceruloplasmin in activated than in nonactivated U937 cells, is likely due to the contribution of nonceruloplasmin factors in LDL oxidation by activated U937 cells. This result suggests that in addition to stimulation of ceruloplasmin production, zymosan increases U937 cell production of the nonceruloplasmin factor(s) that is also required for maximal cellular oxidant activity.

Treatment with antisense oligonucleotide targeted at ceruloplasmin blocks LDL oxidation by human peripheral blood monocytes as well as by U937 cells. The higher amount of oligonucleotide required for inhibition of blood monocytes may be due to a less efficient uptake mechanism or a higher rate of degradation by oxidative or nucleolytic processes in monocytes. The rate of ceruloplasmin production (and LDL oxidation) by freshly prepared, zymosan-stimulated peripheral blood monocytes is highly variable in our experiments (not shown), thus our evidence that cell-derived ceruloplasmin is involved in monocyte oxidative processes is less conclusive than that for U937 cells. However, others have shown ceruloplasmin production by activated pulmonary macrophages (27) and ceruloplasmin gene expression (by in situ hybridization) in 10–15% of peripheral blood monocytes (57), consistent with a role in oxidative processes in these cells.

The specific mechanism(s) by which ceruloplasmin increases cell-mediated oxidation of LDL has not been resolved, but the activity of the ceruloplasmin-bound copper is likely and is supported by its suppression by Chelex-100. In one mechanism, cell-derived ceruloplasmin may simply function as a source of free copper for oxidation reactions, or alternatively, a ceruloplasmin/lipoprotein complex may be required for the action of the specific oxidant copper. In any case, ceruloplasmin production by monocytic cells may provide a cellular mechanism by which metal ions are "presented" in a regulatable way for participation in oxidative processes, e.g., by the Haber-Weiss reactions (5) or by copper ion–dependent modification of lipid peroxides to reactive peroxyl and alkoxyl radicals (58). In summary, our experiments are consistent with a role for cell-derived ceruloplasmin in LDL oxidation by U937 monocytic cells. The results also indicate that cellular factors in addition to ceruloplasmin, possibly reactive oxygen species and/or lipoxygenases, are essential and act synergistically with ceruloplasmin in regulating cellular oxidant processes. The role of macrophage-derived ceruloplasmin in lipid oxidation processes in the vessel wall and in other inflammatory responses in vivo has yet to be determined.

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