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Products of lipid peroxidation induce missorting of the principal lysosomal protease in retinal pigment epithelium

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Abstract

Phagocytosis of photoreceptor outer segments (OS) by retinal pigment epithelium (RPE) is essential for OS renewal and survival of photoreceptors. Internalized, oxidatively modified macromolecules perturb the lysosomal function of the RPE and can lead to impaired processing of photoreceptor outer segments. In this study, we sought to investigate the impact of intracellular accumulation of oxidatively damaged lipid–protein complexes on maturation and distribution of cathepsin D, the major lysosomal protease in the RPE. Primary cultures of human RPE cells were treated with copper-oxidized low density lipoprotein (LDL) and then challenged with serum-coated latex beads to stimulate phagocytosis. Three observations were noted to occur in this experimental system. First, immature forms of cathepsin D (52 and 46 kDa) were exclusively associated with latex-containing phagosomes. Second, maturation of cathepsin D was severely impaired in RPE cells loaded with oxidized LDL (oxLDL) prior to the phagocytic challenge. Third, pre-treatment with oxLDL caused sustained secretion of pro-cathepsin D and the latent form of gelatinase A into the extracellular space in a dose-dependent manner. These data stimulate the hypothesis that intracellular accumulation of poorly degradable, oxidized lipid–protein cross-links, may alter the turnover of cathepsin D, causing its mistargeting into the extracellular space together with the enhanced secretion of a gelatinase.

Keywords: Pigment epithelium of retina; Lipid peroxidation; Lysosome; Cathepsin D; Membrane transport; Phagocytosis

1. Introduction

Lipid peroxidation has been implicated in many age-associated disorders including macular degeneration of the retina [1–3]. The retinal pigment epithelium (RPE) plays a crucial role in the turnover of photoreceptor cell membranes [4,5], which can be a substrate for peroxidation [6]. It is reported that oxidatively modified macromolecules can perturb processing of photoreceptor outer segments (OS) by RPE, leading to intracellular accumulation of OS breakdown products and lipofuscin formation [7–10]. Proteins damaged by products of lipid peroxidation have also been identified in drusen, a term used to describe subRPE deposits which are the hallmark of age-related macular degeneration (AMD) [11]. AMD shares the phenomenon of accumulation of subRPE debris with Sorsby's fundus dystrophy, an autosomal dominant degenerative disease of the macula caused by mutations in the tissue inhibitor of metalloproteinase-3 (TIMP-3) gene [12]. Recent findings that the S156C mutant of TIMP-3 has diminished ability to block gelatinase activity [13] suggest that the abnormal subretinal deposits can by viewed as a result of the imbalance between buildup and breakdown of the extracellular matrix (ECM).

Remodeling of the ECM may be dependent on redox regulation, as in the case of blood vessels. Oxidized glutathione activates matrix metalloproteinases (MMPs) in cardiac fibroblasts [14], oxidized low density lipoprotein (oxLDL) induces collagen synthesis in arterial smooth muscle cells [15] and upregulates expression of MMP-9 in macrophages [16], and the antioxidant *N*-acetyl-cysteine abolishes gelatinase activity and MMP-9 secretion by aortic macrophage-derived foam cells [17]. Since abnormal secretion of lysosomal proteases has been implicated in oxidative

Abbreviations: AMD, age-related macular degeneration; ECM, extracellular matrix; LDL, low density lipoprotein; oxLDL, oxidized LDL; MMP, matrix metalloproteinase; OS, photoreceptor outer segments; RPE, retinal pigment epithelium; hRPE, human RPE

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activation of MMPs [17], we hypothesized that intracellular accumulation of oxidatively damaged, cross-linked and poorly degradable lipid–protein complexes may be a common mechanism for intracellular lipofuscin formation and extracellular buildup of ECM.

We have previously developed an experimental model of loading cultured macrophages or RPE cells with non-toxic amounts of copper-oxidized LDL as a source of a complex mixture of oxidatively damaged lipids and proteins [7,18]. OxLDL mimics A2E, a major retinoid-containing lipid fluorophore of lipofuscin, and provides a physiologic toxin of oxidative origin that can initiate cytotoxicity and lysosomotropy [19–22]. Cells pre-treated with oxLDL exhibited selective impairment in processing of internalized macromolecules through severe inhibition of lysosomal proteolysis with little effect on surface binding and rate of internalization of a variety of tested proteins, including lipoproteins, and bovine photoreceptor OS. These findings suggest that the lysosomal enzyme activity and/or transport is affected by oxLDL. It is reported that oxLDL is a potent inhibitor of the thiol protease cathepsin B, but not the aspartic protease cathepsin D [18]. The latter enzyme is the principal lysosomal protease in RPE [23] and is responsible for the proteolysis of internalized OS disks [24,25]. Transgenic mice that overexpress mutant (inactive) cathepsin D acquire features of AMD, including geographic atrophy, accelerated photoreceptor death, and presence of basal laminar deposits [26].

The proper maturation of cathepsin D requires cleavage of its pro-domain by a thiol protease [27–30]. Since products of lipid peroxidation are potent inhibitors of thiols, including thiol proteases [31], we asked whether internalized oxLDL perturbs maturation and distribution of cathepsin D during RPE phagocytosis. We report that treatment of primary cultures of human RPE (hRPE) cells with oxLDL impairs intracellular trafficking of cathepsin D by blocking its maturation, inducing secretion of procathepsin D into the extracellular space. In addition to the mistargeting of cathepsin D, oxLDL caused an increase in secretion of extracellular gelatinase. These data suggest a novel mechanism for oxidation-induced remodeling of Bruch's membrane.

2. Materials and methods

2.1. Reagents

Cell culture media and culture media additives were purchased from Invitrogen, Carlsbad, CA. Heat-inactivated CELLect Gold fetal bovine serum (FBS) was purchased from ICN Biomedicals, Irvine, CA. All protein gel electrophoresis reagents and instruments were from Bio-Rad, Hercules, CA and Invitrogen. All chemicals were from Sigma Chemical Co., St. Louis, MO unless specified otherwise.

2.2. RPE cell culture

Primary cultures of human RPE cells were a gift from Dr. Janice Burke (Medical College of Wisconsin), and were obtained as confluent cell monolayers at early passages ranging from 3 to 8. Cultures of RPE cells from three different donors, ages 55–80, were used in the study. Cells were maintained and passaged as described previously with biweekly feedings of minimum essential medium (MEM) containing 10% FBS, 500 U/ml penicillin, 500 μg/ml streptomycin, 125 ng/ml fungizone, and 50 μg/ml gentamicin [32].

2.3. LDL isolation and oxidation

Low density lipoprotein (LDL) was isolated from fresh plasma obtained from the Cleveland Clinic Blood Bank by sequential ultracentrifugation as a $1.019 < d < 1.063$ g/ml fraction using the procedure of Hatch and Lees [33]. The LDL was dialyzed against 0.15 M NaCl containing 0.5 mM Na_2EDTA, pH 8.5, filter-sterilized and stored at 4 °C. Oxidation of LDL was performed in the presence of 10 μM $CuSO_4$ in 0.15 M NaCl at a protein concentration of 500 μg/ml for 24 h at 20 °C [18,21]. The concentration of EDTA in LDL preparations was reduced prior to oxidation by overnight dialysis against 0.15 M NaCl. Oxidation was terminated by dialyzing samples into 0.15 M NaCl, 0.3 mM Na_2EDTA, and 20 mM Na phosphate pH 7.4, for 24 h. Butylated hydroxytoluene dissolved in ethanol was added at a final concentration of 40 μM to prevent oxidation.

2.4. Phagosome isolation

hRPE cells were plated into 100 mm Petri dishes and were grown for 1 week until confluency. After appropriate pre-treatments of the hRPE performed in fresh culture media, and specified in the figure legends, the phagocytosis was initiated by adding 50 or 200 μl of 2% suspension of carboxylated paramagnetic latex beads (2 μm in diameter) (Polysciences, Warrington, PA), which were coated with FBS and washed prior to the addition to cell cultures [34]. Cells were then incubated for the indicated periods of time at 37 °C in a CO_2 incubator, the experiment was stopped by placing the dishes on ice, and gentle scraping and pelletting of cells at $200 \times g$. Cells were then resuspended in homogenization buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES, pH 7) containing a cocktail of protease inhibitors Complete (Roche, Indianapolis, IN), and homogenized by passing the cell suspension five times through a 26-gauge needle. Phagosomes containing latex beads were isolated using magnet extraction (Dynal, Oslo, Norway). Phagosomal fraction was resuspended in homogenization buffer and magnetic isolation of phagosomal as well non-phagosomal fractions was repeated two times to ensure purity of these subcellular fractions.

2.5. Western blot analysis

Laemmli buffer (2 ×) supplemented with 2 × cocktail of protease inhibitors Complete but without reducing agents was used to elute hRPE proteins associated with latex beads, as well as to solubilize proteins of non-phagosomal fractions or total cell homogenates. Protein concentration in extracts was determined using BCA reagent (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were loaded and resolved by SDS-PAGE using 4–20% gradient precast minigels (Invitrogen). Proteins separated by SDS-PAGE were electro-transferred on a PVFD membrane and probed with one of the following antibodies: mouse anti-human LAMP-1, clone H4A3 (Development Studies Hybridoma Bank, Iowa City, IA); goat anti-cathepsin D, clone C-20; and rabbit anti-Rab5, clone (S-19) (both from Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected with a corresponding second anti-IgG antibody conjugated with HRP (Jackson ImmunoResearch, West Grove, PA), followed by Renaissance reagent (NEN Life Science, Boston, MA) as a chemiluminescent substrate.

2.6. Proteolytic activity of cell extracts

Bovine serum albumin (BSA) was modified by maleic anhydride as described by Goldstein et al. [35], and labeled with $Na^{125}I$ using the iodine monochloride procedure as described by Bilheimer et al. [36]; the specific activity of labeled preparations was between 1000 and 2000 cpm/ng protein. ^{125}I-Maleylated BSA (mBSA) was used as a protein substrate for measuring proteolytic activity in RPE-conditioned culture media. After appropriate pre-incubations of hRPE cells, conditioned media was harvested as specified in the figure legends, and subjected to centrifugation at 10,000 × *g* for 10 min, to remove detached cells and cellular debris. Fifty-microliter aliquots of conditioned media were mixed with 50 μl of either 0.2 M phosphate buffer, pH 7.4, or 0.2 M acetate buffer, pH 4.5. The reaction was started by adding 2 μg of ^{125}I-mBSA in a 2 μl aliquot. Following a 5-h incubation at 37 °C, the degradation was stopped by placing the reaction mixture on ice, then adding 10 μl FBS and 20 μl of 100% trichloroacetic acid (TCA). Formation of TCA-soluble, non-iodine degradation products was measured according to the procedure described previously [21]. Results are presented as micrograms of ^{125}I-mBSA degraded by an aliquot of conditioned media and normalized to the amount of RPE cell protein.

2.7. Zymography

After pre-incubation of hRPE cells with oxLDL, conditioned culture media was collected, cellular debris was removed by centrifugation at 10,000 × *g* for 10 min, and equal aliquots of media were loaded on a 10% precast polyacrylamide gel with 1 mg/ml gelatin (Invitrogen). Following electrophoresis, gels were agitated (30 min, two times) in a Renaturating Buffer (Invitrogen) to remove SDS and to promote renaturation of proteases. The gels were then incubated for 20 h in the Developing Buffer (Invitrogen), and stained with 5 mg/ml Coomassie Blue R-250 in acetic acid/methanol/water (1:3:6) for 2 h and destained with acetic acid/methanol/water (1:3:6) as described by Qi et al. [13].

3. Results

Cathepsin D is initially synthesized as an inactive pro-enzyme (52 kDa) that is subsequently converted into an active intermediate (46 kDa) in the endosomal compartment [25,37]. A final cleavage in the lysosome generates the mature 31-kDa form of cathepsin D [25,37]. We first sought to establish the presence and distribution of cathepsin D isoforms in the cultured human RPE during non-specific phagocytosis of latex beads. Addition of serum-coated magnetic carboxylated polystyrene microspheres to hRPE cells resulted in their efficient internalization by the cell cultures (not shown) followed by a time-dependent increase of phagosome-associated LAMP-1, cathepsin D, and Rab5 (Fig. 1A). Rab5 is a small GTPase essential for membrane fusion events during the early steps of phagocytosis [38,39]. LAMP-1 is an abundant lysosomal membrane-associated protein that exists as a collection of multiple heavily glycosylated forms with molecular weights ranging from 90 to 120 kDa [40]. Compartments containing latex beads acquired lower molecular weight and less heterogeneous LAMP-1 species compared to the non-phagosomal cellular fraction (Fig. 1A). Immature forms of cathepsin D were virtually absent from the phagosomal fractions, and only very low levels of the proenzyme were detected outside of phagosomes. However, when the pH of intracellular compartments was raised by chloroquine, pro-cathepsin D was readily detected in hRPE cell extracts (Fig. 1B). Chloroquine efficiently blocked the formation of highly glycosylated species of LAMP-1 in hRPE cells (Fig. 1B), which is consistent with the reported pH-dependency of final maturation of this glycoprotein [41].

Since less mature forms of LAMP-1 tend to associate with newly formed phagosomes (Fig. 1A), we hypothesized that a greater phagocytic challenge may result in more internalized particles in the RPE, thereby increasing the demand for phagosomal constituents, which in turn may stimulate additional production of cathepsin D. In synchrony with this idea, challenge of RPE cells with larger amounts of latex particles (200 μl/100 mm Petri dish instead of 50 μl/dish) caused the appearance of immature cathepsin D in hRPE extracts. The 46-kDa form was predominantly found in the non-phagosomal cellular fraction, whereas the 52-kDa form was exclusively associated with phagosomes (Fig. 1C). This result indicated that excessive phagocytic load exceeded the capacity of hRPE cells to supply mature

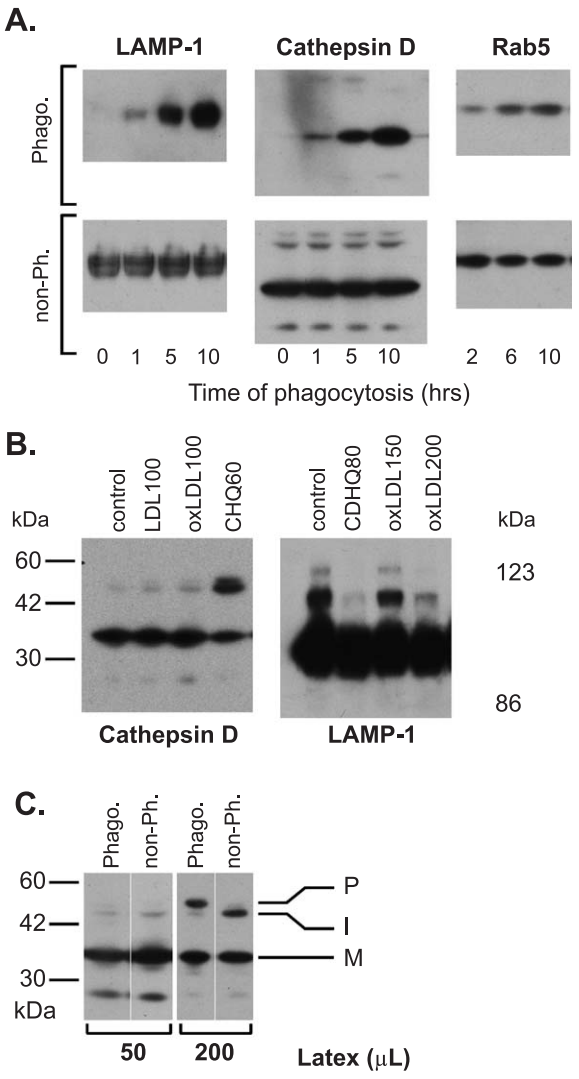

Fig. 1. Effect of latex beads internalization by the hRPE on intracellular distribution of LAMP-1, cathepsin D and Rab5. (A) Confluent cultures of hRPE cells were incubated with FBS-coated magnetic latex beads (50 μl/100 mm dish) for the indicated periods of time, followed by cell homogenization and separation of the beads from the rest of the cellular homogenate (see Materials and methods for details). (B) The hRPE were incubated for 48 h in culture media alone (control), or in the presence of the following additives: 100 μg/ml LDL (LDL100); 60 or 80 μM chloroquine (CHQ60 or CHQ80); 50, 100, or 200 μg/ml oxLDL (oxLDL50, oxLDL100, or oxLDL200). (C) Separate cultures of the hRPE grown in 100 mm Petri dishes received either 50 or 200 μl/dish of FBS-coated magnetic latex beads, followed by the 6-h incubation and phagosome isolation. Phagosomal (Phago.), non-phagosomal (non-Ph.) proteins (A and C) or total cell homogenates (B) were solubilized in Laemmli sample buffer, resolved on the gradient 4–20% SDS-PAGE, and analyzed by Western blotting using antibodies against LAMP-1, cathepsin D, or Rab5 as described in Materials and methods. Positions of pro-, intermediate, and mature forms of cathepsin D are indicated as P, I, and M, respectively.

enzyme to phagolysosomes suggesting that pro-cathepsin D associated with latex beads represents a newly synthesized enzyme.

To determine whether cathepsin D maturation is affected by oxLDL, we analyzed the phagosomal association of various cathepsin D isoforms in hRPE cell cultures. Confluent RPE were pre-incubated with 100 μg/ml oxLDL for 24 h followed by the removal of oxLDL-containing media and extensive washing of cell monolayers. Cells were then challenged with latex beads for 4 h. Western blot analysis revealed that oxLDL impedes formation of the intermediate 46-kDa form of cathepsin D, while inducing phagosomal accumulation of the proenzyme (Fig. 2). At the same time, no changes in cathepsin D distribution was found in the non-phagosomal fraction, indicating that only a specific fraction of the lysosomal enzyme, i.e., recruited by phagosomes, is sensitive to oxLDL, suggesting that phagosomal cathepsin D is a result of active transport and maturation. Due to the fact that both oxLDL-treated and control cells demonstrated time-dependent increase of cathepsin D association with latex beads, it seems unlikely that the observed block of maturation is the result of a non-specific toxic effect due to oxidative stress. It is noteworthy that treatment of the hRPE with high doses of oxLDL resulted in the disappearance of highly glycosylated forms of LAMP-1 from total cell extracts, suggesting that LAMP-1 maturation was also impaired by oxLDL (Fig. 1B).

Suppression of lysosomal maturation by inhibitors with different modes of action often occurs in association with changes in enzyme sorting, namely instead of lysosomal targeting, enzymes are secreted extracellularly [42,43]. To assess whether oxLDL-mediated blockade of cathepsin D maturation is also coupled to the enzyme secretion, we used Western blot to determine the protein level of cathepsin D in media conditioned by hRPE cells during a 24-h treatment with varied concentrations of oxLDL. Fig. 3 demonstrates that only the 52-kDa form of pro-cathepsin D was found in the conditioned media. Pre-loading the hRPE with oxLDL but not with native LDL significantly increased amounts of the secreted proenzyme in a dose-dependent fashion (Fig. 3A). The enhanced secretion of cathepsin D by the hRPE was sustained for at least 48 h, even when oxLDL was no longer present in the culture medium (Fig. 3B). As a positive control, we also tested whether a known inhibitor of lysosomal function, chloroquine, perturbed protease maturation. Chloroquine, which raises the pH in intracellular compartments [42], stimulated a significant increase of immature 52-kDa cathepsin D in the culture media of hRPE (Fig. 3A), which correlated with a reduction of the intracellular, mature 31-kDa form (Fig. 3C). To test whether secreted pro-cathepsin D is functionally competent, we measured the proteolytic activity in culture media conditioned by hRPE cells pre-treated with increasing amounts of oxLDL. Media obtained from untreated RPE cells was able to demonstrate only low levels of degradation of ^{125}I-labeled substrate protein, measured by the change of maleylated BSA to TCA soluble fragments (Fig. 3D). Pre-incubation of the hRPE with oxLDL did not significantly change basal levels of proteolysis in the extracellular space when the reaction mixtures were kept at neutral pH, but was markedly increased in a dose-dependent manner upon

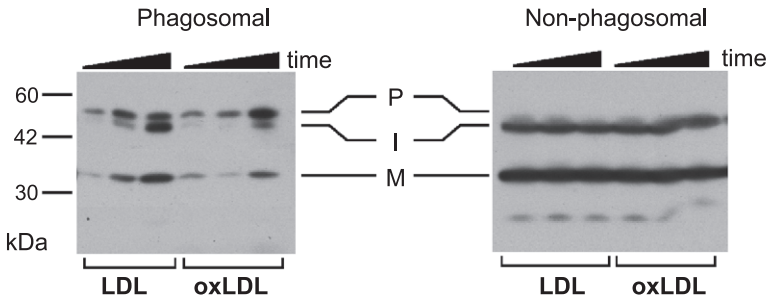

Fig. 2. Effects of oxLDL on the phagosomal distribution of pro- and mature cathepsin D in the hRPE. Confluent cultures of hRPE cells were pre-treated for 20 h with either LDL or oxLDL at 100 μg/ml, followed by the removal of lipoprotein-containing media and extensive washings. FBS-coated magnetic latex beads were then added for another 1, 4, or 8 h (200 μl/100 mm dish), after which cell homogenates were prepared and separated into phagosomal and non-phagosomal fractions. RPE proteins were then subjected to SDS-PAGE and Western blot analysis using anti-cathepsin D antibody.

changing the pH of the assay to that of 4.5. Together, these results indicate that oxLDL promotes enhanced secretion by the hRPE of the functionally latent pro-cathepsin D, which is readily activated by acidification of the environment.

We have previously reported that that the membrane integrity of hRPE cells was not compromised by oxLDL [7]. Using concentrations of oxLDL as high as 100 μg/ml did not result in any elevation of [^{14}C]adenine release from hRPE cell monolayers pre-loaded with the label [7] when compared to cells treated with oxLDL. Moreover, no mature 31-kDa cathepsin D was observed in culture media of either control, LDL-, or oxLDL-treated cells (Fig. 3A), indicating that extracellular pro-cathepsin D was not the result of a non-specific increase in RPE membrane perme-

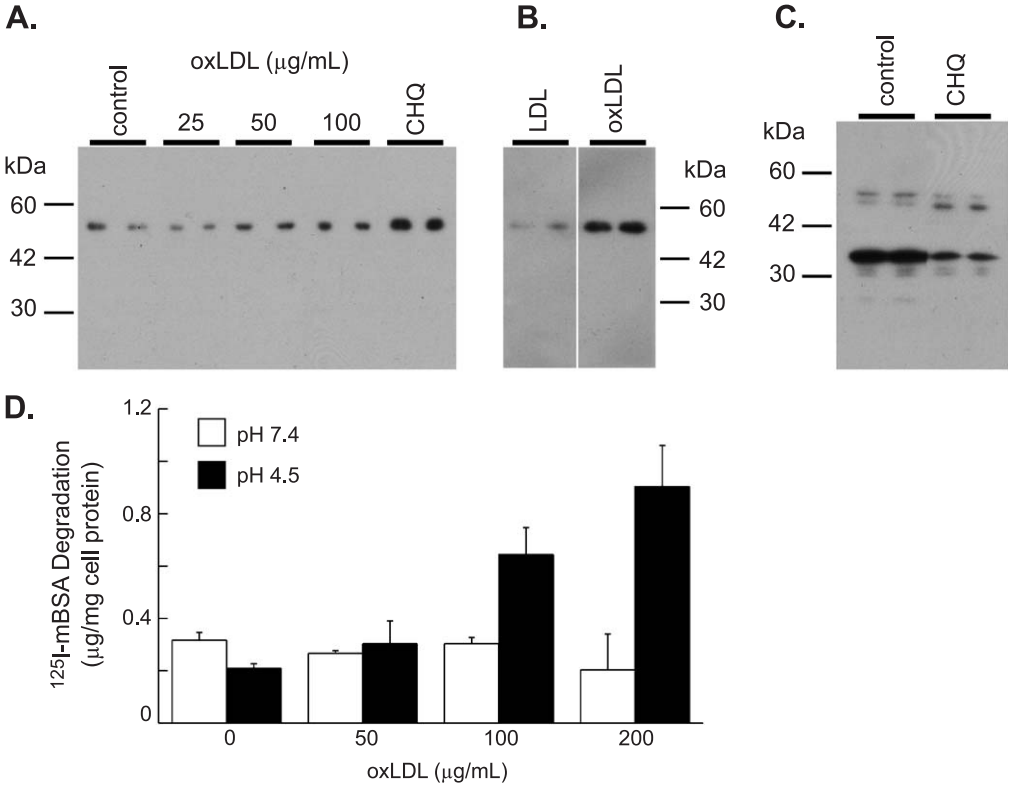

Fig. 3. Pro-cathepsin D in conditioned media of hRPE cells treated with oxLDL. (A) Confluent hRPE cells were cultured in the serum-free MEM alone, in the presence of indicated amounts of oxLDL, or 70 μM chloroquine (CHQ). In the end of the 24-h incubation period, the conditioned culture media were collected, resolved by SDS-PAGE, and analyzed by Western blotting using anti-cathepsin D antibody. (B) Confluent hRPE were first incubated with either LDL or oxLDL at 100 μg/ml, followed by the removal of lipoprotein-containing media, and extensive washings, and addition of serum-free MEM for another 48 h. In the end of the 48-h incubation period the presence of cathepsin in RPE-conditioned media was analyzed as above. (C) The hRPE were treated with 70 μM chloroquine (CHQ) for 24 h as above, after which cell proteins were solubilized in Laemmli buffer, resolved by SDS-PAGE, and analyzed for the presence of cathepsin D by Western blot. (D) The hRPE was first pre-incubated for 48 h in the serum-free MEM alone, or in the presence of indicated concentrations of oxLDL, followed by the removal of lipoprotein-containing media, and extensive washings, and addition of serum-free MEM for another 48 h. Aliquots of RPE-conditioned media were then adjusted to pH 7.4 with 0.2 M phosphate buffer or to pH 4.5 with 0.2 M acetate buffer, mixed with 2 μg of ^{125}I-mBSA, and assayed for the formation of TCA-soluble fragments as described in Materials and methods.

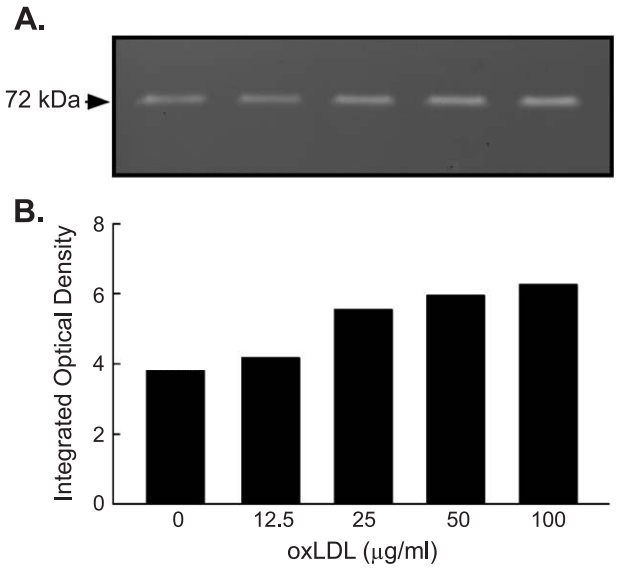

Fig. 4. Gelatinase activity in culture media conditioned by RPE cells incubated with oxLDL. Confluent hRPE cells were first pre-incubated for 24 h in the serum-free MEM alone, or in the presence of indicated concentrations of oxLDL, followed by the removal of lipoprotein-containing media, and extensive washings, and addition of serum-free MEM for another 9 h. RPE-conditioned was media subjected to zymographic analysis using gelatin-embedded gels as described in Materials and methods. (A) Zymogram gel stained with the Coomassie G-250 Blue dye. (B) Integrated optical density of the 72-kDa band on the zymogram analyzed by densitometry after black–white reversal of the gel image.

ability, but rather originated from the altered Golgi-to-lysosome transport.

Secreted lysosomal enzymes have been shown to participate in ECM remodeling via their direct collageno- [44,45] and elastolytic activity [46,47] or by activating MMPs [48–52]. To determine whether oxLDL-induced increase in cathepsin D secretion is associated with changes in metalloproteinase activity in the culture media of hRPE, we performed a zymography analysis of the samples of media conditioned by hRPE cultures pre-incubated with increasing amounts of oxLDL. Gelatin zymograms shown in Fig. 4 revealed a dose-dependent increase of a 72-kDa band in the conditioned media of oxLDL-loaded hRPE. Gelatinase A or MMP-2, the major MMP in the human RPE [53], is secreted as an inactive 72-kDa proenzyme, which requires proteolytic cleavage to a 62-kDa form for its activation. No gelatinase activity was observed at 62 kDa, suggesting that only a latent form of gelatinase A was secreted by the RPE in response to oxLDL. Consistent with the finding of procathepsin D secretion, these data implies that oxLDL affected trafficking, but not activity of RPE-derived MMPs.

4. Discussion

Cathepsin D is the most abundant lysosomal protease in RPE cells and a key enzyme responsible for proteolytic degradation of photoreceptor OS membranes [23–25]. Our data indicate that phagocytic challenge stimulated an increase of intracellular pro-cathepsin D, and that this inactive form of the immature lysosomal enzyme was found exclusively in phagosomes. This infers that cathepsin D maturation in actively phagocytizing RPE may take place in phagolysosomes and does not occur en route to this compartment prior to the fusion of the proenzyme-containing transport vesicles with phagosomes. A similar phenomenon was reported earlier for mouse bone marrow macrophages ingesting IgG-coated latex beads or mycobacteria [54]. Since the acidification below pH 4.5 is the major limiting factor for activation of lysosomal proteases [30,43], our data suggest that phagocytosis-induced biosynthesis and transport of cathepsin D exceeds the rate of phagolysosome acidification. Furthermore, the lack of procathepsin D in non-phagosomal subcellular fraction suggests a direct delivery of de novo synthesized lysosomal enzymes from trans-Golgi to phagolysosomes. Two distinct pools of lysosomes have been described in rat RPE by electron microscopy. The first is a regular size (2 μm) electron-dense vesicle which was present in RPE at all times; the second is a larger cathepsin D-positive compartment detected only during massive internalization of shed photoreceptor membranes [55]. Lysosomal compartments of phagocytizing RPE were devoid of the immature lysosomal enzyme and therefore consistent with the idea that the second larger structures are formed "on demand" due to the excessive phagocytic challenge via the fusion of pre-existing mature lysosomes.

The mechanism of oxLDL-mediated block of cathepsin D maturation is likely to be linked to the high potency of oxLDL in direct inactivation of thiol-containing proteins [56,57] and lysosomal thiol enzymes in particular [18,31]. A thiol protease cathepsin S was identified in RPE cells as an enzyme required for maturation of cathepsin D [27], confirming earlier findings that cysteine-dependent proteolytic activity is necessary for normal processing and trafficking of cathepsin D [28–30]. OxLDL contains a variety of products of lipid peroxidation and oxidative lipid–protein cross-links, which are poorly degradable by cultured cells [21,58]. We have demonstrated earlier that aldehydic adducts on oxLDL are relatively unreactive at neutral pH, but when exposed to acidic pH, become more capable of modifying thiols on neighboring proteins such as cathepsin B in lysosomes, inducing cross-linking of proteins and enzyme inactivation [31]. Thiol reactivity of oxidized lipids appears to play a key role in the mechanism of lipofuscin formation, because inactivation of thiol protease activity by the specific inhibitors, leupeptin or E64, leads to the development of lipofuscin-like fluorescence in the cultured RPE [59]. Moreover, intraocular injections of leupeptin or E64 resulted in accumulation of undigested rod OS discs in the RPE followed by time-dependent conversion of accumulated substances into lipofuscin-like granules [60].

Our results demonstrate that concomitantly with its inhibitory effect on cathepsin D maturation, accumulation of oxidized products in RPE lead to an active secretion of pro-cathepsin D into the extracellular space via the mechanism that cannot be explained by general toxicity or compromised plasma membrane integrity of RPE. Li at al. [61] have reported that oxLDL induced intracellular relocation of lysosomal enzymes in macrophages, implying a trafficking defect caused by accumulation of oxidized lipids. Efficient processing of phagosomal content relies on vesicular transport of a number of de novo synthesized macromolecules including hydrolytic enzymes, proton pump subunits, and lysosomal membrane structural proteins [62]. Our previous studies on inhibition of OS processing in cultured RPE have suggested that oxLDL interfered with normal phagosomal maturation [7], possibly via blocking membrane fusion events such as proper activation of the Rab5 [34], a small GTPase required for membrane fusion during the early steps of endocytosis and phagocytosis [38]. Another possible mechanism is that oxLDL induces a trafficking defect by interfering with the lipid membrane components of the vesicle transport machinery [63], or by affecting lipid phosphatases and/or kinases [64]. For example, phosphatidylinositol 3-kinase has been show to mediate multiple effects of oxLDL, e.g., activation of NFκB [65], apoptosis [66], and cell proliferation [67]. We have observed that oxLDL treatment of RPE mimicked the action of phosphatidylinositol 3-kinase inhibitors, including cathepsin D secretion (this study), actin polymerization, and membrane recruitment of the p85 subunit (Hoppe et al., manuscript in preparation).

Secretion of ECM-remodeling enzymes by the RPE represents a plausible mechanism for the development of sub-pigment epithelial deposits [13]. A distinct localization of MMPs and their inhibitors was reported recently, namely that TIMP-3 but not MMP-1, -2 or -3 were detected in the core of drusen, while active MMPs were found widespread in RPE and choroid [68], supporting the idea that RPE-derived ECM buildup originates at the cell–matrix interface. While the secretion of pro-cathepsin D seems to be mediated by abnormal membrane trafficking leading to mistargeting of newly synthesized enzyme, the mechanism of MMP secretion is likely to involve pro-inflammatory activation of RPE by oxLDL [69]. One common feature was that both molecules were secreted as inactive or latent proenzymes raising the possibility of regulation via proteolytic cleavage in the extracellular milieu. As we have shown here, lowering the pH alone will cause maturation of the secreted cathepsin D, which in turn can facilitate activation of MMP by proteolytic cleavage. Lysosomal dysfunction induced by products of lipid peroxidation may lead to abnormal turnover and mislocalization of hydrolytic enzymes increasing ECM-degrading potential of the cells that accumulate lipofuscin.

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