Immune complex formation in human diabetic retina enhances toxicity of oxidized LDL towards retinal capillary pericytes

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ABSTRACT

Recently it has been shown that levels of circulating oxidized LDL immune complexes (ox-LDL-IC) predict the development of diabetic retinopathy (DR). This study aimed to investigate whether ox-LDL-IC are actually present in the diabetic retina, and to define their effects on human retinal pericytes vs. ox-LDL. In retinal sections from people with type 2 diabetes, co-staining for ox-LDL and IgG was present, proportionate to DR severity, and detectable even in the absence of clinical DR. In contrast, no such staining was observed in retinas from non-diabetic subjects. In vitro, human retinal pericytes were treated with native (N-) LDL, ox-LDL, and ox-LDL-IC (0-200 mg protein/l), and measures of viability, receptor expression, apoptosis, ER and oxidative stresses, and cytokine secretion were evaluated. Ox-LDL-IC exhibited greater cytotoxicity than ox-LDL towards retinal pericytes. Acting through the scavenger (CD36) and IgG (CD64) receptors, low concentrations of ox-LDL-IC triggered apoptosis mediated by oxidative and ER stresses, and enhanced inflammatory cytokine secretion. The data suggest that IC formation in the diabetic retina enhances the injurious effects of ox-LDL. These findings offer new insights into pathogenic mechanisms of DR, and may lead to new preventive measures and treatments.

Keywords: apoptosis, blood retinal barrier, diabetes, dyslipidemia, ER stress, immunohistochemistry, lipoprotein, oxidative stress, scavenger receptor
Abbreviations:
ATF6, activating transcription factor 6
BRB, blood retina barrier
CHOP, C/EBP-homologous protein
DCCT, Diabetes Control and Complications Trial
DR, diabetic retinopathy
EDIC, Epidemiology of Diabetes Interventions and Complications
ER, endoplasmic reticulum
GRP78, 78kDa glucose-regulated protein
Ig, immunoglobin
IL-6, interleukin-6
LDL, low-density lipoprotein
N-LDL, native LDL
NPDR, non-proliferative diabetic retinopathy
Ox-LDL, oxidized LDL
Ox-LDL-IC, oxidized LDL immune complex
PARP, cleaved poly ADP ribose polymerase
PDR, proliferative diabetic retinopathy
PEDF, pigment epithelium-derived factor
p-IRE1α, phospho-inositol requiring enzyme 1 alpha
p-PERK, phospho-protein kinase-like endoplasmic reticulum kinase
ROS, reactive oxygen species
SFM, serum-free medium
sICAM-1, soluble intercellular adhesion molecule-1
sVCAM-1, soluble vascular cell adhesion molecule-1
TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF, vascular endothelial growth factor
Δψm, mitochondrial membrane potential
Introduction

Diabetic retinopathy (DR) remains a leading cause of vision loss in working-age adults. Early disease is characterized by vascular abnormalities, including pericyte loss, basement membrane thickening, microaneurysm formation, and capillary leakage (1, 2). Pericytes are critical to vascular integrity (3), and their loss is considered an initiating event in DR.

Dyslipidemia has been implicated in DR, and associations between plasma lipoprotein profiles and disease severity have been observed in large cohort studies (4-6), but were not of sufficient strength to define risk for individuals. The term ‘dyslipidemia’ may include qualitative as well as quantitative alterations in plasma lipoproteins. The qualitative abnormalities include modification by oxidation, but oxidized LDL (ox-LDL) constitutes only a small fraction of total plasma LDL, ranging from 0.001% in healthy people to 5% in the presence of cardiovascular disease (7). We therefore hypothesized that the dominant effects of lipoproteins in DR occur not in plasma, but rather after extravasation through leaking blood-retina barriers (BRBs) (8-15); the extent of this leakage may be more important than plasma concentrations of lipoproteins. We further hypothesized that extravasated, intra-retinal lipoproteins undergo extensive modification (glycation, oxidation), rendering them cytotoxic (8). Thus in DR, as in atherosclerosis (16, 17), extravascular modified LDL could play an important role in disease progression; but in contrast
to the artery, extravasation of LDL in the retina does not occur unless BRB leakage is present, and thus particularly affects people with diabetes.

Supporting these hypotheses, we demonstrated that apolipoprotein B and ox-LDL were present in diabetic human retinas, even before the development of clinical retinopathy, but were absent in non-diabetic retinas (8). Consistent with a general neurovascular retinal insult in DR (18), we also found evidence of a broad array of cytotoxic effects: “highly oxidized, glycated LDL” promoted oxidative stress, endoplasmic reticulum (ER) stress, inflammation and abnormal gene expression, and decreased viability, not only in retinal vascular cells (endothelial cells, pericytes), but also in Müller glia and retinal pigment epithelial cells (RPEs) (9-15).

It is now well known that modification of LDL may render it immunogenic, leading to the formation of LDL immune complexes (LDL-IC) (19). Recently, in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) cohort, we found that increased levels of circulating ox-LDL-IC predicted risk for severe non-proliferative (NPDR) and proliferative diabetic retinopathy (PDR) in type 1 diabetes (20). Others have shown that plasma levels of antibodies to malondialdehyde-modified apolipoprotein B-100 were correlated with DR severity in type 2 diabetes (21). Further emphasizing the importance of ox-LDL-IC, it is now established that in plasma, only a minor proportion of ox-LDL circulates free; 95% circulates in immune complexes (7, 19). For ox-LDL-IC, as with ox-LDL, changes
observed in plasma may provide only an indirect index of events in tissue. Thus in the retina, effects of ox-LDL-IC could predominate over those of ox-LDL, and it is important to define whether immune-complex formation alters the known toxicity of ox-LDL towards retinal cells. In the present study, we aimed to determine whether ox-LDL-IC are indeed present in the diabetic retina, and to define their effects on human retinal pericytes vs. ox-LDL.

Methods and Materials

The study was approved by the Institutional Review Boards of the University of Oklahoma Health Sciences Center and the Medical University of South Carolina, and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from each living participant.

Immunohistochemistry of human retinas

Retinas were obtained post-mortem from 15 type 2 diabetic and 5 non-diabetic humans through the National Disease Research Interchange (NDRI; Philadelphia, PA) as described previously (8). Subjects were grouped as follows: non-diabetic, diabetic without clinical DR, NPDR, and PDR (n=5), based on the diagnosis provided by NDRI. Ages were similar across the groups (non-diabetic, 62.6±14.9 years; diabetic without DR, 69.4±9.9 years; NPDR, 70.6±8.3
years; and PDR, 57±6.5 years; mean±SD, p>0.05 by one-way ANOVA). The eyes were obtained and fixed in 10% neutral-buffered formalin within 12h after death. For immunohistochemistry, retinal sections (5 µm) were incubated overnight with rabbit polyclonal anti-ox-LDL or goat polyclonal anti-human IgG antibody (Abcam, Cambridge, MA), followed by detection with fluorescence-conjugated anti-rabbit or -goat antibodies (Life Technologies, Carlsbad, CA) and confocal microscopy (Olympus, Japan) as described (8). Absence of non-specific tissue binding by secondary antibodies was confirmed.

**Human LDL preparation**

N-LDL was isolated by sequential ultracentrifugation (density 1.019-1.063) of fresh plasma pooled from 4-6 fasted, healthy volunteers; ox-LDL was prepared as before (12). For preparation of insoluble ox-LDL-IC, human ox-LDL antibodies were isolated using a two-step protocol involving affinity chromatography with immobilized protein G (Protein G-Sepharose 4 Fast Flow; Amersham-Pharmacia Biotech, Piscataway, NJ) and fractionation by affinity chromatography in Sepharose-linked ox-LDL (22). Isolated ox-LDL antibodies were centrifuged (90,000g, 45min) to eliminate aggregates, sterilized, and confirmed free of endotoxin using the Etoxate kit (detection limit 0.005-0.01 ng/mL; Sigma-Aldrich, St. Louis, MO). Subsequently,
human ox-LDL-IC were prepared (22, 23), with the ratio of ox-LDL and IgG antibody approximately 1:3.3 (w/w).

**Human retinal pericyte culture**

Human retinal pericytes (Cambrex, Walkersville, MD) were cultured in EBM-2 medium (glucose 5.5 mM) supplemented with the EGM-2 SingleQuot kit (Lonza, Allendale, NJ). Cells of passages 3-9 (80-85% confluence) were pretreated with serum-free medium (SFM) for 18-24h before being treated with lipoprotein preparations (50-200 mg protein/l). In some experiments, 50 mg/l monoclonal antibodies (Ancell Corp., Bayport, MN) were employed to block CD16, CD32, CD36 and CD64 receptors. Tunicamycin, 4-phenylbutyric acid (4-PBA), tauroursodeoxycholic acid (TUDCA), Tempol, and n-acetyl cysteine were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell viability**

Pericyte viability was determined in 96-well plates (1×10^4 cells/well) using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD) (15).

**Flow cytometry for cell surface receptors**
Cultured pericytes were detached from the flask with trypsin, which was neutralized using a Lonza ReagentPack, per vendor’s instruction (Lonza, Walkersville, MD). Cells were then incubated with FITC-labeled anti-CD16, -CD32, -CD36 and -CD64 antibodies (R&D, Minneapolis, MN) for 25 min on wet ice, washed with PBS/2%BSA. Cells were re-suspended in 300 μl FACS solution, immediately followed by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA).

*Phosphatidylserine externalization* was measured using an Annexin V-FITC Apoptosis Detection Kit II (BD Pharmingen, Franklin Lakes, NJ) according to the manufacturer’s manual. In brief, pericytes were trypsinized, washed twice with cold PBS, and re-suspended in a binding buffer (10mM HEPES/NaOH, pH 7.4, 140mM NaCl, 2.5mM CaCl₂) at a density of 1x10⁶ cells/ml. 100 μl of the cell suspension was transferred to a 5ml tube, to which were added 5 μl Annexin V-FITC and 5 μl propidium iodide. After 20 min incubation under room temperature in the dark, an additional 400 μl of binding buffer was added, followed by immediate analysis by flow cytometry.

*Caspase-3 activity* was measured by a caspase-3 activity assay kit (Cell Signaling Technology, Danvers, MA) per manufacturer’s instruction. Briefly, extracted pericyte lysates containing 10 μg
protein were added to a black 96-well plate containing 200μl fluorogenic substrate solution (25mM HEPES, pH 7.5, 100mM NaCl, 1mM EDTA, 0.1% CHAPS, 10mM DTT, 20μM Ac-DEVD-AMC), then incubated at 37°C for 2h in the dark. Fluorescence was quantified by a VICTOR3 microplate reader (PerkinElmer, Waltham, MA), with excitation at 380nm and emission at 460nm.

Mitochondrial membrane potential (Δψm)

DiOC6(3) (Life Technologies, Carlsbad, CA) was used to evaluate the Δψm changes (15). Pericytes were harvested and resuspended in 400μl PBS containing 20nM DiOC6(3) followed by 15min incubation at 37°C in the dark, then analyzed by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA). The results are shown as the proportion of cells exhibiting low Δψm as indicated by reduced DiOC6(3) uptake.

Western blot

Cells were homogenized (Complete Lysis Buffer, Roche, Indianapolis, IN) and protein concentrations determined (BCA assay; Pierce, Rutherford, IL). Protein (30μg) was resolved by SDS-PAGE and blotted with antibodies against 3-nitrotyrosine, glutathione peroxidase 1, 78kDa glucose-regulated protein (GRP78), phospho-inositol requiring enzyme 1 (p-IRE1α) (all from
Abcam, Cambridge, MA), phospho-protein kinase-like endoplasmic reticulum kinase (p-PERK), C/EBP-homologous protein (CHOP), activated caspase-3, and cleaved poly ADP ribose polymerase (PARP) (all from Cell Signaling Technology, Danvers, MA). Blots were stripped and re-blotted with antibody against β-actin for standardization.

**Immunofluorescence for ATF6 and TUNEL assay** was performed as previously (15). In brief, pericytes were fixed (4% paraformaldehyde, 20min), permeabilized, and incubated overnight at 4°C with primary antibody against activating transcription factor 6 (ATF6; Abcam, Cambridge, MA), followed by the secondary antibody for 1h. Apoptosis was assessed by the TUNEL assay (In-Situ-Cell-Death-Detection Kit; Roche, Indianapolis, IN).

**Measurement of intracellular reactive oxygen species (ROS)**

Intracellular ROS were measured by the dichlorodihydrofluorescein method using CM-H$_2$DCFDA (Invitrogen, Carlsbad, CA), as per manufacturer’s instruction. Pericytes were washed, incubated with 2μM CM-H$_2$DCFDA in phenol red-free medium (37°C, 20min), and fluorescence was measured (VICTOR3; PerkinElmer, Waltham, MA).

**Measurement of cytokines**
IL-6, sICAM-1, sVCAM-1, PEDF and VEGF were assayed in cell supernatants (DuoSet ELISA, R&D, Minneapolis, MN), per manufacturer’s instruction.

Data analysis

Data were expressed as means±SD. Statistical significance was determined by one-way ANOVA followed by Dunnett’s post-hoc test (Prism 5, GraphPad, La Jolla, CA). P≤0.05 was considered significant.
Results

**Ox-LDL and IgG were present and co-localized in diabetic retina**

Fig. 1 shows the representative ox-LDL and IgG immunostaining in retinal sections from non-diabetic and three categories of type 2 diabetic subjects (no clinical DR, NPDR, PDR). No signal was detectable in non-diabetic retinas. Staining for ox-LDL (red) and IgG (green) was observed in all three diabetic groups, increasing with DR severity. Co-localization of ox-LDL and IgG was clearly seen in the photos merging the two stains, consistent with the presence of ox-LDL-IC in diabetic retina.

**Ox-LDL-IC caused greater reductions in pericyte viability than ox-LDL**

As shown in Fig. 2, both ox-LDL-IC and ox-LDL decreased pericyte viability in a dose- (0-200 mg/l) and time- (0-48h) dependent manner. There was a significant leftward shift of the dose-response relationship for ox-LDL-IC vs. ox-LDL, indicating much higher potency of ox-LDL-IC in triggering cell death. At 50 mg/l, ox-LDL-IC elicited cell death much earlier that ox-LDL (6 vs. 48h).

**CD36 and CD64 receptors mediated effects of ox-LDL-IC**
To identify the receptors mediating ox-LDL-IC-induced toxicity, we examined the expression of CD36 (receptor for ox-LDL as well as other molecules and multi-molecular complexes), CD16 and CD32 (low-affinity IgG receptors, FcγRIII and FcγRII respectively) and CD64 (high-affinity FcγRI). CD36 and CD64 were expressed on pericyte surface, but neither CD16 nor CD32 was detectable (Fig. 3A). Only ox-LDL-IC, but not ox-LDL, induced CD64 upregulation vs. SFM and N-LDL. To determine the role of CD36 and CD64, cells were pre-treated with relevant blocking antibodies, and viability was determined. Both anti-CD36 and anti-CD64 attenuated ox-LDL-IC-induced cell death, and when combined, evidence suggesting additional protection was observed (p<0.1 vs. anti-CD36 alone; Fig. 3B).

**Ox-LDL-IC caused pericyte death by apoptosis**

Since 50 mg/l of ox-LDL was not lethal to pericytes over 24h, this concentration was selected for additional studies. Flow cytometry showed that ox-LDL-IC dramatically increased apoptosis (annexin V-positive and propidium iodide-negative cells) vs. SFM, N-LDL or ox-LDL (Fig. 4A); apoptosis was attenuated by antagonism of CD36 or CD64, or both combined (all p≤0.05). There was a trend towards a greater effect by combined antagonism than by either alone (p=0.06). TUNEL assay yielded similar results (Fig. 4B). Ox-LDL-IC decreased Δψm (Fig. 4C-D). Ox-LDL-IC also increased protein levels of both the cleaved PARP and activated
caspase-3 (Fig. 4E), as well as caspase-3 activity (Fig. 4F). In contrast, for all endpoints, 50 mg/l ox-LDL produced minimal effects vs. N-LDL. The data support apoptosis mediated by CD36 and CD64 receptors as the mechanism of pericyte death induced by ox-LDL-IC, and demonstrate that ox-LDL-IC have greater cytotoxicity than ox-LDL.

**ER stress was implicated in ox-LDL-IC-induced apoptosis**

Upon treatment with ox-LDL-IC, pericytes expressed higher levels of GRP78, an ER stress chaperon, PERK phosphorylation, and CHOP, a pro-apoptotic factor activated in ER stress (Fig. 5A). ATF6 nuclear translocation was increased (Fig. 5B). These findings indicate increased ER stress; however, phosphorylation of IRE1α was unchanged.

Tunicamycin (an ER stress inducer) was used to determine whether ER stress was sufficient to induce apoptosis. After 12h exposure, increased GRP78 and CHOP confirmed ER stress (Fig. 5C), while caspase-3 activation and PARP cleavage confirmed apoptosis (Fig. 5D). The ER stress inhibitors, 4-PBA and TUDCA were also employed prior to ox-LDL-IC challenge (Fig. 5E-H). They attenuated ER stress (Fig. 5E), reduced apoptosis (Fig. 5F), and mitigated mitochondrial dysfunction (Fig. 5G-H). Again, CD36 and CD64 blockade inhibited apoptosis and improved mitochondrial function (Fig. 5F-H). The data support a role for ER stress in ox-LDL-IC-induced apoptosis, implicating CHOP.
Increased intracellular ROS by ox-LDL-IC preceded ER stress

Ox-LDL-IC significantly increased intracellular ROS vs. N- or ox-LDL in a time-dependent fashion (Fig. 6A). The increase was attenuated by pre-treatment with the antioxidant Tempol, and by blockade of CD36 and CD64. Ox-LDL-IC also increased levels of 3-nitrotyrosine-positive proteins, and decreased glutathione peroxidase 1, whereas N- and ox-LDL did not (Fig. 6B). Antioxidants Tempol and n-acetyl cysteine decreased ER stress markers (Fig. 6C-D), suggesting that oxidative stress is upstream of ER stress. Again, blockade of CD36/CD64 was protective.

Ox-LDL-IC had greater effects on pericyte cytokine secretion than ox-LDL

Secretion of cytokines related to inflammation, oxidative stress, and angiogenesis was determined (Fig. 7). Compared with SFM, ox-LDL-IC significantly increased cellular secretion of IL-6 and sICAM-1, decreased PEDF, and had no effect on sVCAM-1 or VEGF. Ox-LDL affected only IL-6, while N-LDL was without effect.
Discussion

We present the first evidence for the presence/formation of ox-LDL-IC in human diabetic retinas. Immunostaining for ox-LDL, detectable prior to the onset of clinical DR, confirmed earlier observations (8), while co-staining for IgG supported the concept of ox-LDL-IC formation in the retina. We therefore compared effects of ox-LDL-IC vs. ox-LDL (and N-LDL) on human retinal capillary pericytes in vitro.

We found that ox-LDL-IC exerted greater toxicity towards retinal capillary pericytes than ox-LDL; low concentrations, at which ox-LDL itself was almost without effect, induced oxidative and ER stresses, apoptosis, increased secretion of inflammatory cytokines, and reduced secretion of a key anti-angiogenic factor, PEDF. The scavenger receptor CD36 and Fcγ receptor I (CD64) were implicated.

Previously, we demonstrated the presence of intra-retinal apolipoprotein B and ox-LDL, attributing it to vascular leakage (no LDL was detectable in the absence of diabetes) (8). Such leakage is likely: in diabetes the BRB becomes permeable to particles that are much larger than LDL (24). Intra-retinal ox-LDL was associated with apoptosis, and in proliferative DR, with macrophage infiltration (8). Consistent with a generalized retinal injury (18), in vitro-modified LDL was toxic towards Müller cells and RPEs as well as towards vascular cells (9-15). In the
present work, we have demonstrated that formation of ox-LDL-IC might further enhance the toxic effects of ox-LDL, and explored some of the mechanisms involved.

Our current findings complement our recent clinical observations in the type 1 diabetes DCCT/EDIC cohort that increased plasma ox-LDL-IC predicted DR progression years in future (20). In plasma, ox-LDL represents only a small fraction of total LDL (7), and also, most ox-LDL is incorporated in immune complexes (19). In atheromatous plaque, ox-LDL concentration may be as much as 70-fold higher than in plasma (16). Human plasma has considerable antioxidant capacity, and therefore LDL oxidation is most likely to happen in the extravascular compartment, and circulating ox-LDL may be derived largely through “reflux” from the sub-endothelial space back into plasma (25). It should also be noted that estimates of intra-plaque ox-LDL concentrations represent averages of a non-uniform distribution; local concentrations could be much higher. This consideration also applies to the retina, given the patchy distribution of apolipoprotein B and ox-LDL seen in our earlier study (8). Overall, we consider the concentrations of ox-LDL and ox-LDL-IC employed in our present work (10-30% of total plasma LDL) to be conservative estimates of local intra-retinal concentrations in diabetes.

We now demonstrate co-localization of immunoglobulins and ox-LDL, providing strong circumstantial evidence for the presence of intra-retinal ox-LDL-IC. We observed this co-localization in all three diabetic patient groups, including the ‘no clinical DR’ group, consistent
with a role for ox-LDL-IC in initiating retinal injury. While ox-LDL was found throughout the retina in diabetes, IgG appeared initially in the inner ganglion cell layer (i.e. in subjects with no clinical DR), but permeated all layers in those with overt DR. This is consistent with intra-retinal ox-LDL-IC formation, and suggests that ox-LDL-IC may contribute strongly to the propagation of DR, including eventually to macrophage activation in PDR.

We recognize that intra-retinal immunoglobulins may bind to antigens other than ox-LDL. Antibodies against vascular cells (26), collagens (27), and phospholipids (28) have been reported in patients with DR. Furthermore, since the retina is lipid-rich, oxidation of other retinal lipids or lipid-protein structures could yield epitopes similar to modified LDL. Finally, BRB leakage in DR implies a general leakage of plasma immunoglobulins into the retina.

Ox-LDL-IC was more potent than ox-LDL in reducing human retinal pericyte viability via caspase-dependent apoptosis, as measured by phosphatidylserine exposure, TUNEL staining, mitochondrial membrane potential, activated caspase-3, and PARP. Apoptosis was mitigated by cyclosporine A, an inhibitor of mitochondrial permeability transition pore opening, and Z-VAD-FMK, a pan-caspase inhibitor (data not shown). While these effects are similar to those of (non-immune-complexed) modified LDL (8, 15), ox-LDL-IC exhibited toxic effects at much lower concentrations.
To define receptor mechanisms, we hypothesized that putative receptor(s) could be related to ox-LDL and/or IgG. We assessed cell surface expression of the scavenger receptor CD36 (23) and three IgG Fc receptors: high-affinity CD64 (FcγRI), and low-affinity CD32 (FcγR II) and CD16 (FcγR III) (29). Only CD36 and CD64 were detectable in pericytes. Ox-LDL-IC induced CD64 membrane expression, suggesting that IC-pericyte interaction may mediate toxicity. Toxicity was attenuated by blockade of CD36 or CD64, and more effectively when both were blocked simultaneously. Cellular ROS levels, ER stress, and apoptosis were likewise attenuated by CD36/CD64 blockade. Compared with mesangial cells (30), two differences were noted: first, CD36 mediated the interaction with ox-LDL-IC in pericytes, but not in mesangial cells; second, blockade of CD16 inhibited ox-LDL-IC effects in mesangial cells, but expression of this receptor was undetectable in pericytes. CD64 played a role in both cells.

ER stress has been implicated in DR, and can be elicited by ox-LDL (12, 15, 31, 32). We found ox-LDL-IC to be more potent inducers of ER stress than ox-LDL, increasing expression of GRP78, activating ER stress sensors PERK and ATF6, and increasing CHOP, a pro-apoptotic protein triggered by ER stress. We did not observe alterations of p-IRE1α, suggesting that it may not be involved; however expression of this protein was low and so this tentative conclusion merits further investigation. ER stress was manifest 3-6h after treatment, peaking at 12h; whereas mitochondrial dysfunction and apoptosis were manifest after 6h, with the peak at 24h.
The data suggest that ER stress precedes apoptosis, with CHOP serving as the link between the two (33). Induction of ER stress by tunicamycin was sufficient to cause apoptosis, whereas ER stress inhibitors, TUDCA and 4-PBA, attenuated mitochondrial dysfunction and apoptosis elicited by ox-LDL-IC. The data suggest that ER stress mediated apoptosis induced by ox-LDL-IC in pericytes. While (non-immune-complexed) ox-LDL exhibited similar effects to those of ox-LDL-IC in pericytes (and in other human retinal cell types including RPEs (14) and Müller cells (12), as well as in mouse retina after intravitreal administration of ox-LDL (34)), it did so only at much higher concentrations.

Oxidative stress, an established risk factor for DR (35), may operate partly through induction of ER stress. In the current study, ox-LDL-IC induced oxidative and nitrosative stresses and decreased the expression of glutathione peroxidase at much lower concentrations than previously observed with ox-LDL (12, 14, 15). It also reduced PEDF levels, perhaps further compromising antioxidant capacity (13). Attenuation of oxidative stress mitigated ox-LDL-IC induced ER stress, suggesting that oxidative stress was, at least partly, responsible for the ER stress.

Pericytes play an important role in maintaining vascular integrity (3), and alterations in their secretory behaviour may contribute to DR. We previously showed that ox-LDL modulates pericyte cytokine secretion, enhancing inflammation and oxidative stress (13). We now
demonstrate that ox-LDL-IC produced even more robust pro-inflammatory and pro-angiogenic effects, increasing IL-6 and sICAM-1, and decreasing PEDF at concentrations where ox-LDL had little effect.

In summary, our findings support the presence (and probable formation) of ox-LDL-IC in human diabetic retina, beginning before the clinical onset of DR, and accumulating to a degree proportionate to DR severity. In vitro, ox-LDL-IC induced more pronounced toxic effects to pericytes than ox-LDL, mediated via CD36 and CD64 receptors. Oxidative stress and subsequent ER stress mediated apoptosis. Ox-LDL-IC altered the secretory pattern of pericytes, potentially contributing to inflammation and angiogenesis in diabetic retina. Conceivably, therapeutic strategies to reduce immune complex-mediated retinal injury could include plasma lipid lowering, reduction of oxidative stress and ox-LDL production (e.g. antioxidants and glycemic control), antagonism of CD36 and CD64 receptors, inhibition of IgG synthesis (e.g. rituximab), and anti-inflammatory treatments e.g. by corticosteroids. In practice, the benefits of reducing immune complex-mediated complications need clearly to outweigh the risks inherent in immuno-suppression, which has the potential for severe side effects. The development of new therapeutic agents to prevent BRB leakage could be the most effective means to reduce LDL extravasation and immune complex formation in the retina.
Acknowledgements

D.F., M.W., M.D., Y.C., and T.J.L. designed and conducted experiments. D.F., J.Y.Y., M.W. and T.J.L. discussed/analyzed the data and wrote the manuscript. G.V. and M.F.L. selected individuals with high antibody levels, supervised the separation of antibodies against ox-LDL from serum, and supervised the preparation of ox-LDL-IC and tested the fresh preparations before each experiment. S.A.A. and Y.L. provided technical assistance in the separation of the ox-LDL antibodies and in the preparation of ox-LDL-IC. M.E.B. provided the human retina sections from NDRI. J-X.M. supervised the immunohistochemistry work. G.V., M.E.B, J-X.M, M.F.L. and J.C. reviewed the manuscript and contributed to discussion. T.J.L. initiated the study, contributed to the study conception and design, oversaw the project, wrote the manuscript and approved the final version for publication. T.J.L.is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Duality of interest

The authors declare no duality of interest associated with this manuscript.
References


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Figure Legends

Figure 1. Immunostaining for ox-LDL and IgG in human retinas. Immunohistochemistry for ox-LDL (red) and IgG (green) in retinal sections from four groups: non-diabetic (no DM), diabetic without clinical retinopathy (DM w/o DR), non-proliferative DR (NPDR), and proliferative DR (PDR). Merged images revealed co-localization of ox-LDL and IgG (yellow), suggesting formation of ox-LDL-IC. ONL, INL: outer, inner nuclear layers; GCL: ganglion cell layer. DAPI staining was partially superimposed on the “merged” images to show the cell layers.

Figure 2. Ox-LDL-IC reduced pericyte viability (CCK-8) more than ox-LDL. A-C: Dose responses to N-LDL, ox-LDL or ox-LDL-IC (0-200 mg/l for 24h). D-F: Time-course responses to 50 mg/l N-LDL, ox-LDL or ox-LDL-IC for 0-48h. Data show percentage vs. SFM or pre-dose baseline (means±SD, n=3). *P<0.05, **p<0.01, ***p<0.001 vs. SFM or pre-dose.

Figure 3. Pericyte surface receptors and effects of ox-LDL-IC. A: Receptor expression in pericytes. Cells were treated for 6h with 50 mg/l N-LDL, ox-LDL, ox-LDL-IC, or SFM, and labeled with FITC-tagged antibodies against CD16, CD32, CD36 or CD64 for flow cytometry. IgG or IgM were used, as appropriate, as non-binding baseline controls. Mean fluorescence intensities (MFI) in response to N-LDL, ox-LDL or ox-LDL-IC were compared to non-binding baseline control and SFM (means±SD, n=3; *p<0.05 vs. non-binding baseline, indicative of receptor expression; ‡p<0.05 vs. SFM). CD36 and CD64 were expressed, but not CD16 or CD32.
Ox-LDL-IC increased CD64 (but not CD36) expression vs. SFM. Neither ox-LDL nor N-LDL altered CD64 or CD36 expression. 

**B: Receptor-mediated cytotoxicity.** Cell viability was measured by the CCK-8 assay after 24h treatment with 50 mg/l N-LDL, ox-LDL or ox-LDL-IC vs. SFM. Ox-LDL-IC significantly reduced pericyte viability; the effects were attenuated by the blocking antibodies against CD36 and CD64. Blockade of both receptors together offered additional protective effects. Data are expressed as percentage of SFM control (means±SD, n=3). **P<0.01 vs. SFM; ‡p<0.05 vs. ox-LDL-IC treatment alone.

**Figure 4. Ox-LDL-IC was more potent in inducing pericyte apoptosis than ox-LDL. A-B:**

*Early- and late-stage apoptosis.* Effects of 50 mg/l N-LDL, ox-LDL or ox-LDL-IC ± 1h pre-treatment with 50 mg/l blocking antibodies against CD36 and CD64, individually or combined.

A: Early apoptosis: annexin V-positive/propidium iodide-negative cell numbers relative to SFM quantified by flow cytometry after 12h treatment. Means±SD, n=3; ***P<0.001 vs. SFM, ‡p<0.05 vs. ox-LDL-IC treatment in absence of blocking antibodies. Treatment with combined antibodies produced greater attenuation of apoptosis than either individually. B: Late apoptosis: Quantitative analysis of the TUNEL staining after 24h treatment: data expressed relative to SFM (mean±SD, n=3). **P<0.01 vs. SFM, ‡p<0.05 vs. ox-LDL-IC treatment without blocking antibodies. C: Mitochondrial Δψm: pericytes were treated with a) SFM, b) 50mg/l N-LDL, c) ox-LDL (each for 24h), or d-g) 50mg/l ox-LDL-IC (3-24h), then analyzed by the DiOC6(3)
method with flow cytometry. The % total events (i.e. M1) denote low membrane potential corresponding to low fluorescence intensity, indicating mitochondrial dysfunction. D: Quantitative analysis of mitochondrial $\Delta \psi_m$ assay. Mean±SD, n=3; *p<0.05, **p<0.01 vs. SFM.

E: Cleaved PARP and activated caspase-3 by Western. Data are representative of three independent experiments. F: Caspase-3 activity using Ac-DEVD-AMC as a substrate. Means±SD, n=3; ***p<0.001 vs. pre-dose.

**Figure 5. Ox-LDL-IC induced greater ER stress than ox-LDL in pericytes LDL.** A: GRP94, GRP78, p-IRE1$\alpha$, p-PERK and CHOP expression by Western after treatment with 50 mg/l N-LDL, ox-LDL or ox-LDL-IC. B: ATF6 nuclear translocation at 12h (a, SFM; b, N-LDL; c, ox-LDL; d, ox-LDL-IC all at 50 mg/l). C-D: GRP94, GRP78, CHOP, cleaved PARP and activated caspase-3 expression after treatment with tunicamycin (TM, an ER stress inducer) for 12h. E: Pre-treatment with ER stress inhibitors, TUDCA and 4-PBA for 1h, reduced the expression of GRP94, GRP78, and CHOP as induced by ox-LDL-IC (50 mg/ml, 12h). F-H: Cells were pre-treated with TUDCA (2 mg/l), 4-PBA (10 mg/l) or anti-CD36+CD64 (50 mg/l each) for 1h, followed by exposure to 50 mg/ml ox-LDL-IC for 24h. F: Expression of cleaved PARP, activated caspase-3, and CHOP by Western. G: Mitochondrial $\Delta \psi_m$ assay: pericytes were treated as indicated, followed by %M1 determination as above. H: Quantitative analysis of the mitochondrial $\Delta \psi_m$ data. Means±SD, ***P<0.001 vs. SFM, ‡p<0.05 vs. ox-LDL-IC. All
experiments were confirmed in three separate occasions. The data implicate ER stress in ox-LDL-IC induced apoptosis in pericytes: effects of ox-LDL-IC differ significantly from ox-LDL at this concentration.

**Figure 6. Oxidative stress was proximal to ox-LDL-IC-induced ER stress in pericytes LDL.**

Cells were exposed to 50 mg/l N-LDL, ox-LDL or ox-LDL-IC for various durations, with or without 1h pre-treatment with the antioxidants Tempol (10 mM) or n-acetyl cysteine (NAC; 100 μM), or combined anti-CD36+CD64 blocking antibodies (50 mg/l) (n=3 for all experiments). **A:** Intracellular ROS levels measured by the dichloro-dihydrofluorescein method. Data relative to SFM baseline (means±SD; **p<0.01 vs. N-LDL, †††p<0.001 vs. N-LDL and ox-LDL, ‡‡p<0.01 vs. ox-LDL-IC). **B:** Increased expression of 3-nitrotyrosine (3-NT) and decreased expression of glutathione peroxidase 1 (GPX-1) following exposure to ox-LDL-IC. In both experiments A and B, the effects of ox-LDL-IC differ significantly from ox-LDL; the latter produced minimal changes. **C:** Effects of antioxidants and receptor antagonism on ox-LDL-IC-induced ER stress. Cells were pre-treated with n-acetyl cysteine, Tempol, or combined anti-CD36+CD64 for 1h, then exposed to ox-LDL-IC for 12h. ER stress markers GRP94, GRP78, p-PERK and CHOP were determined by Western. **D:** ATF6 nuclear translocation induced by a) ox-LDL-IC (12h) was inhibited by pre-treatment with b) NAC, c) Tempol, or d) anti-CD36+CD64 combined. Overall, data suggest that inhibition of oxidative stress decreases both ER stress and apoptosis.
Figure 7. Ox-LDL-IC exert greater effects on pericyte cytokine secretion than ox-LDL.

Pericytes were treated with 50mg/l N-LDL, ox-LDL or ox-LDL-IC vs. SFM for up to 24h. Supernatants were serially harvested to measure the levels of IL-6, sICAM-1, sVCAM-1, PEDF, and VEGF by ELISA. Data are expressed as means±SD, n=3. ***P<0.001 vs. SFM, ‡‡‡p<0.001 vs. ox-LDL. Ox-LDL-IC significantly increased pericyte secretion of IL-6 and sICAM-1, and decreased secretion of PEDF. No changes were observed for sVCAM-1 or VEGF. In contrast, while ox-LDL increased cellular secretion of IL-6, it had no effect on other cytokines.
Fig. 1

DAPI  Ox-LDL  IgG  Merged

No DM

DM w/o DR

NPDR

PDR

GCL  INL  ONL

GCL  INL  ONL

GCL  INL  ONL

GCL  INL  ONL

20 μm
**Fig. 2**

(A) N-LDL 24h
(B) Ox-LDL 24h
(C) Ox-LDL-IC 24h
(D) N-LDL 50mg/l
(E) Ox-LDL 50mg/l
(F) Ox-LDL-IC 50mg/l
Fig. 4

A

DiOC6(3)

B

Counts

SFM

N-LDL 24h

Ox-LDL 24h

Ox-LDL IC 3h

Ox-LDL IC 6h

Ox-LDL IC 12h

Ox-LDL IC 24h

M1 (% low membrane potential events)

SFM 24h

N-LDL 24h

Ox-LDL IC 3h

Ox-LDL IC 6h

Ox-LDL IC 12h

Ox-LDL IC 24h

Total PARP

Active PARP

Activated caspase 3

β-actin

F

DEVDase activity vs. SFM (%)
Fig. 5

A

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H

M1 (% low membrane potential events)