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HIGHLY UP-REGULATION OF *FAS LIGAND* GENE EXPRESSION AFTER INCREASING IN OXIDIZED LOW-DENSITY LIPOPROTEIN

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Abstract: Oxidized low-density lipoprotein (ox-LDL) shows many harmful effects such as induction of apoptosis on function of endothelial cell (EC). Fas ligand (FASL) induces apoptosis in divergent pathological conditions. EC apoptosis which induced by ox-LDL is related with FASL. In this study, we aimed to determine how different ox-LDL levels affect *FASL* expression in ECs. We treated human umbilical vein endothelial cells (HUVECs) with two different concentration of ox-LDL. *FASL* gene expression in groups was detected by quantitative polymerase chain reaction (qPCR). We compared *FASL* gene expression level between the groups according to ΔΔCt method. *FASL* gene expression was statistic significantly up-regulated in the group treated higher amounts of ox-LDL ($P<0.001$). We found that treatment with higher amounts of ox-LDL in HUVECs increased *FASL* gene expression dramatically. According to our findings, we concluded that increasing the amount of ox-LDL may be critical in inducing the FASL-dependent apoptotic pathway in ECs.

Keywords: Oxidized low-density lipoprotein, *Fas ligand*, Gene expression, Quantitative polymerase chain reaction, Human umbilical vein endothelial cell

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1. Introduction

Oxidized low-density lipoprotein (ox-LDL) is composed result from oxidation of low-density lipoprotein (LDL) under oxidative stress (Khatana et al., 2020). Ox-LDL interacts with molecular targets of endothelial cells (ECs) through various mechanisms and play physiological or pathological roles (Poznyak et al., 2021). Fas ligand (FASL) is a membrane-bound protein that recognizes Fas receptor which is transmembrane, death receptor protein (Tian et al., 2017). Death of a cell is occured through contact of soluble FASL to membrane-bound Fas. The functional association of FASL is initiating death signals in the cells (Malarkannan, 2020). FASL binds to death receptor protein at the cell membrane and induces the recruitment of Fas-associated death domain proteins. These proteins interact with other apoptotic proteins to activate the downstream effects of the cell. Then formation of death-inducing signaling complex activates the cell apoptosis (Tian et al., 2017). Ox-LDL binds to ECs and induces apoptosis. Ox-LDL-induced apoptosis activates a death receptor, Fas, when it binds to its ligand FASL. It has been demonstrated that ox-LDL increases expression of the FASL in vascular cells triggering the apoptosis (Munno et al., 2024). Cells expressing FASL bind the Fas receptor on a target cell before apoptotic pathway initiates in target cells (Malarkannan, 2020). It was implicated that FASL-neutralizing antibodies decrease ox-LDL-induced apoptosis (Munno et al., 2024).

It was shown that ox-LDL treatment restrict human umbilical vein endothelial cells (HUVECs) viability, cell proliferation, and accelerate apoptosis of HUVECs (Cao et al., 2024). Studies to date have shown that ox-LDL induces FASL and FASL-mediated apoptosis (Sata and Walsh, 1998; Li et al., 1998; Imanishi et al., 2002, Li et al., 2014; Tian et al., 2017). The hypothesis of this study was created to determine the effect of higher amounts of ox-LDL treatment on FASL gene expression. Within the scope of this hypothesis, it was aimed to compare *FASL* gene expression in HUVECs treated with different amounts of ox-LDL through quantitative polymerase chain reaction (qPCR) method.

2. Materials and Methods

2.1. Materials

HUVECs provided from ATCC were used to cell line experiments. EC growth medium/DMEM (CAPRICORN) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin was culture medium for HUVECs at 5% CO_2, 37°C, and 95% relative humidity. HUVECs were separated from the EC growth medium/DMEM using 0.25% trypsin. HUVECs obtained from logarithmic growth phase were utilized for experiments (Liu et al., 2020). HUVECs at ~80-90% confluence were used for ox-LDL treatments. HUVECs were divided into two groups. Former group was group 1, in which HUVECs were treated 25 µg/ml ox-LDL (invitrogen LOT2160046, L34357); and

latter group was group 2 in which HUVECs were treated with 40 µg/ml concentration of ox-LDL for 24 hour. Our cell density decreased significantly after the application of 40 µg/ml ox-LDL in HUVECs. Therefore, we determined our experimental group as HUVECs treated with 40 µg/ml ox-LDL. It was determined by reviewing the literature that the ox-LDL concentration level at which cell viability began to be affected was 25 µg/ml, and experiments were carried out with these two ox-LDL concentrations (Ma et al., 2024). Five biological repetitions were made for each ox-LDL concentration.

2.2. Methods

Firstly, total RNA was extracted from HUVECs through RNeasy Mini Kit (QIAGEN, catalog no:74104) for RNA isolation step. Then we synthesized complementary DNA (cDNA) by using reverse transcription kit (A.B.T.™ with RNase Inh. High Capacity, Catalog No:C03-01-20). Finally, amplification of FASL gene expression was detected qPCR utilizing SYBR Green dye (A.B.T.™ 2X qPCR SYBR-Green MasterMix kit, Catalog No:Q03-02-01 ve Q03-02-05), cDNA, RNase free water (nzytech, MB11101) and *FASL* qPCR primers. Spesific human *FASL* gene primer sequences are forward:5'ACAGCATCATCTTTGGAGAAGC3' (A.B.T, ID:20220609/1-117); reverse:5'ACCTTGAGTTGGACTTGCCT3' (A.B.T, ID:20220609/1-118). on qPCR instrument (LightCycler 96 Real-Time PCR Instrument, Roche). *GAPDH* was used as housekeeping control gene in qPCR. Forward and reverse primer sequences of *GAPDH* are forward:5'ACAACTTTGGTATCGTGGAAGG3' and reverse:5'GCCATCACGCCACAGTTTC3', respectively. Three biological repetitions were made for qPCR experiments.

2.3. Statistical Analysis

We used "GeneGlobe Data Analysis Center" (https://geneglobe.qiagen.com/us/analyze QIAGEN, Hilden, Germany) to analyze the gene expression data. qPCR data was uploaded to the analysis system. Threshold cycle (Ct or Cq) values are obtained after qPCR experiments. Analysis system gives to fold change (FC) value obtained from the groups' comparison according to the qPCR results. Gene expression ratios (FC calculations) are calculated performing method of ΔΔCt. FC was calculated as the ratio of the relative gene expression between the group 1 (HUVECs treated 25 µg/ml ox-LDL) and group 2 (HUVECs were treated 40 µg/ml ox-LDL). Numbers higher than 1 show increased gene expression (up-regulation), numbers between 0 and 1 show decreased gene expression (down-regulation), and a FC value of 1 shows no change (Livak and Schmittgen, 2001). P-value was calculated based on a Student's t-test of the replicate $2^{-\Delta Ct}$ values for each gene in each control group (group 1) and experiment group (group 2) comparison (RT2 Profiler PCR Arrays & Assays Data Analysis Handbook, 2019). P-value results less than 0.05 were accepted significant.

3. Results

In this study, we performed qPCR to compare FASL gene expression level between group of HUVEC treated with 25 µg/ml ox-LDL (group 1) and group of HUVECs treated with 40 µg/ml ox-LDL (group 2). Ct values in both group 1 and group 2 were measured to calculate changes in gene expression. FASL expression was up-regulated 179.77 fold ($P<0.001$) in HUVECs treated with 40 µg/ml ox-LDL when compared to HUVEC treated with 25 µg/ml ox-LDL (Table 1).

Table 1. *FASL* gene expression change of in HUVECs after different concentration of ox-LDL treatment

Genes	HUVECs treated with 25 µg/ml ox-LDL	HUVECs treated with 40 µg/ml ox-LDL	FC	FR	P value
FASL					
Mean of Ct	25.34	16.7	179.77	Up-regulation	<0.001*
GAPDH					
Mean of Ct	28.54	27.39	1.00	1.00	Nan

HUVECs= human umbilical vein endothelial cells; ox-LDL= oxidized low-density lipoprotein; FC= fold change; FR= fold regulation; Ct/Cq= threshold cycle; HUVECs+ox-LDL:HUVECs induced with ox-LDL; *GAPDH* (control gene)= g*lyceraldehyde 3-phosphate dehydrogenase*; *:P value<0.001

4. Discussion

Death receptor proteins of apoptotic pathway have critical role in apoptosis. The pathway involving FASL is significant and widely recognized in this process (Tian et al., 2017). In vascular smooth cells, FASL pathway is involved in ox-LDL-induced apoptosis (Li et al., 2021). In HUVECs, FASL-mediated apoptosis is sensitives to ox-LDL. Induction of Fas-related mRNA expression such as FASL expression following ox-LDL treatment in HUVECs were examined previously (Imanishi et al., 2002). Cell death enzyme-linked immunosorbent assay was applied for investingating ox-LDL effect on viability of HUVECs after HUVECs had been treated with 40µg/ml ox-LDL. It was found that treatment of ox-LDL to the HUVECs did not significantly change apoptosis (Imanishi et al., 2002). In our study, we investigated effect of ox-LDL addiction to FASL gene expression in HUVECs. We detected that FASL gene expression was highly up-regulated in HUVECs treated with 40µg/ml ox-LDL. ECs are resilient to Fas-mediated cell death in response to binding of cell surface

Fas with FAS antibody. HUVECs were cultured with combinations of agonistic anti-Fas antibody in the presence or absence of the ox-LDL for evaluating whether ox-LDL induces the Fas death pathway. It was found that incubation with ox-LDL dose dependently triggered apoptosis. Then, Imanishi et al (2002) examined whether influence of the ox-LDL on Fas-induced-apoptosis was related with the change in expression of the death-regulating genes. The amounts of these genes such as FASL mRNA in HUVECs following ox-LDL treatment were evaluated via RT-Multiplex PCR (Imanishi et al., 2002). We used qPCR to assess FASL gene expression in current study. FASL expression did not significantly change incubation of HUVECs with ox-LDL for 6 hours according to the RT-Multiplex PCR analysis results (Imanishi et al., 2002). We found that FASL expression statistic significantly differed after ox-LDL treatment for 24 hours. It had been reported that FASL expression was up-regulated after treatment with ox-LDL and thereby this leads to ox-LDL-induced apoptosis (Sata and Walsh, 1998). But in other study, it was not detected that FASL expression found to be increased after treatment of the ox-LDL (Imanishi et al., 2002). A potential explanation for the difference between these two studies is the ox-LDL concentrations (150 µg/ml vs 40 µg/ml, respectively). Another study had demonstrated that a 20 µg/ml ox-LDL significantly inreased apoptosis of cultured human coronary arterial endothelial cells (Li et al., 1998). In our study, we treated HUVECs with 25 and 40 µg/ml concentrations of ox-LDL. We found that 40 µg/ml concentrations of ox-LDL caused approximately 180 fold in FASL gene expression when we compared to the group which treated 25 µg/ml ox-LDL (Table 1). This up-regulation of *FASL* gene expression may play a significant role in apoptotic process. It was also implicated that overproduction of ox-LDL triggers apoptosis (Wu et al., 2017). Ox-LDL-induced apoptosis of ECs is also related to the FASL (Sata and Walsh, 1998). Vascular ECs have ability to resist Fas-mediated apoptosis. However, high ox-LDL concentration increases Fas-mediated apoptosis of the EC (Imanishi et al., 2002). In addition, ox-LDL effects ox-LDL-induced Treg apoptosis via the FASL pathway (Li et al., 2014). Ox-LDL activated apoptosis, and the up-regulation of death pathway-related proteins, including FASL in RAW264.7 macrophages (Tian et al., 2017). Cells were cultured with 25, 50 and 100 mg/l ox-LDL for 24 hours. The protein levels of Fas/FASL pathway-related molecules were analyzed by western blot protein expression analysis. Ox-LDL considerably raises Fas/FASL pathway-related molecule level, altough pretreatment with an antagonist antibody that blocks Fas/FASL interaction inhibits ox-LDL-induced apoptosis in macrophages (Li et al., 2006). In the other study, it was shown that ox-LDL activated apoptosis and up-regulated the expression of Fas/FASL pathway-related proteins in RAW264.7 cells, although silencing of Fas by FAS siRNA suppressed ox-LDL-induced macrophage apoptosis (Tian et al., 2017). Findings of the current study generally is compatible with results of the previous studies in the literature. The effect of increased ox-LDL levels on *FASL* gene expression in HUVECs was investigated using the qPCR method and the results of this study may guide future comprehensive molecular function studies on ox-LDL-induced FASL-mediated apoptosis in ECs.

5. Conclusion

Our analysis results suggest that highly up-regulation of the *FASL* originated from increased level of the ox-LDL may regulate apoptosis of the ECs.

Author Contributions

The percentages of the authors' contributions are presented below. The authors reviewed and approved the final version of the manuscript.

	B.B.	S.A.
C	70	30
D	70	30
S	40	60
DCP	70	30
DAI	70	30
L	90	10
W	70	30
CR	70	30
SR	70	30
PM	40	60
FA	40	60

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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