Inhibition of Copper-Mediated Oxidation of LDL by Rat Serosal Mast Cells

A Novel Cellular Protective Mechanism Involving Proteolysis of the Substrate Under Oxidative Stress

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Rat serosal mast cells, when stimulated to exocytose their cytoplasmic granules, effectively blocked the copper-mediated oxidation of low density lipoproteins (LDLs) in vitro. This effect depended on the proteolytic activity of the formed extracellular granule remnants, since specific inhibition of chymase, the neutral protease that they contain, blocked the protective effect of the mast cells. The mechanism of this chymase-mediated inhibition of LDL oxidation was found to be binding of the copper ions present in the incubation medium by peptides released from LDL on proteolytic degradation of their apolipoprotein B (apoB) component. This was verified by demonstrating that addition of such peptides to LDL–copper ion mixtures completely prevented oxidation of LDL and that this protective effect could be overcome by adding copper ions in excess. Furthermore, proteolytic degradation of the apoB of LDL, with concomitant release of copper-containing peptides, left the partially degraded apoB without the copper ions necessary for propagation of LDL oxidation. These observations provide the first evidence for cell-mediated inhibition of LDL oxidation. (Arteriosclerosis and Thrombosis 1993;13:23–32)

Key Words • chymase • exocytosis • granules • atherosclerosis

The cholesterol found in atherosclerotic lesions is derived mainly from low density lipoproteins (LDLs) that have penetrated into the intima, the inner layer of the arterial wall. Native LDLs are not effectively metabolized by macrophages, which are the precursors of most of the cholesteryl ester–filled foam cells found in atherosclerotic lesions. Therefore, it has been postulated that LDL must be first modified in the intima to be recognized and taken up by macrophages.

One cell type present in atherosclerotic lesions in both animals and humans is the mast cell. Recent studies with rat serosal mast cells both in vitro and in vivo have disclosed a specific mechanism by which mast cells may modify LDL and render it susceptible to uptake by macrophages. The mast cell–induced uptake of LDL by the macrophages is initiated by a strictly ordered sequence of events in which stimulation of mast cells first leads to exocytosis of their cytoplasmic granules, followed by binding of LDL to the heparin proteoglycan component of the formed extracellular granule remnants and proteolytic modification of the granule remnant–bound LDL by the granule-neutral proteases, chymase and carboxypeptidase A. The two granule remnant–bound neutral proteases act in sequence: chymase first cleaves peptides from the apolipoprotein (apo) B, and then carboxypeptidase A cleaves the carboxy-terminal aromatic or branched-chain aliphatic amino acids from the peptides so formed. The peptides cleaved by chymase and liberated from LDL particles are a heterogeneous group of relatively small peptides having $M_r$ of less than 5,000. The result of the binding and degradation of LDL by mast-cell granule remnants is a modified LDL particle characterized by selective loss of protein and increased particle size from 20 nm up to 100 nm through fusion. Finally, granule remnants loaded with the modified LDL particles are phagocytosed by macrophages, within which the cholesteryl ester droplets characteristic of foam cells are formed.

Another type of lipoprotein modification, oxidation, appears to play a major role in generating a modified LDL that leads to foam cell formation in atherosclerotic lesions in both animal models and humans. Experiments conducted in vitro have demonstrated that LDL may be oxidized by many types of intimal cells, e.g., endothelial cells, smooth muscle cells, or macrophages. The oxidized LDL present in atherosclerotic lesions may be formed by several mechanisms: release of superoxide anions from the cells, direct action of membrane-bound enzymes on LDL, or transfer to LDL of lipid peroxides generated within cell membranes by such enzymes as cellular lipoxygenases.

After the initial “seeding” of LDL with peroxides, ions of transition metals such as copper and iron have been shown to play a crucial role in the further oxidative modification of LDL. Indeed, the prototype of oxidized LDL has been “copper-oxidized LDL,” a modification generated in vitro in the absence of cells. More recently, it has been realized that only if ions of either copper or iron are present at nanomolar concentrations will the cell-mediated oxidation of LDL be of sufficient magnitude to induce uptake of the modified LDL.
particles by macrophages. Most importantly, it has been reported that the LDL particles themselves are able to bind three to eight copper ions, suggesting the presence in vivo of LDL-copper ion complexes.

Most cells may also be involved in the oxidative metabolism of LDL. The mast cells, on stimulation, secrete superoxide anions. However, at the same time they secrete a superoxide-scavenging system composed of secretory granule-bound superoxide dismutase (SOD) and peroxidase. These earlier observations suggested that mast cells were potential candidates for involvement in the oxidative metabolism of LDL as either prooxidants or antioxidants. To study the role of these cells in the oxidation of LDL, we incubated LDL with copper ions in the presence of rat serosal mast cells. In contrast with the other intimal cell types studied so far, mast cells totally inhibited copper ion-mediated oxidation of LDL. Unexpectedly, these experiments disclosed that the mechanism for inhibition of the copper-mediated LDL oxidation was the proteolytic degradation of the apoB moiety of LDL by granule remnants exocytosed from stimulated mast cells.

Methods

Materials and Animals

Sodium [125I]iodide (13–17 mCi/μg) and [U-14C]sucrose (>350 Ci/mmol) were purchased from Amersham International. Compound 48/80, diethyldithiocarbamic acid (DDC), chymotrypsin, and bovine serum albumin were obtained from Sigma. Human serum albumin was purchased from Kabi Diagnostica. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Boehringer Mannheim. Malonaldehyde bis(dimethyl acetal) was purchased from Malinaldehyde (d=1.019-1.050 g/ml) were isolated by iodine monochloride method as previously described, and labeled with [U-14C]sucrose as described by Pittman et al. To obtain EDTA-free LDL, either labeled or unlabeled LDLs were dialyzed against three changes (5 l each) of buffer A. Dulbecco's phosphate-buffered saline (PBS) was obtained from Gibco. Male Wistar rats (200–500 g) and female NMRI mice (25–35 g) were purchased from Orion (Espoo, Finland).

Preparation and Labeling of Lipoproteins

Human LDLs (d=1.019–1.050 g/ml) were isolated from plasma by sequential ultracentrifugation, iodinated by the iodine monochloride method as previously described, and labeled with [U-14C]sucrose as described by Pittman et al. To obtain EDTA-free LDL, either labeled or unlabeled LDLs were dialyzed against three changes (5 l each) of buffer A (150 mM NaCl and 5 mM tris(hydroxymethyl)aminomethane [Tris]-chloride, pH 7.4). In experiments the labeled lipoproteins were diluted with unlabeled LDL to give the specific activities indicated in the figure legends. The amounts and concentrations of LDL are expressed in terms of protein.

Isolation and Treatment of Mast Cell Granules

Mast cells and mast cell granules were isolated as described. Briefly, cells were collected from the peritoneal and pleural cavities of rats by lavage with isolation buffer (PBS supplemented with 0.5 mg/ml bovine serum albumin and 5.6 mM glucose, pH 7.3). The cells were washed and resuspended in RPMI 1640 supplemented with 10 mg/ml human serum albumin, 5% fresh rat serum, 25 mM NaCl, 100 IU/ml penicillin, and 2 mM L-glutamine. The cells were seeded into plastic Petri dishes and incubated in a humidified CO₂ (5% CO₂ in air) incubator at 37°C for 1 hour. Nonadherent cells, chiefly mast cells (90–95% purity), were removed for use in experiments. Mast cell granules were then isolated by stimulating the cells at 37°C for 15 minutes with compound 48/80, a positively charged small-molecular-weight compound that causes specific and noncytotoxic degranulation of mast cells. The supernatant, containing the secreted granules, was removed and centrifuged at 12,000 g for 15 minutes to sediment the granule remnants. Proteolytically inactive granule remnants, i.e., PMSF-treated granule remnants, were prepared as described. The quantity of granule remnants is expressed in terms of granule remnant protein.

Purification of Mast-Cell Granule Chymase and Heparin Proteoglycan

Chymase from rat serosal mast cells was purified by hydrophobic chromatography on octyl-Sepharose CL-4B as described by Kido et al. Mast-cell granule heparin proteoglycan was purified by treating the soluble proteoglycan fraction released on mast cell stimulation with chondroitinase ABC as described. After incubation at 37°C for 18 hours the heparin proteoglycan was dialyzed against three changes (5 l each) of buffer A. The amount of heparin proteoglycan was determined by the Alcian blue method, with commercial heparin as the standard.

Isolation of Mouse Peritoneal Macrophages

Macrophages were harvested from unstimulated NMRI mice in PBS containing 1 mg/ml bovine serum albumin as described by Goldstein et al. The peritoneal cells were resuspended in RPMI 1640 culture medium containing 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded into plastic Petri dishes (35×10 mm, Falcon) and incubated in a humidified CO₂ incubator (5% CO₂) at 37°C for 1 hour. Three rinses of PBS (2 ml) were used to remove nonadherent cells. For experiments the dishes received 1 ml culture medium (RPMI 1640 with L-glutamine containing 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin).

Preparation of LDL Degradation Products

LDL (500 μg) was incubated in 300 μl buffer A containing 20 μg granule remnants at 37°C for 18 hours. An aliquot was treated with trichloroacetic acid to determine the degree of proteolytic degradation of LDL. The low-molecular-weight degradation products were separated from the proteolyzed LDL particles and granule remnants by ultrafiltration through a Centricon 10 filter (Amicon) with a 10,000-d cutoff point. For this purpose the filter was centrifuged four times at 1,400 g for 30 minutes, and the ultrafiltrates were pooled and analyzed for protein concentration.

Copper-Mediated Oxidation of LDL

In a standard assay EDTA-free LDL (100 μg) was incubated in 300 μl PBS containing 7 μM CuSO₄ or mast cell-derived material at 37°C for 3 hours. The extent of lipid peroxidation of LDL was measured as thiobarbituric acid (TBA)–reactive materials and expressed as malondialdehyde (MDA) equivalents.
Mast Cell–Mediated Inhibition of LDL Oxidation

In a standard assay, mast cells (10⁶) in 250 μl PBS were stimulated with compound 48/80 (5 μg/ml) for 5 minutes at 37°C. After incubation the cells were centrifuged at 150g for 10 minutes. The supernatant containing the exocytosed material was removed (150g supernatant). The cell pellet was washed twice and resuspended in 250 μl PBS. Aliquots of the exocytosed material (the 150g supernatant) derived from 4 × 10⁶–1 × 10⁸ mast cells and the corresponding cell pellets were then tested for their effects on the copper-mediated oxidation of LDL as described above.

Uptake of Modified LDL by Mouse Peritoneal Macrophages

In a standard assay, [U¹⁴C]sucrose-LDL (100 μg) was incubated in 300 μl PBS containing 7 μM CuSO₄ and 10 μg untreated or PMSF-treated granule remnants at 37°C for 3 hours. After incubation the granule remnants were sedimented by centrifugation at 12,000g for 15 minutes. The supernatant was removed, and a 100-μl sample was analyzed for the presence of TBA-reactive material and a 10-μl sample for electrophoretic mobility. The remaining material was dialyzed against two changes (5 l each) of buffer (150 mM NaCl, 5 mM Tris-chloride, and 1 mM EDTA, pH 7.4) and applied to the macrophage dishes. After incubation for 18 hours the uptake of [¹⁴C]sucrose-LDL was determined as described.³⁵ Uptake is expressed as micrograms of LDL protein per milligram of cell protein.

Determination of Copper Ions in the Reaction Mixture

The concentration of copper ions was determined by atomic absorption spectrophotometry (Perkin Elmer AAS-5000) by using the graphite furnace method according to the manufacturer’s recommendations or according to the DDC method described by Marciani et al.³⁶ For analysis with the latter method, samples or copper sulfate standards (0–100 μM) were mixed into 400 μl buffer A containing 1.5 μM DDC. The absorbance of the copper–DDC complex was measured with an automatic microplate reader (Multiskan MCC, Labsystems) at 450 nm. In an additional experiment we used atomic absorption spectrophotometry to measure the concentration of copper ions in the granule-remnant fraction and found that the granule remnants were unable to bind copper ions (data not shown).

Other Assays

Protein was determined by the procedure of Lowry et al³⁷ with bovine serum albumin as the standard. The electrophoretic mobility of the oxidized LDL was determined on cellulose acetate plates (Helena Laboratories) stained with Ponceau red.

Results

The effect of mast cells on the copper-mediated oxidation of LDL was measured by incubating LDL with copper ions in the presence of either unstimulated mast cells or mast cells that were stimulated with compound 48/80. The formation of TBA-reactive material in the incubation medium was taken as an index of lipid peroxidation of LDL. In the presence of unstimulated mast cells, copper ions caused rapid and extensive oxidation of LDL (Figure 1, column 2). In contrast, the copper-induced oxidation of LDL was totally abolished if the mast cells were stimulated at the beginning of incubation (column 3). A control experiment showed that in the absence of copper ions, stimulated mast cells themselves did not trigger the formation of TBA-reactive material (column 4).

To study whether the capacity of stimulated mast cells to protect against oxidation of LDL resided in the cells or in the material released from them on stimulation, we sedimented the stimulated mast cells by low-speed centrifugation and made separate studies of the effects of the cell pellet and the supernatant on the copper-mediated oxidation of LDL. As shown in Figure 2, the protective effect resided in the supernatant, which contained the material exocytosed from the stimulated mast cells. In the incubation system containing 330 μg/ml LDL protein and 7 μM CuSO₄, the exocytosed material caused a concentration-dependent inhibition of LDL oxidation. 85% inhibition being achieved with 25 μl exocytosed material, corresponding to the amount of material released from approximately 100,000 mast cells.

To locate the protective agent present in the material released from stimulated mast cells (150g supernatant), the granule remnants were sedimented by centrifugation of the supernatant at 12,000g.³² The two fractions formed (granule remnants and granule-free supernatant) were then incubated separately with LDL in the presence of copper sulfate. It was found that most of the protective capacity was located in the granule-remnant fraction (Figure 3). Indeed, addition of increasing vol-

FIGURE 1. Bar graph shows the effect of stimulated rat serosal mast cells on copper-mediated oxidation of low density lipoprotein (LDL). Mast cells (10⁶) were incubated at 37°C in 300 μl Dulbecco’s phosphate-buffered saline containing 100 μg EDTA-free LDL (column 1) and the following additions: 7 μM CuSO₄ (column 2); 7 μM CuSO₄ and 5 μg/ml compound 48/80 (column 3); or 5 μg/ml compound 48/80 (column 4). After incubation for 3 hours the amount of thiobarbituric acid (TBA)―reactive material was determined as described in the text. Each value is the average of duplicate incubations. MDA, malondialdehyde.
The cells were stimulated with compound 48/80 (5 ng/ml) for 5 minutes. Cells were then sedimented by centrifugation at 150g for 10 minutes. The exocytosed material (150g supernatant) was removed, and the cell pellet was washed twice and resuspended in 300 μl PBS. The indicated amounts of mast cell pellet (A) and 150g supernatant (•) were then incubated in a final volume of 300 μl PBS containing 100 μg LDL and 7 μM CuSO₄. After incubation at 37°C for 3 hours, the amount of thiobarbituric acid (TBA)–reactive material was determined as described in the text. Each value is the average of duplicate incubations. MDA, malondialdehyde.

The two major components of the granule remnants of rat serosal mast cells are heparin proteoglycans and the neutral protease chymase:8 the former binds apoB and the latter degrades it.10 We isolated and purified both components of the granule remnants for separate studies of their effects on the copper-induced oxidation of LDL. We found that heparin proteoglycans did not prevent the formation of TBA-reactive material (Figure 3). In contrast, addition of increasing amounts of purified chymase inhibited the oxidation of LDL in a concentration-dependent fashion, maximal inhibition being achieved with approximately 6 μg/ml purified chymase.

To test whether the protective capacity of the granule chymase was due to its proteolytic action on the apoB component of LDL, chymase was specifically inhibited by treating the granule remnants with PMSF.8 As shown in Figure 5A, incubation of LDL with copper ions caused extensive oxidation of LDL, i.e., more than a 10-fold increase in the concentration of TBA-reactive material (columns 1 versus 2). The copper-induced LDL oxidation could be inhibited by adding granule remnants to the incubation system (column 3). In contrast, if the granule remnants were first treated with PMSF and then added to LDL along with copper sulfate, no such inhibition of LDL oxidation could be observed (column 4). After the incubations, aliquots were taken from the incubation mixtures to determine the electrophoretic mobilities of the LDL particles. As shown in the inset of Figure 5A, the LDL incubated with PMSF-treated granule remnants and copper ions showed the increased electrophoretic mobility typical of oxidized LDL (lanes 4 and 2, respectively). On the other hand, the LDL incubated with proteolytically active granule remnants and copper ions showed electrophoretic mobility similar to that of untreated native LDL (lanes 3 and 1, respectively). The effect of the granule remnants on oxidative modification of LDL was also studied with a bioassay in which the ability of cultured mouse peritoneal macrophages to internalize modified LDL was tested.3 As shown in Figure 5B, oxidized LDL was taken up avidly by mouse peritoneal macrophages (column 2). Addition of proteolytically active granule remnants to the incubation system totally blocked the copper-induced increment in LDL uptake, so that such LDLs were taken up by macrophages at approximately the same very low rate as native LDL (columns 3 and 1, respectively). Finally, inhibition of chymase activity with PMSF blocked the protective effect of the granule remnants and thus permitted the copper-mediated oxidation of LDL, leading to increased uptake of LDL by macrophages (column 4).
To quantitatively relate the protective effect of chymase to its ability to proteolyze the apoB component of LDL, we measured the formation of TBA-reactive material as a function of the degree of apoB degradation. For this purpose, LDLs were first incubated in the presence of granule remnants for various periods of time, after which the proteolytic degradation of apoB was stopped by addition of PMSF. Oxidation of the granule remnant–treated LDL was then initiated by addition of copper sulfate, and the amounts of TBA-reactive material were measured after a standard incubation of 3 hours. As shown in Figure 6A, incubation of LDL in the presence of granule remnants resulted in progressive degradation of apoB, measured as production of trichloroacetic acid–soluble material. It was found that the more extensively apoB was proteolyzed, the less susceptible it was to oxidation (Figure 6B). Indeed, after degradation for 60 minutes, LDL was practically resistant to copper-mediated oxidation.

The above results suggest that either the degradation products, i.e., the peptides and amino acids released from apoB,11 possessed a protective effect or that the proteolytically degraded LDL particles themselves are more resistant to oxidation than are native LDLs. The first alternative was tested by measuring the protective effect of the proteolytic degradation products released from apoB. For this purpose LDL was incubated with granule remnants, and the low-molecular-weight degradation products formed were separated from the proteolyzed LDL particles by ultrafiltration through a filter with a 10,000-d cutoff point. Increasing amounts of the degradation products were then added to a reaction mixture containing incubation medium, intact LDL particles, and copper sulfate. It was found that the degradation products of apoB prevented the copper-mediated oxidation of LDL in a dose-dependent fashion (Figure 7A). Addition of copper sulfate in excess overcame the protective effect of the proteolytic degradation products (Figure 7B).
After incubation at 37°C for 3 hours, the amount of thiobarbituric acid (TBA)–reactive material was determined as described in the text. Each value is the average of duplicate incubations. MDA, malondialdehyde.

FIGURE 6. Line graphs show inhibition of copper-mediated low density lipoprotein (LDL) oxidation as a function of LDL degradation. 125I-LDL (100 μg; 1,800 counts per minute/μg protein) was incubated at 37°C in 100 μl Dulbecco’s phosphate-buffered saline (PBS) containing 10 μg granule remnants for the indicated time intervals. Proteolysis was stopped by adding phenylmethylsulfonyl fluoride (final concentration, 250 μg/ml). A sample of each reaction mixture was taken to determine the amount of 125I-labeled trichloroacetic acid–soluble degradation products (panel A) as described in the text. Another sample of each reaction mixture was incubated in a final volume of 300 μl PBS containing 7 μM CuSO4. After incubation at 37°C for 3 hours, the amount of thiobarbituric acid (TBA)–reactive material was determined (panel B) as described in the text. Each value is the average of duplicate incubations. MDA, malondialdehyde.

μg/ml apoB degradation products was required for inhibition of the oxidation of 330 μg/ml LDL induced by 13 μM CuSO4 in the given buffer.

The stoichiometry between the apoB peptides and copper sulfate in the above experiment suggested direct interaction between the divalent copper ions and the peptides. This hypothesis was tested by investigating the effect of apoB peptides on the elution of copper sulfate on a Bio-Gel P-2 column. As shown in Figure 8A, a control solution containing copper sulfate alone eluted at the position corresponding to the total volume of the column. In contrast, if the peptides produced by proteolytic degradation of apoB by chymase were incubated with copper sulfate and the reaction mixture was chromatographed on a Bio-Gel P-2 column, part of the copper ions coeluted with these peptides (Figure 8B–C, fractions 10–38), indicating that the apoB-derived peptides bound copper ions.

The second possibility for the protective effect of apoB degradation is that the release of copper-containing peptides from apoB on proteolytic degradation of LDL lowers the copper ion content of the apoB, thereby rendering LDL resistant to oxidation. This hypothesis was tested as follows: LDL (400 μg) was incubated in 400 μl buffer A containing 20 μM CuSO4 at 37°C for 15 minutes. Unbound copper ions were separated from LDL-bound copper ions by ultrafiltration. The remaining LDLs were resuspended in buffer A; analysis showed they contained approximately two thirds of the added copper ions. These copper-containing LDLs (100 μg) were incubated in 300 μl buffer A with or without 5 μg purified chymase at 37°C for 18 hours. The peptides formed were separated from the degraded LDL by ultrafiltration. Protein and copper ion concentrations in the degraded copper-containing LDL fraction and in the ultrafiltrate containing the degradation products were determined. Treatment with chymase led to the release of not only low-molecular-weight degradation products of apoB (43% of total apoB mass) but also to substantial (87%) amounts of the LDL-bound copper ions into the incubation medium, thus producing copper-deficient LDL particles (Table 1).

Finally, we tested whether the proteolytic modification of LDL by chymase treatment would render the LDL particles resistant to oxidation. For this purpose, copper-containing LDLs were prepared by first incubating LDLs with copper sulfate and then removing unbound copper ions as described above. Subsequently, the copper-containing LDL particles were incubated at 37°C with purified mast cell chymase up to 6 hours. As shown in Figure 9A, such incubation led to progressive degradation of apoB. More importantly, the proteolyzed LDL, i.e., the copper-deficient LDL particles, did not become oxidized like the LDLs that were not incubated with chymase and, consequently, contained all the copper ions bound to them during the initial incubation with copper sulfate (Figure 9B). However, addition of copper sulfate (7 μM) to the proteolyzed copper-deficient LDL particles resulted in formation of TBA-reactive material in amounts that equaled those observed when copper sulfate was added to untreated LDL (data not shown).

Discussion

The present in vitro study demonstrates that stimulation of rat serosal mast cells leads to inhibition of copper-mediated oxidation of LDL. This inhibition depends on the extracellular generation of copper-binding apoB peptides from LDL by chymase, the neutral protease of mast cells. Since chymase is a matrix-bound component of the cytoplasmic granules of mast cells, this enzyme does not gain access to the apoB component of LDL present in the extracellular fluid unless the granules are exocytosed in response to mast cell stimulation. In previous studies, we have shown that even after exocytosis the granule-bound chymase does not degrade all of the LDL particles present in the incubation medium, but only the very small fraction of the particles that are bound by the granule remnants.11
These remnant-bound particles appear to form a barrier that shields the enzyme molecules from contact with the apoB of the unbound LDL particles. Accordingly, only a small fraction of the apoB molecules present in the incubation system are degraded by the granule remnants and act as the source of the copper-binding peptides. Nevertheless, the quantity of peptides released was found to be sufficient to protect both the bound and unbound fractions of LDL from copper-mediated oxidation.

The peptides released from the apoB portion of granule-remnant-bound LDL on proteolytic degradation by chymase have been found to have molecular weights of less than 5,000 d. The present report on the ability of these small peptides to bind copper ions is the first biological function assigned to them. The mechanism by which these peptides inhibit the copper-induced oxidation of LDL was found to be the binding of copper ions present in the aqueous phase, with formation of apparently redox-inactive copper ion-peptide complexes. The amino acids histidine, cysteine, and tryptophan have been shown to form complexes with copper ions. Since these amino acids are among the peptides released from the apoB of LDL by the proteolytic action of mast-cell granule remnants, it appears likely that they were the agents responsible for the observed binding of copper ions. However, isolation and sequencing of the apoB peptides released by chymase are required to identify the copper-binding sites on apoB.

Furthermore, amino acids such as histidine and tryptophan are also strong inhibitors of copper-mediated LDL oxidation, suggesting that other proteolytic peptides besides those derived from the apoB of LDL could act as copper-ion chelators. Indeed, we have shown that high density lipoprotein degradation products are capable of inhibiting the copper-mediated oxidation of LDL (P.T. Kovanen et al, unpublished observations).

Copper-induced oxidation of LDL was also prevented by another mechanism. During chymase-mediated degradation of LDL, the gradual proteolytic cleavage of apoB led to the release not only of peptides but also of copper ions. Since the released peptides had an affinity for copper ions, it is conceivable that the copper ions, known to have affinity for apoB, had initially been bound to the peptide segments and were then released passively along with them. Thus, the second protective mechanism was the generation of proteolytically modified, copper-deficient LDL particles. However, since chymase is a granule-remnant-bound protease and only the granule remnant-bound LDL particles are degraded by chymase, only a minor fraction of LDL could potentially benefit from this type of protection against copper-catalyzed oxidation.

The major protective mechanism of mast cells against copper-mediated oxidation of LDL could be assigned to the proteolytic action of granule-remnant chymase. However, a slight inhibition of LDL oxidation was still observable after the granule remnants (and chymase) had been removed from the mast cell-conditioned medium (Figure 3, granule-free supernatant). This granule-free fraction of the medium contains all the histamine released from the exocytosed mast cell granules. When copper ions are immobilized in chelating Sepharose beads, a column of such Sepharose is then able to adsorb histamine from the eluent. This ability of histamine and copper ions to form complexes is not surprising, since histamine contains the ring structure of histidine that is responsible for the binding of copper ions. Thus, copper ions are effectively bound not only by the apoB-derived peptides but also by histamine, which likewise protects LDL from oxidation. Indeed, by
binding of copper ions to LDL and release of copper-containing peptides by granule chymase

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<tr>
<th>Fraction</th>
<th>Protein (mg/l)</th>
<th>Copper (µmol/l)</th>
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<tr>
<td>LDL degradation products</td>
<td>212 (43)</td>
<td>2.80 (87)</td>
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<tr>
<td>Proteolyzed LDL</td>
<td>286 (57)</td>
<td>0.42 (13)</td>
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<tr>
<td>LDL</td>
<td>498 (100)</td>
<td>3.22 (100)</td>
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LDL, low density lipoprotein. The values for LDL are calculated values and represent the sums of LDL degradation products plus proteolyzed LDL. Each value is the average of duplicate incubations. Values in parentheses represent percentages of control values. For further details, see “Methods.”

adding sufficient commercial histamine to mixtures of copper ions and LDL, we could inhibit oxidation of LDL completely (data not shown).

The cytoplasmic secretory granules of mast cells contain SOD, another matrix-bound enzyme and a potent scavenger of superoxide anions. Therefore, this granule enzyme must also be regarded as a potential candidate for the protective defense system of stimulated mast cells. PMSF, a specific inhibitor of the granule chymase that had no effect on the SOD activity of granule remnants (data not shown), inhibited the protective effect of mast-cell granule remnants by about 80% (Figure 5). Whether the residual inhibition of LDL oxidation (approximately 20%; 0–20% in different experiments) observed with granule remnants was due to an SOD effect remains to be shown. It is noteworthy that Parthasarathy and coworkers have shown that human recombinant SOD effectively inhibits copper-induced oxidation of LDL. In addition, these workers have found that SOD effectively blocks oxidation of LDL by smooth muscle cells. Stimulated mast cells could be able, by means of their granule SOD, to effectively inhibit cell-mediated oxidation of LDL, notably oxidation by smooth muscle cells and monocytes, in which the superoxide pathway predominates.

In terms of human disease, oxidation of LDL in the arterial intima, the site of atherogenesis, appears to be of major importance. Thus, the various cell types of the arterial intima studied so far, i.e., the endothelial cell, monocyte-macrophage, and smooth muscle cell, all have the ability to oxidize LDL in vitro. The mast cell, a cell type also present in the arterial intima, clearly seems to be an exception among the intimal cells. Indeed, the mast cell is the first cell type of the arterial intima studied that prevents rather than promotes oxidation of LDL. This unique property of mast cells is a consequence of their ability to induce extracellular degradation of proteins that have an affinity for heparin proteoglycan such as apoB, an ability that appears not to be shared by the other cell types present in the arterial intima.

Initiation of the oxidative modification of LDL in the arterial intima may result both from cellular oxidative reactions and from cell-independent spontaneous oxidative reactions in the extracellular fluid. After initiation, the propagation of the lipid peroxidation necessary to modify LDL to a form that can be recognized by the scavenger receptors of macrophages is greatly facilitated by trace amounts of transition metal ions. In the presence of such metal ions, stimulation of mast cells with ensuing apoB degradation and release of histamine

Figure 8. Line graphs show binding of copper ions by apolipoprotein B–derived peptides. Low density lipoprotein degradation products were obtained as described in the text. Copper sulfate (1 mM) was incubated either alone (panel A) or with 100 µg 125I-labeled peptides (383 counts per minute/µg) (panels B and C) in 200 µl buffer A at 37°C for 15 minutes. After incubation, the reaction mixtures were applied to a Bio-Gel P-2 column (1.0×17 cm). The column was eluted with buffer A at a flow rate of 4 ml/hr at room temperature. Fractions of 400 µl were collected into tubes containing 25 µl of 25 mM diethyldithiocarbamic acid. The fractions were measured for their copper ion concentration and 125I radioactivity as described in the text. The void volume (V₀) and the total volume (Vₜ) of the column are indicated by arrows (panel A). Note the different scales in absorbance in panels A and B.
could act locally as a defense mechanism against the oxidative stress impinging on the intimal LDL. Can intimal mast cells prevent the oxidative modification of LDL in the arterial intima? Current evidence for the presence of oxidized LDL in atheromatous lesions of the arterial intima does not favor this possibility. Whether the absence of oxidized LDL from the nonatheromatous areas reflects a lesser oxidative stress or, perhaps, a sufficiency of protective activity emanating from mast cells remains to be shown.

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References

8. Kokkonen JO, Kovanen PT: Stimulation of mast cells leads to cholesterol accumulation in macrophages in vitro by a mast cell granule-mediated uptake of low density lipoprotein. Proc Natl Acad Sci U S A 1987;84:2287-2291
lipoprotein phospholipids. Proc Natl Acad Sci USA 1984;81:3883–3887
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