Intraretinal Leakage and Oxidation of LDL in Diabetic Retinopathy

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Purpose. The pathogenesis of diabetic retinopathy (DR) is not fully understood. Clinical studies suggest that dyslipidemia is associated with the initiation and progression of DR. However, no direct evidence supports this theory.

Methods. Immunostaining of apolipoprotein B100 (ApoB100, a marker of low-density lipoprotein [LDL]), macrophages, and oxidized LDL was performed in retinal sections from four different groups of subjects: nondiabetic, type 2 diabetic without clinical retinopathy, diabetic with moderate nonproliferative diabetic retinopathy (NPDR), and diabetic with proliferative diabetic retinopathy (PDR). Apoptosis was characterized using the TUNEL assay. In addition, in cell culture studies using in vitro-modified LDL, the induction of apoptosis by heavily oxidized-glycated LDL (HOG-LDL) in human retinal capillary pericytes (HRCPs) was assessed.

Results. Intraretinal immunofluorescence of ApoB100 increased with the severity of DR. Macrophages were prominent only in sections from diabetic patients with PDR. Merged images revealed that ApoB100 partially colocalized with macrophages. Intraretinal oxidized LDL was absent in nondiabetic subjects but present in all three diabetic groups, increasing with the severity of DR. TUNEL-positive cells were present in retinas from diabetic subjects but absent in those from nondiabetic subjects. In cell culture, HOG-LDL induced the activation of caspase, mitochondrial dysfunction, and apoptosis in HRCPs.

Conclusions. These findings suggest a potentially important role for extravasated, modified LDL in promoting DR by promoting apoptotic pericyte loss. (*Invest Ophthalmol Vis Sci.* 2008;49:2679–2685) DOI:10.1167/iovs.07-1440

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Diabetic retinopathy (DR) is a leading cause of visual impairment and blindness in the world, especially in people younger than 50.[1] DR develops gradually, and its earliest detectable features are believed to result from damage to the specialized retinal capillaries, causing leakage of the blood retinal barrier and apoptotic pericyte loss.[2–5] Recently, early damage to the neural retina, especially to the region where synapses and neuronal branching occur, has also been implicated.[6]

Sophisticated grading systems exist to define the severity of DR.[7] Briefly, DR progresses from background disease, through nonproliferative diabetic retinopathy (NPDR), to proliferative diabetic retinopathy (PDR).[8] Development and progression of DR are closely associated with glycemic control,[9,10] and subclinical damage to the retina may be established early in poorly controlled diabetes, thus setting the stage for further progression.[11,12]

With regard to pathogenesis, dyslipidemia has been associated with severity of DR in clinical studies.[7,13] Specifically, DR was positively associated with serum triglycerides and with serum concentrations of low-density lipoprotein (LDL), LDL particle concentration, and apolipoprotein B (ApoB), the principal lipoprotein component of LDL.[7] In atherosclerosis, modified lipoproteins, particularly oxidized LDL, promote vascular damage through complex inflammatory and immunologic mechanisms in the arterial intima.[14–16] We hypothesize that analogous effects of extravasated and modified LDL in the retina promote DR. Supporting this, we have shown adverse effects of in vitro–modified LDL (glycated and oxidized) on retinal capillary cells, specifically in mediating cytotoxicity and altered gene expression.[17–20] However, to date there is little direct evidence of the presence of modified LDL in the diabetic retina. In the present study, using immunohistochemistry, we demonstrate the presence of extravasated ApoB100 and oxidized LDL in retinas from diabetic patients, correlating with the severity of DR. Further, in PDR, the presence of macrophages and their colocalization with ApoB was observed. In related cell culture experiments, we confirmed that LDL that has been glycated and then oxidized induced apoptosis in human retinal capillary pericytes (HRCPs) through the activation of caspase pathways and mitochondrial dysfunction. These findings support the notion that oxidized, glycated LDL is implicated in the initiation and development of DR.

Materials and Methods

The study was approved by the Institutional Review Board at the University of Oklahoma Health Sciences Center.

Sample Preparation

Retinal sections (5 μm) from four different groups of human subjects (five subjects per group) were obtained from National Diseases Research Interchange (NDRI; Philadelphia, PA). Subject groups were as follows: nondiabetic, type 2 diabetic without clinical retinopathy, type 2 diabetic with moderate NPDR, and type 2 diabetic with PDR. Tissue acquisition was undertaken as previously described.[21] Eyes were enucleated and fixed in 10% neutral-buffered formalin within 12 hours of death. Subject

ages were similar in the different groups (nondiabetic, 62.6 ± 14.9 years; type 2 diabetic without clinical retinopathy, 69.4 ± 9.9 years; type 2 diabetic with moderate NPDR, 70.6 ± 8.3 years; type 2 diabetic with PDR, 57 ± 6.5 years). Ophthalmologic records recorded DR status and the absence of other retinal diseases, including macular edema. Diabetic neuropathy and nephropathy were documented as absent. Other clinical characteristics of diabetes, including glycemic control, HbA1c, and lipid profile, were not available from NDRI.

Immunohistochemistry: ApoB100, Macrophages, Oxidized LDL, and TUNEL Assay

Positive staining for ApoB100 was used to identify LDL. ApoB100 is also present in very low-density lipoprotein (VLDL), but because VLDL particles are larger than LDL particles, we interpreted ApoB100 in the retina as evidence of LDL extravasation, though not excluding VLDL extravasation.

After deparaffinization and rehydration, sections were treated with sodium citrate buffer (pH 6.0, 10 minutes) and blocking buffer (10% normal goat serum, 0.05% Triton X-100 in PBS; 1 hour room temperature). In the first series of experiments, ApoB100 and macrophages were identified by incubation with goat anti-human ApoB100 (1:200; Abcam, Cambridge, MA) and mouse anti-human CD68 (1:400; Abcam) overnight at 4°C. In the second series of experiments, oxidized LDL was detected with rabbit anti-human oxidized LDL (the antibody was raised against copper-oxidized LDL; 1:400; Abcam). In the third series of experiments, apoptosis and oxidized LDL were double stained using TUNEL assay (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) and rabbit anti-human oxidized LDL antibody in retinal sections from nondiabetic and diabetic subjects. Sections were washed in PBS and incubated with either Alex Fluor 594-conjugated donkey anti-goat (1:200; Molecular Probes, Eugene, OR) or Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:200; Molecular Probes) in blocking buffer (1 hour, room temperature), counterstained with DAPI, and mounted in mounting medium (Fluoromount-G; Vector Laboratories, Burlingame, CA). Signals were visualized by fluorescence microscopy (20× objective lens). Four independent experiments were performed on sections from different subjects.

Human Retinal Capillary Pericyte Culture

Methods for pericyte culture have been described previously.[17–20] Briefly, HRCPs (Cambrex, Walkersville, MD) were cultured at 37°C with 5% CO_2 in medium containing 5% fetal bovine serum, 0.1% human epithelial growth factor (hEGF), 0.04% hydrocortisone, 0.1% vascular epithelial growth factor (VEGF), 0.4% human fibroblast growth factor (hFGF)-B, 0.1% R^3-insulinlike growth factor (IGF)-1, 0.1% ascorbic acid, and 0.1% GA-1000 (Clonetics, Walkersville, MD). HRCP from passages 4 to 13 were used. Pericytes at 85% confluence were treated in serum-free medium (SFM) for 24 hours to induce quiescence and then were treated for 24 hours with SFM or SFM containing native-LDL (N-LDL) or heavily oxidized-glycated (HOG)-LDL.

Preparation, Modification, and Characterization of LDL

In brief, human LDL was isolated from pooled plasma obtained from four to six healthy, nonsmoking, volunteer subjects aged 20 to 40 years who did not take prescribed medications or antioxidant vitamins. Native LDL (N-LDL) was prepared by sequential ultracentrifugation and quantified and characterized as described in our previous studies.[17,18] HOG-LDL was prepared by first glycating N-LDL in the presence of freshly prepared 50 mM glucose for 72 hours at 37°C under anti-oxidant conditions (1 mM N,N-bis{2-(bis[carboxymethyl]-amino)ethyl}glycine (DTPA) and 270 μM EDTA, under nitrogen) and subsequently oxidizing the glycated LDL in the presence of 10 μM $CuCl_2$ (24 hours, 37°C). (Note that the antibodies used to detect intraretinal oxidized LDL in the experiments described were raised against in vitro–copper-oxidized LDL.) Protein in LDL preparations was quantified with BCA protein assay (Pierce, Rockford, IL). N-LDL and HOG-LDL preparations were characterized by gel electrophoresis (Paragon LIPO Gel; Beckman, Fullerton, CA) by measuring fluorescence at 360 excitation/430 emission (Fluorometer IV; Gilford, Oberlin, OH) and absorbance at 234 nm using a spectrophotometer (model DU650; Beckman).

Apoptosis Assays

After 24 hours in SFM, HRCPs were treated with N-LDL or HOG-LDL at 100 or 200 mg/L or remained in SFM at 37°C for a further 24 hours. Cells were harvested using trypsin and were washed with PBS. The percentage of apoptotic cells was determined by flow cytometry using the Annexin-V–FITC apoptosis detection kit according to the manufacturer's instructions (Calbiochem, San Diego, CA).

Caspase-3 and -7 Cleavage Assays

HRCPs were treated with growth medium (GM), SFM, N-LDL at 200 mg/L, or HOG-LDL at 50, 100, and 200 mg/L for 24 hours in 96-well plates (Greiner, Longwood, FL). Activity of caspase-3 and -7 was assessed by analyzing the cleavage of caspase-3 and -7, as described.[22] Cells were lysed and centrifuged, and supernatants were transferred to a black 96-well plate (Corning, Corning, NY) and were incubated with 2.5 μM Z-DEVD-Alexa 488 (30 minutes, 37°C). Fluorescence was read on a plate reader (BMG, Durham, NC) using the fluorescein channel. Lysates of cells prepared at the same time and in an identical manner were used to determine cell protein (BCA protein assay; Pierce). Data were expressed as picomole substrate per milligram protein per hour.

TUNEL Assay

Three round, glass coverslips (12-mm diameter) coated with poly-L-lysine were put into each well in six-well cell culture plates. HRCPs were incubated with SFM, 200 mg/L N-LDL, or 50, 100 or 200 mg/L HOG-LDL for 24 hours. Subsequently, cells in the coverslips were fixed with 4% paraformaldehyde. DNA strand breaks were detected by enzymatically labeling terminal deoxynucleotidyl transferase (TdT) with modified nucleotides (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Pittsburgh, PA). Signals were visualized by light microscopy (40× objective lens).

Western Blot Analysis

HRCPs were treated with N-LDL (200 mg/L) or HOG-LDL (50–200 mg/L) for 24 hours. HRCPs were washed three times with ice-cold phosphate-buffered saline (PBS) and then exposed to a detergent buffer (1% Triton X-100, 0.5% Tween 20, 0.5 M NaCl, 50 mM HEPES, pH 7.5), containing a protease inhibitor mixture (EDTA-free; Roche, Mannheim, Germany). HRCPs were harvested and lysed with lysis buffer. Protein concentrations in extracts were determined by BCA protein assay. Aliquots of 20 μg protein were run on 12% SDS-PAGE gels. After transferring the samples onto polyvinylidene difluoride membranes, proteins were probed with polyclonal anti-Bax antibody (Abcam).

Statistical Analysis

Differences were examined with a one-way ANOVA followed by Tukey multiple comparisons test. The level of significance was set at $P < 0.05$.

Results

Detection of ApoB100, Macrophages, Oxidized-LDL, and Apoptosis in Retina

Retinas from all four groups were immunopositive for ApoB100 (Fig. 1a, red). In nondiabetic eyes, this staining was very faint and homogenous. Among diabetic eyes, immunofluorescence increased with the severity of retinopathy and showed evidence consistent with lipoprotein aggregation. The presence and aggregation of ApoB100 was evident in diabetes even in the absence of clinical retinopathy, consistent with a role in the early development of DR,

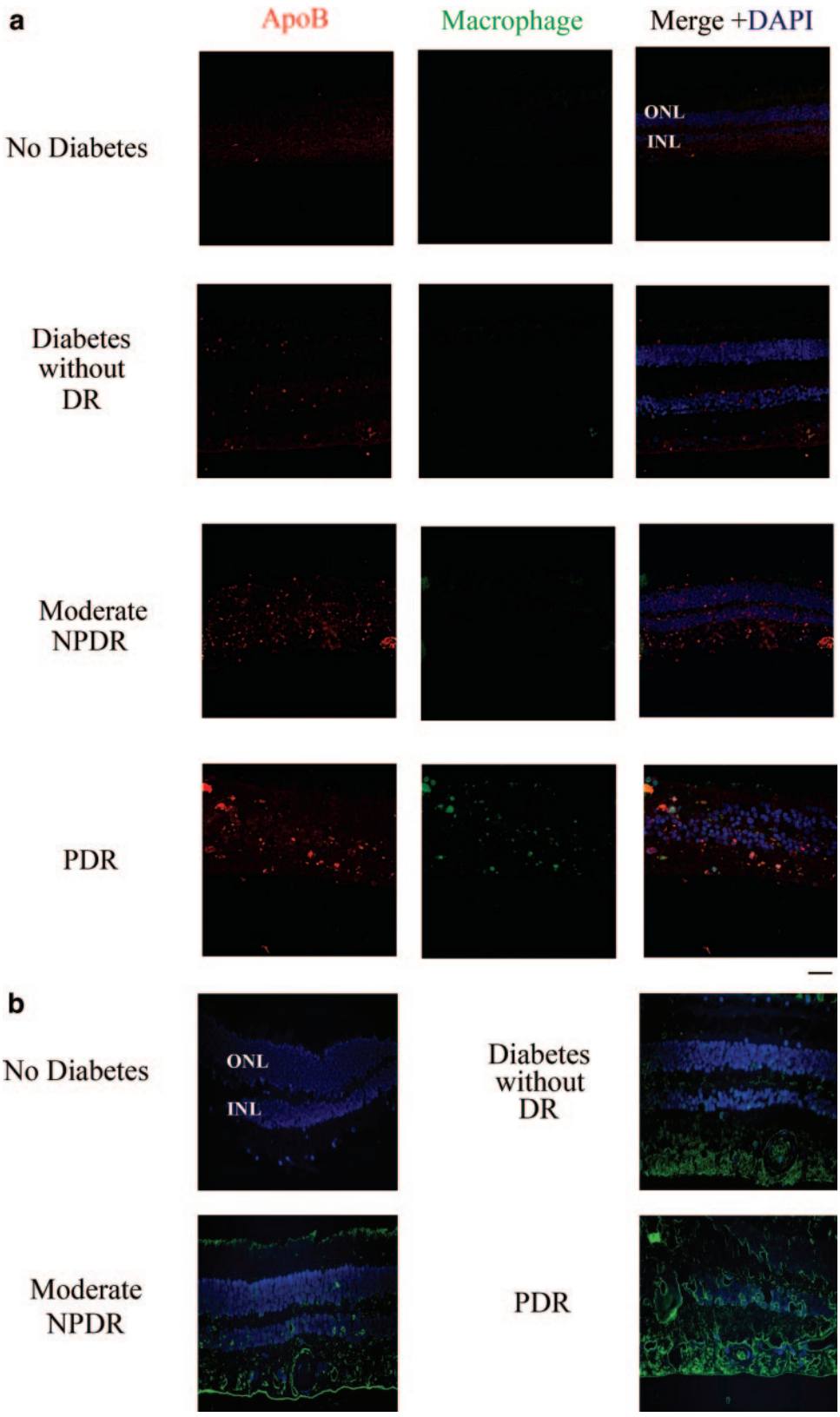

FIGURE 1. Presence of ApoB, macrophages, and oxidized LDL in retinal sections of human subjects with four different categories of diabetes: no diabetes, type 2 diabetes without clinical retinopathy, type 2 diabetes with moderate NPDR, and type 2 diabetes with PDR. (**a**) Immunostaining for ApoB100 and macrophages. Retinal sections were stained with anti-ApoB100 antibody (*red*), anti-macrophage antibody (*green*), and DAPI (*blue*). (**b**) Immunostaining for oxidized LDL. Retinal sections were stained with anti-oxidized LDL antibody (*green*), and DAPI (*blue*). These images are representative of four independent experiments. Scale bar, 20 μm.

and was more marked in the presence of more severe DR. Intraretinal macrophages (Fig. 1a, green) were prominent only in PDR, where they were found throughout all layers of the retina. Merged images revealed that ApoB100 partially colocalized with macrophages (Fig. 1a). Intraretinal immunostaining for oxidized LDL (Fig. 1b, green) was observed in all three diabetic groups, again correlating with the severity of DR, but was absent in nondiabetic subjects. In the three diabetic groups, immunostaining for oxidized LDL was prominent in the inner retina (ganglion cell layer) where most of the blood supply is derived from the central retinal artery. In the PDR group, immunostaining was also detected

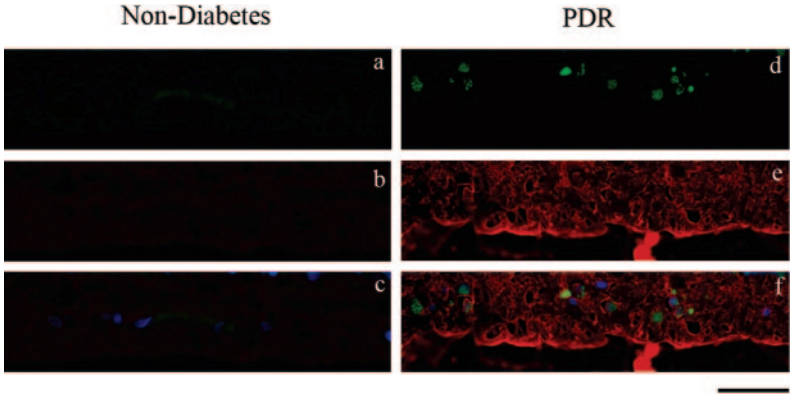

FIGURE 2. Presence of oxidized LDL and TUNEL-positive cells in the ganglion cell layer of the inner retina from a nondiabetic subject (**a–c**) and a type 2 diabetic subject with PDR (**d–f**). Retinal sections were stained with anti–TUNEL antibody (*green*, **a**, **d**), antioxdized LDL (*red*, **b**, **e**), and merged staining with DAPI (*blue*, **c**, **f**). Scale bar, 20 μm.

in the outer retina, close to the pigment epithelium, where the choroidal circulation provides blood supply. The findings suggest that leakage of lipoproteins through the inner and outer blood retinal barriers occurs in the late stages of DR.

Consistent with a role for modified LDL in the mediation of injury to retinal blood vessels, we observed oxidized LDL adjacent to retinal capillaries (Fig. 1b). Double staining for TUNEL and oxidized LDL demonstrated that TUNEL-positive cells were present in association with oxidized LDL—that is, they were present in the diabetic retina according to the severity of retinopathy but absent in the nondiabetic retina. In Figure 2, these findings are illustrated in representative samples of inner retina (the location of pericytes) from a nondiabetic control subject and a subject with PDR. To further demonstrate the roles and mechanisms of oxidized LDL in pericyte apoptotic death, an established feature of "pericyte dropout" in early DR, we investigated the effects of in vitro–modified HOG-LDL on HRCPs in cell culture.

HOG-LDL–Induced Apoptosis in HRCPs

HOG-LDL was prepared by in vitro modification of N-LDL isolated from pooled plasma from healthy human subjects, first by glycation, then by oxidation, to mimic LDL modified by exposure to elevated glucose levels in plasma, then to oxidative stress after extravasation. Flow cytometry data (Fig. 3) showed that HOG-LDL significantly induced apoptosis in HRCPs (100 mg/L; 6.76% ± 0.24%; 200 mg/L, 13.95% ± 0.47%), compared with the effects of N-LDL (100 mg/L; 2.73% ± 0.27%; 200 mg/L; 4.43% ± 0.13%) and SFM (3.30% ± 0.37%). This apoptotic effect of HOG-LDL was also confirmed by detection of DNA fragmentation using the TUNEL assay (Fig. 4).

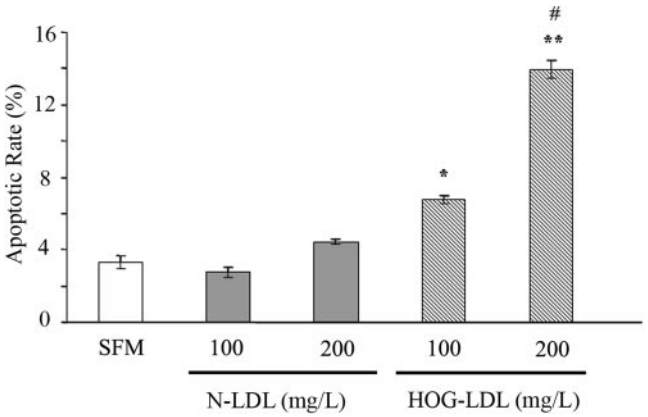

FIGURE 3. Effects of HOG-LDL on the survival of cultured HRCPs. Flow cytometry using Annexin V detected early apoptosis. Data were obtained from cells incubated under different conditions for 24 hours in SFM, and N-LDL did not alter cell growth. HOG-LDL significantly induced apoptosis 24 hours after the treatment. $^{*}P < 0.001$. $^{**}P < 0.0001$, compared with SFM and N-LDL. $\#P < 0.001$, compared with HOG-LDL at 100 mg/L ($n = 4$).

Mechanism of HOG-Induced Apoptosis

To investigate the mechanisms involved in HOG-LDL–induced apoptosis, the expression of activated caspases 3 and 7 and Bax were measured. HOG-LDL at doses of 100 and 200 mg/L significantly increased the expression of activated caspases 3 and 7 compared with SFM, N-LDL at 200 mg/L, or HOG-LDL at 50 mg/L (Fig. 5). An increased level of Bax expression was also detected in HRCPs treated with HOG-LDL compared with N-LDL, each at 200 mg/L ($P < 0.05$; Fig. 6).

DISCUSSION

Dyslipidemia includes quantitative and qualitative abnormalities of lipoproteins, the former including different levels of

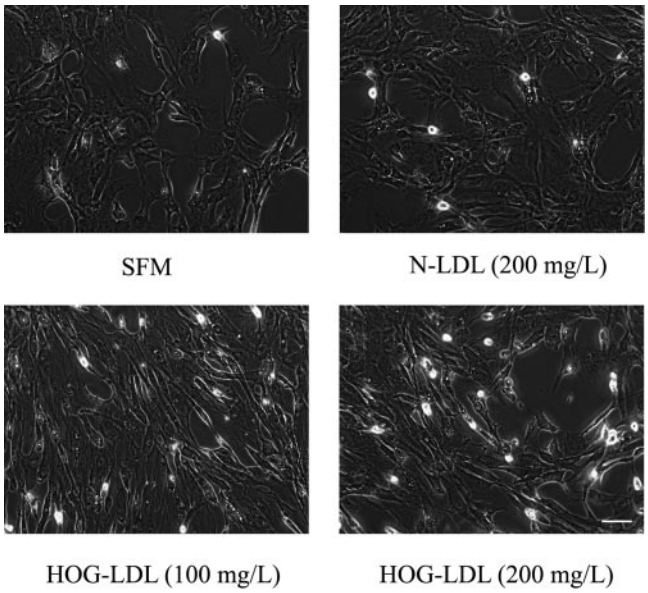

FIGURE 4. HOG-LDL–induced DNA fragmentation in cultured HRCPs. Apoptosis was ascertained by detecting DNA fragmentation using a TUNEL assay. Bright spots represented positive staining of DNA fragmentation in the nucleus. Cells treated with HOG-LDL, particularly at 200 mg/L for 24 hours, showed a significantly increased number of DNA fragmentations. Images are representative of three separate experiments. Scale bar, 20 μm.

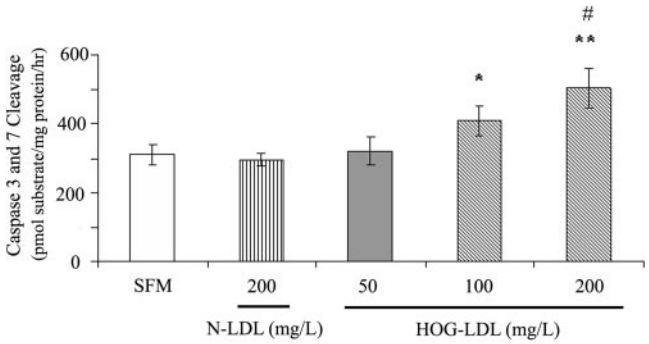

FIGURE 5. HOG-LDL–induced caspase-3 and -7 cleavage in cultured HRCPs. Pericytes were incubated for 24 hours under various conditions. Caspase-3 and -7 activation were monitored by cleavage of the fluorogenic peptide Z-DEVD-Alexa 488. SFM did not increase the cleavage of caspase-3 and -7. N-LDL at 200 mg/L also did not significantly increase the cleavage. HOG-LDL at 100 or 200 mg/L, but not at 50 mg/L, greatly enhanced caspase-3 and -7 cleavage in cultured HRCPs. Data are expressed as picomole substrate per milligram protein per hour. $^{*}P < 0.01$. $^{**}P < 0.001$, compared with SFM and N-LDL. $\#P < 0.001$, compared with HOG-LDL at 100 mg/L ($n = 8$).

lipoprotein classes and subclasses and the latter including modification by glycation and oxidation and altered composition and ratios of lipids and proteins.[23] Quantitative dyslipidemia is associated with the initiation and progression of DR.[7,13] Retinal hard exudates are associated with elevated plasma LDL and lipoprotein (a) levels.[13,24–26] Recently, in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications cohort, we found associations between DR and an adverse plasma lipoprotein subclass distribution, especially in men.[7] Similar associations have been found in the Hoorn[27] and the Epidemiology of Diabetic Complications studies.[28] Dramatic regression of retinal hard exudates has been observed after correction of dyslipidemia.[29,30]

We have hypothesized that, in a manner analogous to atherogenesis, extravasation of LDL from retinal capillaries, and its subsequent oxidation, may be implicated in the progression of DR. The extent of capillary leakage of LDL and the efficiency of retinal defense systems against oxidized LDL could then become more important determinants of DR progression than circulating lipoprotein levels. In the present study, we present three new and important pieces of evidence to support a role for extravasated and modified lipoproteins, specifically oxidized and glycated LDL, in the etiology of pericyte loss in early DR. First, we demonstrated the presence of extravasated and oxidized LDL in the diabetic retina using antibodies raised against copper-oxidized LDL. The extents of staining for ApoB and oxidized LDL were proportional to the severity of retinopathy. Even at the earliest stages, before clinical DR was evident, the aggregation of lipoproteins was observed. Second, TUNEL-positive cells were associated with the presence of oxidized LDL throughout all layers of the retina in diabetic subjects, including the ganglion cell layer of the inner retina, where blood capillaries containing pericytes are found. Third, we showed that after in vitro–copper-mediated oxidation of glycated LDL, the lipoprotein became an effective trigger for the apoptotic cell death of human retinal capillary pericytes.

One previous immunohistologic study demonstrated the presence of extravasated ApoB, cholesteryl ester, and macrophages in retinas obtained from two patients with diabetic maculopathy.[29] However, the present study is the first to document the extent of ApoB100 and oxidized LDL immunostaining in relation to the severity of DR in the absence of macular edema. The increased (and punctate) immunostaining for ApoB100 indicates the existence of extravasated and aggregated LDL (and perhaps VLDL) in the diabetic retina. Importantly, increased and aggregated ApoB100 and oxidized LDL were detected in diabetic patients with clinically "normal" retinal capillaries, indicating retinal injury occurs in diabetes before the appearance of clinical DR that can be observed with an ophthalmoscope. Macrophages were detected in the retina only in diabetic patients with PDR, in whom they were found throughout all layers of the retina. This is consistent with PDR representing a different and more severe phase of DR than nonproliferative disease.

In atherogenesis, which is accelerated in diabetes, oxidized LDL is known as a powerful cytotoxin. Diabetes may amplify LDL oxidation for several reasons. Increased glycation of plasma LDL is known to occur in diabetes[31] and to render the lipoprotein more susceptible to oxidation.[32] Once extravasated, glycated LDL is more likely than native LDL to be sequestered (through advanced glycation product cross-linking), to aggregate, and thus to persist in vessel walls, further enhancing the likelihood of oxidation. Finally, hyperglycemia may directly enhance oxidative stress,[33] again promoting LDL oxidation. The results of the present study support the contention that mechanisms parallel to those in atherogenesis may be operative in DR. They show that ApoB and oxidized LDL are widely distributed in the diabetic retina. The differences between the patterns of ApoB100 and oxidized LDL in this report suggest that the protein component of LDL may become dissociated from the lipid component during modification and fragmentation; such an effect has been reported in atherosclerotic regions of the thoracic aorta.[34] Furthermore, the presence of oxidized LDL in the retinas was associated with the presence of TUNEL-positive cells, indicating apoptosis. The apoptotic cells were seen in all layers of the retina in the presence of diabetes, including the ganglion cell layer, where pericytes are found in the capillaries (Fig. 2), and they were more numerous according to the severity of DR. They were absent in the nondiabetic retina (Fig. 2). These findings are consistent with a role for oxidized LDL in mediating apoptotic cell death in the retina in vivo, including in pericytes. However, the results must be interpreted with caution because we did not have full control of sample processing after the death of the eye donors.

Our findings do not define the origin of ApoB or oxidized LDL in the retina, but leakage of the outer and the inner blood retinal barrier may contribute, especially in the later phases of

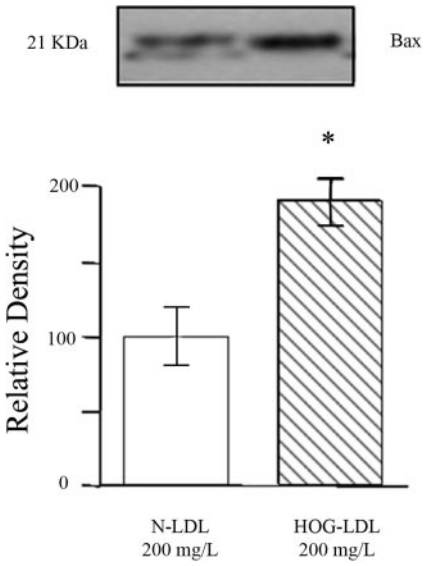

FIGURE 6. Effects of HOG-LDL on Bax expression in cultured HRCPs. Data were obtained from cells incubated with media containing N-LDL or HOG-LDL at 200 mg/L for 24 hours. Bax content was determined by Western blot analysis. The Western blot is representative of four separate experiments. $^{*}P < 0.05$, compared with N-LDL at 200 mg/L.

DR. The injurious effects of oxidized LDL are likely to affect the neural retina and the retinal blood vessels, consistent with recent concepts of a general retinal injury in DR.[2–6] In the present study, we observed that oxidized LDL was expressed mainly in the ganglion cell layer adjacent to retinal blood vessels in NPDR (Fig. 1b), as would be expected if the lipoprotein had leaked from the inner retinal circulation. This is consistent with the possibility that modified LDL may directly injure retinal pericytes. However, oxidized LDL was found throughout the retina in the PDR group (Fig. 1b), implicating it in the more extensive retinal damage characteristic of PDR

In earlier studies using cultured retinal pericytes, we showed that LDL modified by glycation and oxidation decreased the survival of bovine retinal pericytes and endothelial cells.[18] In a single experiment, we documented apoptosis by DNA laddering in bovine pericytes exposed to HOG-LDL.[19] In a subsequent gene expression microarray study, HOG-LDL induced a gene expression pattern markedly distinct from that of either N-LDL or (nonoxidized) glycated-LDL (G-LDL). We identified 60 genes whose expression was altered by more than 1.7-fold by HOG-LDL compared with N-LDL in HRCPs,[17] including some related to fatty acid and eicosanoid metabolism, cell growth and proliferation, and angiogenesis. More recently, we demonstrated that HOG-LDL has unique effects on one of the inhibitors of matrix metalloproteinases, TIMP-3.[20]

It is noteworthy that although glycated LDL has less drastic effects than HOG-LDL on pericytes, as witnessed by our gene array study,[17] modification by glycation is important not only for its own effects but also because it may predispose LDL to oxidative damage and enhance its tendency to become sequestered after extravasation, again increasing the likelihood of oxidation. The sublethal effects of oxidized LDL in the retina merit further study, but our present study addresses its lethal effects on pericytes. For these reasons, we focused in this work on determining the presence of oxidized LDL in the retina and on the effects of HOG-LDL in HRCP culture.

In the present cell culture experiments, we show conclusively that HOG-LDL promotes apoptosis in cultured retinal pericytes in vitro, and we shed some light on the mechanism. Caspases 3 and 7 are major mediators of apoptosis, leading to a cascade of downstream events such as DNA cleavage and cell membrane changes.[35] Both are activated after the treatment of HRCP with HOG-LDL (Fig. 3). There are two pathways of caspase-3 and -7–mediated apoptosis[35]: the extrinsic pathway involving the death receptor Fas and caspase-8 and the intrinsic or mitochondrial pathway involving Bcl-2 proteins and cytochrome *c* release. It has been demonstrated that the extrinsic pathway is involved in oxidized LDL–induced apoptosis in vascular endothelial cells and smooth muscle cells.[36] However, we previously reported that HOG-LDL is taken up mainly by scavenger receptors in rat mesangial cells, indicating that HOG-LDL–induced apoptosis may be mediated through the intrinsic pathway.[37] In this, caspase-3 and -7 pathways are known to be activated by cytochrome *c* leaking from mitochondria and are regulated by the Bcl-2 family of proteins.[38,39] Among the Bcl-2 family, Bcl-2 and Bcl-xL suppress apoptosis, whereas Bax, Bak, and Bid promote it.[40,41] Bax is the best-studied protein among proapoptotic Bcl-2 family members[42] and can form ion-conducting channels in planar lipid bilayers of mitochondrial membrane. These channels are essential for the release of cytochrome *c*, the subsequent activation of caspase-3 and -7, and, hence, the induction of apoptosis.[42] It has been shown that retinal cells of diabetic patients have elevated levels of Bax.[43] In addition, a decreased protein ratio of Bcl-2 to Bax was associated with pericyte loss induced by high levels of glucose.[44] In the present study, we detected that the level of Bax expression was significantly enhanced after HOG-LDL treatment, suggesting HOG-LDL may induce mitochondrial dysfunction by altering Bax level. In future studies, we will measure other proteins, such as bid and bcl2, and the release of cytochrome *c* to understand fully the roles of mitochondrial (intrinsic) pathways in HOG-LDL–induced apoptosis in pericytes.

The possibility that early retinopathy is promoted by interactions of capillary cells with oxidized lipoproteins has potential therapeutic implications. Most obviously, control of plasma lipoprotein levels might limit extravasation, but measures to protect the integrity of the blood retinal barriers, both inner and outer, might be paramount. Other measures could have beneficial effects, even in the presence of LDL extravasation. Antioxidants might mitigate pericyte loss in DR. Apocynin, a nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase inhibitor, reduced apoptosis and the development of avascular retina in an animal model of retinopathy of prematurity, perhaps by affecting pathways triggered by reactive oxygen species (ROS) but upstream from lipid hydroperoxide production.[45] Aminoguanidine is another candidate; it is a well-known inhibitor of advanced glycation end product formation and is beneficial in diabetic retinopathy, as demonstrated in vitro and in vivo.[46–49] Without defining the minimum effective dose, we previously showed that aminoguanidine at concentrations as low as 1 μM significantly blocked the toxicity of modified LDL toward bovine retinal capillary endothelial cells and pericytes.[46] The potential efficacy of aminoguanidine at nanomolar concentrations suggests an action through scavenging of reactive carbonyls (whether generated by oxidative or metabolic processes) and a possible therapeutic target.

In conclusion, to our knowledge, this study provides the first direct evidence that oxidized LDL exists in the diabetic retina and is associated with the severity of DR and the presence and extent of apoptosis. Consistent with a causative role in retinal injury, HOG-LDL significantly induced the activation of caspases, mitochondrial dysfunction, and apoptosis in HRCPs, consistent with the clinical observation of pericyte loss by apoptosis in the early stage of DR. Early intervention to prevent retinal capillary leakage may be needed to prevent DR. Future studies will address receptor mechanisms and signaling pathways activated in pericytes exposed to oxidized LDL, perhaps leading to new treatments to prevent pericyte loss in early DR.

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