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Cholesterol loading augments oxidative stress in macrophages

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Abstract To investigate the molecular consequence of loading free cholesterol into macrophages, we conducted a large-scale gene expression study to analyze acetylated-LDL-laden foam cells (AFC) and oxidized-LDL-laden foam cells (OFC) induced from human THP-1 cell lines. Cluster analysis was performed using 9600-gene microarray datasets from time course experiment. AFC and OFC shared common expression profiles; however, there were sufficient differences between these two treatments that AFC and OFC appealed as two separate entities. We identified 80 commonly upregulated genes and 48 commonly downregulated genes in AFC and OFC. Functional annotation of the differentially expressed genes indicated that apoptosis, extracellular matrix, oxidative stress, and cell proliferation was deregulated. We also identified 87 differentially expressed genes unique for AFC and 31 genes for OFC. The uniquely expressed genes of AFC are associated with kinase activity, ATP binding activity, and transporter activity, while unique genes for OFC are associated with cell signaling and adhesion. To validate the hypothesis that oxidative stress is a common feature for AFC and OFC, we performed a cluster analysis employing the genes related to oxidative stress, but we were unable to distinguish AFC from OFC in this manner. We performed real-time RT-PCR and ELISA on foam cells to examine the transcripts and secreted protein of interleukin 1 beta (IL1β). IL1β was rapidly induced in foam cells, but for AFC both RNA level and protein level dropped immediately and was attenuated. To detect levels of reactive oxygen species in foam cells we conducted hydroethidine staining and observed high levels of superoxide anion. We conclude that loading free cholesterol induces high levels of superoxide anion, increases oxidative stress, and triggers a transient inflammatory response in macrophages.

Keywords: Low-density lipoproteins; Foam cells; Microarray; Atherosclerosis; Macrophages; Oxidized LDL; Acetylated LDL; Apoptosis; Expression pattern; Inflammatory response; Real-time RT-PCR

1. Introduction

The pathogenesis of cardiovascular disease, which is the leading cause of death in developed countries, occurs initially through atherosclerosis [1,2]. High levels of low-density lipoproteins (LDL) and oxidative stress are primary risk factors for the development of atherosclerosis [3–6]. Foam cells, which are the hallmark of an early atherosclerotic lesion, form as a result of a macrophage internalizing oxidized LDL (ox-LDL) through a scavenger receptor without feedback control [7–10]. These foam cells initiate an increase in oxidative stress and stimulate proliferation of smooth muscle cells in vitro [11–16].

The genetic consequences of macrophages taking up ox-LDL have been investigated broadly. Ox-LDL induces pro-inflammatory genes, activates PPARγ, and influences the expression of downstream genes. The induced macrophages secrete interleukin-1 beta (IL1β), which is a potent growth factor for smooth muscle cells (SMCs) [17]. IL1β may stimulate the SMCs to over-express platelet-derived growth factor (PDGF), which may cause migration and proliferation of the SMCs. Ox-LDL is toxic to macrophages; it causes the apoptosis and necrosis of foam cells and the subsequent release of their cellular contents, which may compose the necrotic core found in advanced lesions [18–21]. Large-scale surveys of the gene expression in ox-LDL-loaded foam cells have been performed on genome-wide scale [22–31]. Molecular markers are implicated in the cell growth, survival, migratory, inflammatory, and matrix remodeling of macrophages.

Oxidative modification transforms LDL into a powerful pathogen, but it is not clear whether the oxidative stress of foam cells is due to the oxidative modification of ox-LDL or to the cellular processing that follows the internalization through binding to the scavenger receptor. Acetylated LDL (ac-LDL), a non-oxidatively modified LDL, is readily taken up by macrophages, through a scavenger receptor, to generate foam cells [32–36]. Ac-LDL induces apoptosis and also inflammatory response in macrophages [37]. It is likely, but yet to be proven, that the internalized free cholesterol, analogous to oxidized LDL, triggers cellular pathways that lead to the increase in oxidative stress.

To evaluate the contribution of internalized cholesterol to the atherosclerotic behavior of foam cells, we conducted large-scale expression profiling of foam cells derived from both ox-LDL and ac-LDL [38]. Our results provide insights into the molecular mechanisms that distinguish the intracellular and extracellular oxidative events.

2. Materials and methods

DMEM, FBS, antibiotics, and all other tissue culture reagents were obtained from GIBCO. $CuSO_4$, KBr, thiobarbituric acid, trichloroacetic acid, and other common chemicals were purchased from Sigma or Merck.

2.1. Isolation of LDL and copper-mediated oxidation of LDL

LDL, in the density range 1.019–1.063, was isolated from human plasma through ultracentrifugation as described [39]; the typical protein concentration was 5 mg/ml. Copper-mediated oxidation was performed, as described in Mao et al. [40], through the addition of

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various concentrations of $CuSO_4$ into LDL and subsequent incubation at 37 °C for 16 h. The reaction was terminated upon the addition of excess EDTA.

2.2. Human macrophage cell lines THP-1

Human THP-1 cells were added to six-well tissue culture plates containing glass cover slips at 2×10^6 cells/well in DMEM containing 10% fetal bovine serum and 100 μg/ml penicillin; they were cultured for 4 h at 37 °C in an incubator containing 5% CO_2 at 90% humidity. The non-adherent cells were removed and the monolayers were then placed in DMEM containing 10% fetal bovine serum and supplemented with 100 μg/ml ox-LDL or ac-LDL. Foam cell formation was monitored by Oil Red O staining [41]. Monolayers of macrophages on glass cover slips were fixed with 10% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, stained with Oil Red O, counterstained for 10 min with hematoxylin, and then examined under a microscope.

2.3. Microarray analysis

Inconsistent handling of microarray experiments frequently generates data that is difficult to process, especially for the time course experiment. We carefully initiated all experiments at the same starting point. Once a sample was harvested, we proceeded to cDNA synthesis and stored the labeled cDNA at −80 °C until use. All microarray hybridizations were conducted at the same time.

All microarray procedures – including PCR amplification, spotting, post-spotting processing, RNA extraction, probe preparation, hybridization, and post-hybridization experiments – were performed in a dust/climate controlled laboratory. Microarray design, experimental procedures, data processing, and data presentation were carefully performed according to the MIAME guidelines [42]. A microarray consisting of 9600 sequence-verified human cDNA was utilized in this current study [43].

The microarray experiment for each time point was performed in duplicate. Total RNA was extracted using the protocol supplied with the TRI reagent (Molecular Research Center, Inc., USA). The quality of the RNA was examined through agarose gel electrophoresis; the OD 260/280 ratio was >1.8. In a typical labeling reaction, total RNA (0.5–10 μg) was annealed with polydT(15) (0.5 μg) in a total volume of 20 μL. The cDNA synthesis was performed in 1× Superscript RT II reaction buffer (50 μL) containing annealed RNA, 0.5 mM each of dATP, dGTP, and dTTP, 40 μM of dCTP and Cy3-dCTP or Cy-5-dCTP (Roche, USA), 10 mM DTT, 1 U of RNasin (Invitrogen, USA), and 50 U of Superscript RT II (Invitrogen). The mixture was incubated in the dark for 90 min at 42 °C and the reaction was terminated through heating at 95 °C for 5 min. The RNA was degraded through the addition of 3 N NaOH (5.5 μL) and incubation at 50 °C for 30 min. The mixture was neutralized upon the addition of 3 M acetic acid (5.5 μL) and filter-purified through a Microcon YM-100 (Amicon Co., USA) to a final volume of 30 μL. Using a 50-mL conical tube, the microarray was prehybridized at 42 °C for 1 h in a prehybridization buffer (30 ml) containing 25% formamide, 5× SSC, 0.1% SDS, and 0.1 mg/mL BSA. The labeled probe was mixed with polydA(10) (20 μg) and human Cot-1 DNA (20 μg; Invitrogen) and then denatured at 95 °C for 5 min. The denatured probe was dried and suspended in the prehybridization buffer (20 μL). Hybridization was performed in a Corning hybridization chamber and incubated at 42 °C for 12–16 h. The slide was washed twice with 2× SSC and 0.1% SDS (30 ml) for 5 min at room temperature, followed by three washes (30 ml each; 20 min each) with 0.1× SSC and 0.1% SDS at 42 °C. All washing procedures were performed through gentle shaking in 50-mL conical tubes. Fluorescence scanning was performed using an Axon Genepix 4000B. The fluorescent image was processed by GenePix Pro 3.0 to obtain the raw expression dataset. The mean intensity and mean background intensity were utilized for data processing; the global array intensity was employed for normalization controls. Non-linear normalization was performed using locally weighted linear regression (lowess) [44]. Logarithmic ratios (base 2) were calculated accordingly. Duplicated data were averaged to obtain single dataset for each time point.

2.4. Statistical analysis for microarray datasets

Differentially expressed genes of microarray datasets were derived firstly by significance analysis of microarrays (SAM) [45]. The *P*-values corresponding to each gene were obtained by a standard *t*-test. The multiplicity of *P*-values due to the consequence of performing statistical tests on many genes in parallel was resolved by SAM method. SAM assigns a score to each gene based on change in gene expression relative to standard deviation of measurements. SAM performs permutation for repetitive measurement to estimate false discovery rate (FDR). In the current study, we adjusted the gene expression threshold that gave mean number of false discovery to less than one gene. Additionally, significantly expressed genes derived from SAM were further filtered by the criterion set by twice of the standard deviation calculated from the averaged dataset.

2.5. Real-time reverse transcription polymerase chain reaction (RT^2 PCR)

Real-time PCR analysis was performed, according to the manufacturer's instructions, using an iQ™ SYBR green supermix (Bio-Rad, USA) and the specific primer pairs for selected genes and the primer pairs for ribosomal protein L18 as the reference gene. The threshold cycle number (C_t) was measured using the iCycler and its associated software (Bio-Rad; [46]). Relative transcript quantities were calculated using the $\Delta\Delta C_t$ method with ribosomal protein L18 as the reference gene amplified from the samples. ΔC_t is the difference in the threshold cycles of the sample mRNAs relative to those of the ribosomal protein L18 mRNA. $\Delta\Delta C_t$ is the difference between the values of ΔC_t of the THP-1 cell control and the foam cells. Values for fold-induction were calculated as $2^{\Delta\Delta C_t}$. RT^2PCR was performed in triplicate for each time point.

2.6. Hydroethidine staining

Hydroethidine is one of the best reagents currently available for measuring the intracellular concentration of the superoxide anion ($O2^{\cdot -}$) [47,48]. The superoxide anion reacts with hydroethidine to produce ethidium bromide, which binds to nuclear DNA and emits fluorescence. Cells were washed briefly with PBS, incubated with 5 μM hydroethidine for 30 min, and then washed with PBS to remove any excess dye prior to imaging. Fluorescence was detected using a laser scanning confocal microscope operated at excitation and emission wavelengths of 488 and 610 nm, respectively, and using a 585-nm long-pass filter. The optical density of the ethidium ion was normalized to the number of THP-1 cells and is expressed as the percentage maximal intensity of ox-LDL-derived foam cells.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The secretion of IL1β protein upon stimulation of modified LDL was characterized by ELISA using anti-IL1β IgG. Culture media were measured for IL1β protein content. The acquired reading in optical absorbance unit was normalized by the maximum signal.

2.8. Statistical analysis

All data from RT^2PCR, ELISA, and hydroethidine staining are presented as means ± standard error of the mean (S.E.M.). Data sets were evaluated using one-way analysis of variance (ANOVA). The minimum level of significance was set at $P < 0.05$.

3. Results and discussion

To obtain large-scale gene expression patterns for lipid loaded macrophages, we conducted microarray experiments using cDNA microarray containing 9600 genes of sequence-verified human cDNA. We performed time-course experiments on both ac-LDL-laden foam cells (AFC) and copper-oxidized-LDL-laden foam cells (OFC) while using untreated THP-1 cell lines as the normal control. We harvested the cells 12, 24, 36, 48, and 72 h in duplicates after administration of the modified LDL. Total RNA was fluorescence-labeled and hybridized to the microarray; we then detected the intensities using a laser-excited scanner. After background subtraction, we performed non-linear normalization using lowess procedure [44]. We derived the final expression ratios by comparing the results to those of the untreated cell line; we transformed and the ratios to the $\log_2$ scale for further data processing.