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Original Article

Oxidized low density lipoprotein (oxLDL) promotes mitochondrial dysfunction and induces apoptosis in retinal pigmented epithelium cells

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Abstract: Oxidized low density lipoprotein (oxLDL) may contribute to the capillary injury in diabetic retinopathy. In particular, sustained high oxLDL is closely associated with the dysfunction and death of retinal pigment epithelium (RPE) cells, as may play a vital role in the pathogenesis of age-related macular degeneration (AMD). In the current study, we determined the promotion by oxLDL to the apoptosis in retinal pigmented epithelium ARPE-19 cells, and investigated whether the oxLDL-induced apoptosis depended on the activation of toll-like receptor-4 (TLR-4) signaling pathway. ARPE-19 cells were cultivated with and without oxLDL. Cell apoptosis was evaluated by flow cytometry. Immunofluorescence, western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR) were conducted to assess TLR-4 expression on both protein and mRNA expressions. The RNAi technology was also utilized to examine the dependence of TLR-4 in the oxLDL-induced apoptosis in ARPE-19 cells. Results demonstrated that the incubation of ARPE-19 cells with ox-LDL (more than 50 μg/mL) for 24 hours increased the apoptosis of ARPE-19 cells and promoted the cleaved levels of caspase-3 and 9, which are the active forms of both caspases. The lyzed level of Poly (ADP-ribose) polymerase (PARP), which is mainly lyzed by active caspase 3, was also significantly upregulated by the oxLDL. Moreover, it was demonstrated that TLR-4 expression was also significantly upregulated by the oxLDL treatment with more than 50 μg/mL. However, the RNAi-mediated knockdown of TLR-4 markedly reduced the oxLDL-induced apoptosis in ARPE-19 cells. In conclusion, our findings indicate that the apoptosis was induced by oxLDL in cultured retinal pigmented epithelium ARPE-19 cells at least in part by modulating the TLR-4 signaling pathway. TLR-4 might be a valuable target for the AMD prevention.

Keywords: Oxidized low density lipoprotein (oxLDL), apoptosis, toll-like receptor-4 (TLR-4), ARPE-19 cells

Introduction

Age-related macular degeneration (AMD) is a common cause of blindness in over 65 years' old persons in developed countries [1]. Oxidative stress is believed to be a key factor to the initiation and progression of AMD [2]. The retina is vulnerable to oxidative stress, and antioxidants have been paid attention to recently for the treatment and management of AMD [3]. The damage to the retinal pigment epithelium by oxidative stress involves the mitochondria and mtDNA, which lead to destabilization of mitochondrial function and induction of apoptosis [4, 5]. Further analysis of patients with AMD indicates that apoptosis plays an important role on the death of retinal pigment epithelial cells (RPE) [6].

The RPE is sensitive to apoptosis by several mechanisms such as the high oxidative stress environment [7]. The age-related accumulation of lipids which results from photoreceptor turnover and the internalization of low density lipoproteins (LDLs) is an additional reason causing RPE [8]. Apoptosis is triggered and mediated by caspases, including initiator caspases (caspase-2, -8, -9, -12) and effector caspases (caspase-3, -6, -7) [9]. Additionally, the toll-like receptor-4 (TLR-4) is another signal transducer to induce cell death, which is activated through oxidized low density lipoprotein

(oxLDL) [3, 10]. TLR-4 is a leucine-rich transmembrane protein [11]. They protect host against bacteria, viruses, and toxins through a pattern-recognition capacity [12, 13]. Previous studies revealed that the TLR-4 present in neurons and involves in brain injury [14]. To date, no researches have been performed to investigate the role of TLR-4 in the death of retinal pigmented epithelium cells.

In this study, we tried to determine the effect of oxLDL on the apoptosis in retinal pigmented epithelium ARPE-19 cells, and investigate whether the oxLDL-induced apoptosis depended on the activation of toll-like receptor-4 (TLR-4) signaling pathway. Our findings will indicate that the role of oxLDL in retinal pigmented epithelium cell death, and which signaling pathway are activated. The study will also offer a valuable target for the AMD prevention.

Materials and methods

Cell culture and treatment

The non-immortalized human RPE cell line ARPE-19 was purchased from ATCC. For experiments, ARPE-19 cells were seeded in T-75 flasks containing DMEM with 15 mM Hepes buffer and 10% fetal bovine serum and 2 mM L-glutamine solution at 37°C for 1 week, and medium was replaced with DMEM plus 1% bovine serum albumin for 3 days to make ARPE-19 cells quiescent [15]. Low density lipoproteins (LDL) was oxidized by CuSO4 to generate oxidized LDL (oxLDL) [16]. Serial dosage of oxLDL was added into the medium for 24 h and cells were collected for the further analysis. For siRNA transfection, TLR-4 gene silencing was carried out with siRNA vs rat TLR-4 and GeneSilencer siRNA transfection reagent (Genlantis-BioCat, Germany) [17].

Cell apoptosis assay

Cells apoptotic percentage was performed by Annexin V-FITC Apoptosis Detection Kit (Merk Company, USA) as the tutorial described. Briefly, the oxLDL treated cells were centrifugated at 2000 RPM for 5 minutes and washed twice with PBS, then the cells were suspended in the 400 μl 1× Binding Buffer at a concentration of 1×10^5 cells/ml, then each 5 μl of Annexin V-FITC and Propidium Iodide was added in turn and mixed, the treated cells were placed in the dark at RT for 5-15 minutes to perform flow cytometry analysis.

Western blotting assay

Treated cells were lysed with lysis buffer (Invitrogen, USA) on ice for 20 min, and the cell lysates were centrifugated at 13,000 g at 4°C for 30 min, then the supernatant was collected as the total cellular protein extract. After determining protein concentration using the BCA Protein Assay Kit (Bio-rad, USA), equal amount of each cellular protein was loaded onto 10% SDS polyacrylamide gel. The separated proteins were electrophoretically transferred to PVDF membranes (Bio-rad, USA). The membrane was blocked overnight in blocking buffer containing PBS-T and 5% non-fatty milk. Then the membrane was incubated with primary mouse/goat antibodies against cleaved caspase 3/cleaved caspase 9/lyzed PARP/TLR-4/β-actin antigens for one hour separately and was washed with PBST for 4 times subsequently. Following incubating with the secondary HRP-conjugated antibody for 1 hour, the PVDF membrane was washed for 4 times and was treated with ECL reagent (Pierce, USA) and subjected to X-ray film. Each band was quantified using Image software.

Quantitative real time PCR assays for mRNA expression

Total RNA was extracted from ARPE-19 cells using the RN easy Mini-kit (Qiagen Inc.) according to the manufacturer's recommendation and was reverse transcribed with Super Script II RT (Invitrogen-Gibco). Quantitative real-time PCR was then conducted with SYBR® Green mastermix (Life tech, USA) in a 7500 Fast PCR instrument (Applied Biosystems, USA) using the special primers for TLR-4 mRNA, separately [17]. The cycle threshold (Ct) values of the target gene were normalized to β-actin from the same sample as relative mRNA levels. All samples were run in triplicate in the 96-well reaction plates.

Immunofluorescence assay

Immunofluorescent labeling for ARPE-19 cells was performed as previously described [18]. Briefly, the cells were fixed 10 minutes with 4% paraformaldehyde, permeabilized in 0.1%

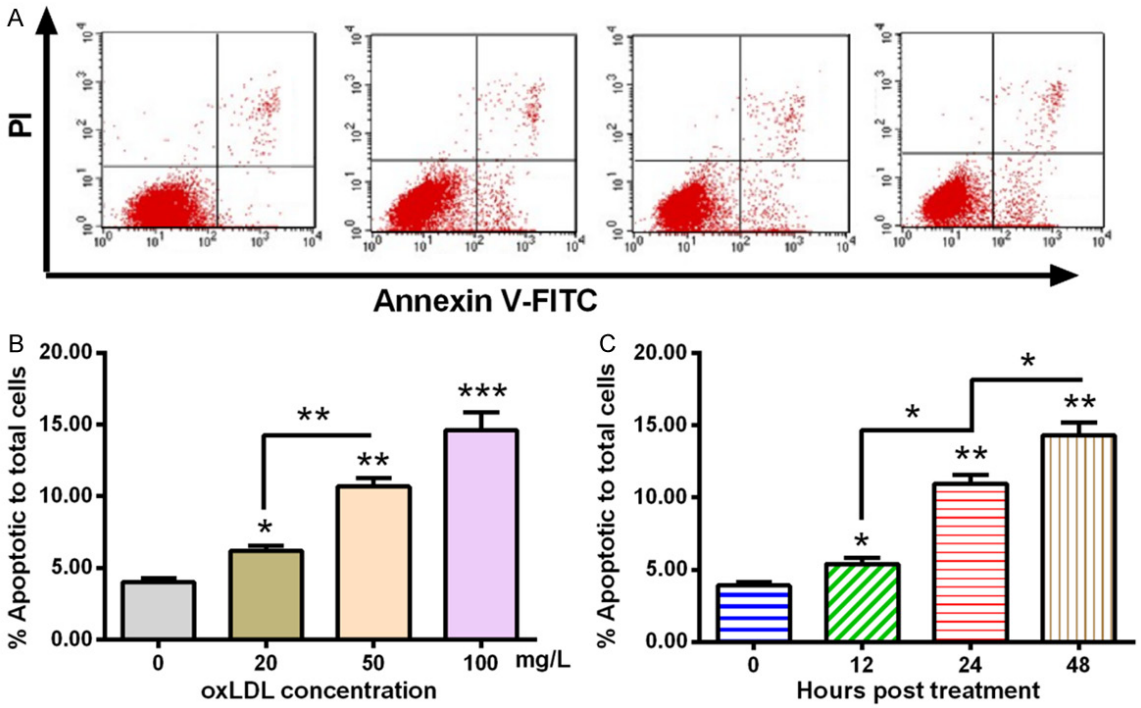

Figure 1. OxLDL induces apoptosis in retinal pigmented epithelium ARPE-19 cells dose- and time-dependently. ARPE-19 cells were treated with 0, 20, 50 or 100 mg/L oxLDL for 0, 12, 24 or 48 hours, then the apoptosis was assessed by flow cytometry using Annexin V-FITC/PI Apoptosis Detection Kit. A: Representative images for oxLDL-induced apoptotic ARPE-19 cells, post the oxLDL treatment with different concentrations (0, 20, 50, 100 mg/L) for 24 h (Later apoptotic cells were denoted in the upper right quadrant, and the early apoptotic cells were denoted in the lower right quadrant). B: Percentage of apoptotic ARPE-19 cells, post the oxLDL treatment with different concentrations (0, 20, 50, 100 mg/L) for 24 h; C: Percentage of apoptotic ARPE-19 cells, post the oxLDL treatment with 50 mg/L ox-LDL for 0, 12, 24 or 48 hours respectively. Data were presented as mean ± standard error of the mean (SEM) (n=3 for each group). *P<0.05, **P<0.01 or ***P<0.001 versus the control group.

Triton X-100/PBS for 10 minutes, and washed three times with PBS. Then cells were blocked with 3% BSA in PBS for one hour. Primary mouse antibody against TLR-4 was incubated with cells in 0.5% BSA-PBS for one hour. After cells were washed with PBS, the secondary antibody incubation was performed for one hour in 0.5% BSA-PBS and cells were washed by PBS. Then confocal microscopy images were obtained with a confocal microscope (Olympus, Japan).

Statistical analysis

All statistical analyses were performed using SPSS 17.0. Data are depicted as the mean ± standard deviation. Student's t-test was used only for a comparison between two groups. One-way analysis of variance was used for multiple comparisons among three or more groups. P<0.05 was considered as a statistically significant difference.

Results

oxLDL induces apoptosis in retinal pigmented epithelium ARPE-19 cells

ARPE-19 cells were treated with 0, 20, 50 or 100 mg/L oxLDL for 0, 12, 24 or 48 hours, then the apoptosis was assessed by flow cytometry using Annexin V-FITC/PI Apoptosis Detection Kit. As intuitively shown in **Figure 1A**, the percentage of apoptotic ARPE-19 cells grows as the increase of oxLDL dose 24 hours post-treatment. Subsequently, we analyzed the apoptotic rate in different doses of oxLDL at serial time points (**Figure 1B** and **1C**), it was found that each group treated with oxLDL presented a higher apoptotic level compared with the control group, with a statistical difference. Specifically, apoptotic rate of group treated with 50 mg/L oxLDL was increased by 50% compared with that of 20 mg/L

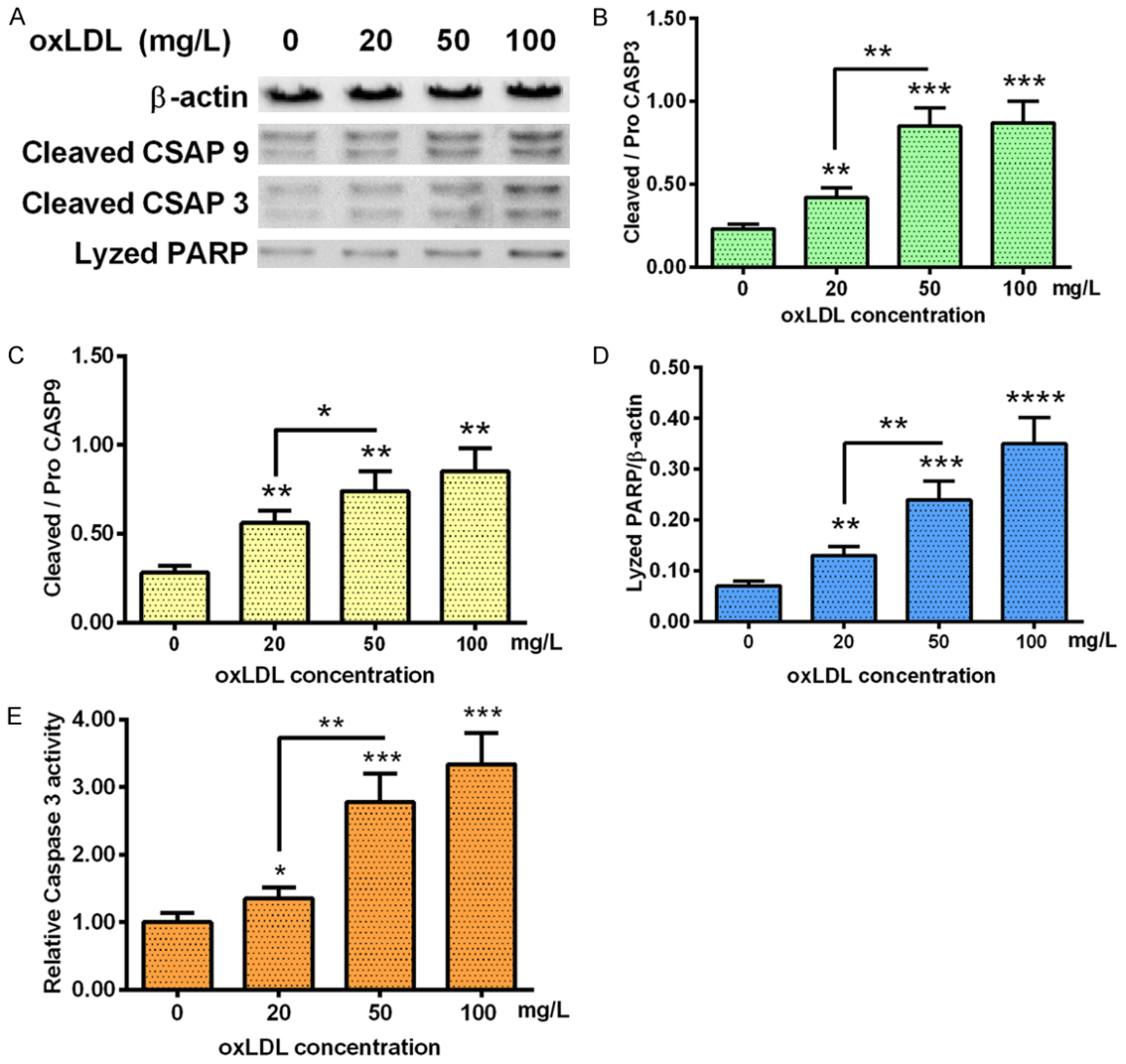

Figure 2. OxLDL promotes the expression of apoptosis-associated markers in ARPE-19 cells. ARPE-19 cells were treated with 0, 20, 50 or 100 mg/L oxLDL for 24 hours, then the western blotting (A) was performed to examine the protein levels of cleaved caspase 3 (Cleaved CASP 3), cleaved caspase 9 (Cleaved CASP 9) and lyzed Poly (ADP-ribose) polymerase (PARP) in each group. The relative levels of Cleaved CASP 3 (B), Cleaved CASP 9 (C) or lyzed PARP (D) to β-actin were presented respectively. (E) Relative caspase 3 activity was examined in the oxLDL-treated ARPE-19 cells, with a Caspase-3 Activity Assay Kit, in which, a fluorogenic substrate (N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin or Ac-DEVD-AMC) was cleaved by active caspase-3 into DEVD and AMC, generating highly fluorescent AMC that can be detected using a fluorescence reader with excitation at 380 nm and emission between 420-460 nm. Data were presented as mean ± SEM (n=3 for each group). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001.

oxLDL treated group (P<0.01). As for the apoptotic rate at sequential time points, a time-dependent increase was observed (**Figure 1C**), with statistical difference between each group.

oxLDL promotes the expression of apoptosis-associated markers in treated cells

After ARPE-19 cells were treated with 0, 20, 50 or 100 mg/L oxLDL for 24 hours, the western blotting was performed to examine the protein levels of pro-caspase 3/cleaved caspase 3, pro-caspase 9/cleaved caspase 9, and lyzed PARP in each group, respectively (**Figure 2A**). As indicated in **Figure 2B-E**, time-dependent increase of ratios of cleaved caspase 3/pro-caspase 3, cleaved caspase 9/pro-caspase 9, and lyzed PARP/β-actin, as well as the relative caspase 3 activity, is observed, separately. Statistical differences also present between each group.

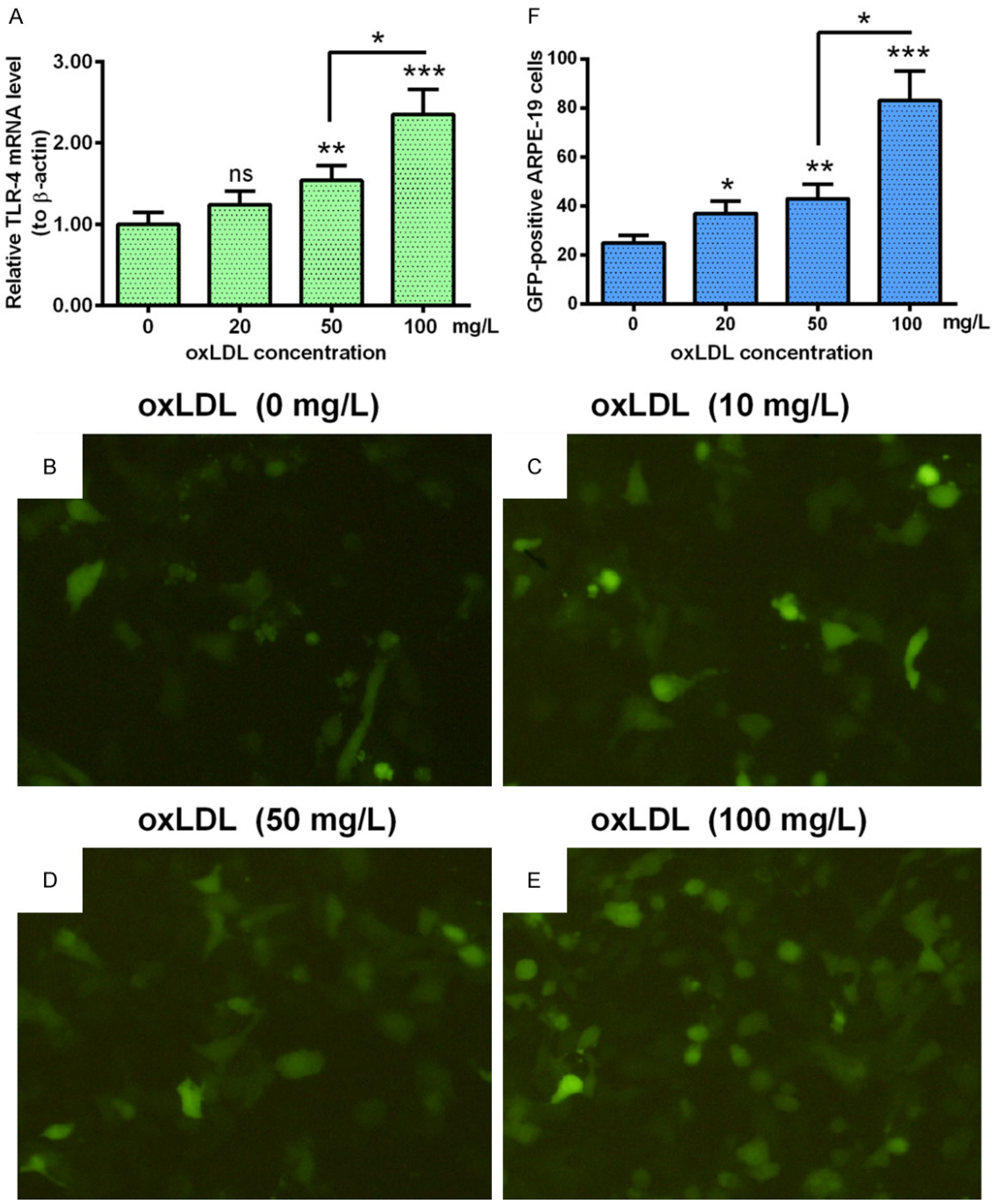

Figure 3. OxLDL promotes TLR-4 expression in ARPE-19 cells. ARPE-19 cells were treated with 0, 20, 50 or 100 mg/L oxLDL for 24 hours, then quantitative real-time PCR was performed to examine the mRNA level of TLR-4 (A) and immunofluorescence assay was performed to determine the protein level of TLR-4 in each group (B-E). The determination of TLR-4 fluorescence intensity was presented as the number of fluorescein isothiocyanate (FITC)-positive cells (F). Data were presented as mean ± SEM. *P<0.05, **P<0.01 or ***P<0.001, ns: no significance.

oxLDL promotes TLR-4 expression in ARPE-19 cells

After ARPE-19 cells were treated with 0, 20, 50 or 100 mg/L oxLDL for 24 hours, then quantitative real-time PCR was performed to examine the mRNA level of TLR-4. As shown in **Figure 3A**, oxLDL stimulated TLR-4 expression at a higher concentration of 50 mg/L (P<0.01), and a statistical difference was observed bet-

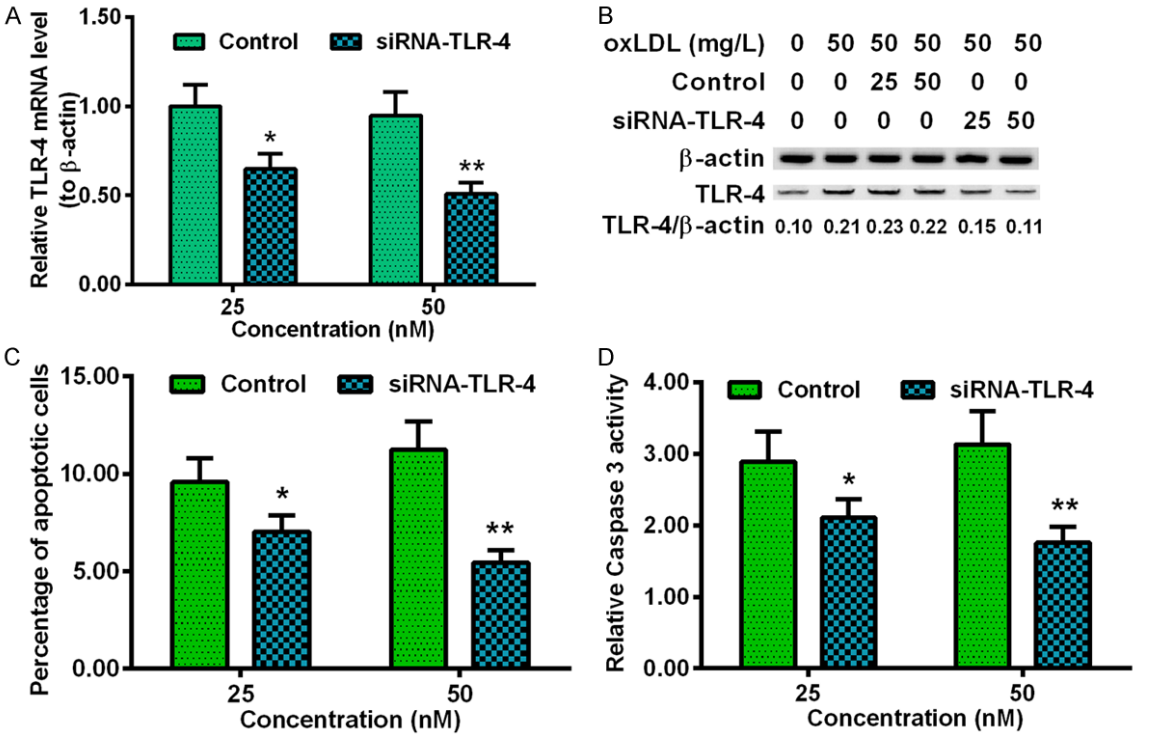

Figure 4. TLR-4 dependence of the oxLDL-induced apoptosis in ARPE-19 cells. ARPE-19 cells were firstly transfected with 25 or 50 nM siRNA-TLR-4 or control siRNA, then were treated with 50 mg/L oxLDL for 24 hours, then quantitative real-time PCR was performed to examine the mRNA level of TLR-4 (A) and western blotting assay was performed to determine the protein level of TLR-4 in each group (B). (C and D) Percentage of apoptotic cells (C) or relative caspase 3 activity (D) in the ARPE-19 cells, which were transfected with 25 or 50 nM siRNA-TLR-4 or control siRNA, after the oxLDL treatment (50 mg/L). Data were presented as mean ± SEM. *P<0.05 or **P<0.01.

ween 50 mg/L oxLDL-treated group and 100 mg/L oxLDL-treated group. Next, immune-fluorescence assay was performed to determine the protein level of TLR4 in each group, each group presented the higher number of GFP-positive cells than the control group, specially, GFP- positive cells number at 100 mg/L-treated group was improved by two times than that of 50 mg/L oxLDL-treated group.

oxLDL induced apoptosis in ARPE-19 cells in a TLR-4 dependent manner

ARPE-19 cells were firstly transfected with 25 nM or 50 nM siRNA-TLR-4 or control siRNA, and then were treated with 50 mg/L oxLDL for 24 hours, quantitative real-time PCR was performed to examine the mRNA level of TLR-4 soon. As shown in **Figure 4A**, cells treated by 25 nM or 50 nM siRNA-TLR-4 presented 40% or 50% lower level than the control, respectively. Then the WB for TLR-4 expression and β-actin expression was performed. As indicated in **Figure 4B**, the TLR4/β-actin ratio of 25 nM or 50 nM siRNA-TLR-4 treated group was 0.15 or 0.11, separately, which is lower than that (0.22 or 0.23) of control group.

Next, we checked the apoptotic rate and relative caspase 3 activity of siRNA-transfected cells, respectively. As indicated in **Figure 4C**, the apoptotic rate is down-regulated by 40% and 70% in comparison with control, when cells were transfected by 25 nM siRNA-TLR-4 or 50 nM siRNA-TLR-4, separately. As for the relative caspase 3 activity, similar result is observed, it is decreased by 40% and 60% compared with control, when treated by 25 nM siRNA-TLR-4 or 50 nM siRNA-TLR-4, respectively (**Figure 4D**).

Discussion

In this research, we determined the promotion by oxLDL to the apoptosis in retinal pigmented epithelium ARPE-19 cells, and investigated whether the oxLDL-induced apoptosis depended on the activation of toll-like receptor-4 (TLR-4) signaling pathway. We found that the

incubation of ARPE-19 cells with ox-LDL (increased the apoptosis of ARPE-19 cells and promoted the cleaved levels of caspase-3 and 9. It was demonstrated that TLR-4 expression was also significantly upregulated by the oxLDL treatment. And the RNAi-mediated knockdown of TLR-4 markedly reduced the oxLDL-induced apoptosis in ARPE-19 cells.

Apoptosis is an important reason causing RPE cell death during early AMD [19, 20]. In our research, we found that some pro-apoptotic genes such as cleaved caspase 3, cleaved caspase 8, and lyzed PARP were up-regulated under the treatment of oxLDL, suggestive of a pro-apoptotic response from oxLDLs. Our experiments also showed a marked increase in apoptotic cells treated with oxLDL compared to controls. At the same time, TLR-4 expression displayed an oxLDL dose-dependent increase, while siRNA-TLR-4 transfection decreased the percentage of apoptotic cells and relative caspase 3 activity. Results above indicate oxLDL at least induce the apoptosis in part by modulating the TLR-4 signaling pathway.

TLR-4 is a proinflammatory sensor of pathogens, which is a potential link between inflammation and diabetic retinopathy [21]. Oxidatively-modified lipoproteins have been identified as TLR-4 ligands suggesting a mechanism for initiation of apoptosis [22]. Evidence from different studies has suggested that TLR-4 can affect the apoptosis of retinal pigmented epithelium cells in multiple ways [23]. In oxLDL-treated cells, TLR-4 blocking decreased apoptosis. These results point strongly to a direct activation of TLR-4 through oxLDL to cause enhanced ARPE-19 cell apoptosis. On the other hand, untreated APRE-19 cells showed TLR-4 expression with statistical differences compared with those treated by oxLDL. This suggests that the TLR-4-dependent APRE-19 cell response seems to depend on a particular ligand fraction and its concentration in the extracellular milieu. Low levels of TLR-4 activity might be necessary for APRE-19 cell survival, but increased TLR-4 activity under oxLDL stimulation then becomes the main inducer of apoptosis.

oxLDL caused a robust transcriptional change in treated ARPE-19 cells including genes implicating a defense and an oxidative stress response. All these responses have been implicated in AMD. Oxidized lipoproteins can potentially induce a pathological phenotype [24], as evidenced by apoptosis identified in this investigation. While this investigation has potential short comings when only performed in environment in vitro, nonetheless, our data support that oxLDL is one trigger that initiates apoptosis of ARPE-19 cells and provide TLR-4 as a tangible treatment target. Future experiments elucidating their specific roles are needed.

In conclusion, we determined the promotion by oxLDL to the apoptosis in retinal pigmented epithelium ARPE-19 cells, and investigated oxLDL-induced apoptosis depended on the activation of toll-like receptor-4 (TLR-4) signaling pathway. A our findings indicate that the apoptosis was induced by oxLDL in cultured retinal pigmented epithelium ARPE-19 cells at least in part by modulating the TLR-4 signaling pathway. TLR-4 might be a valuable target for the AMD prevention.

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Disclosure of conflict of interest

None.

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