Original Article
ABCA1 mRNA expression and cholesterol outflow in U937 cells

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Abstract: Objective: To investigate the oxidized low density lipoprotein (oxLDL) on U937 cell ATP-binding cassette transporter A1 (ABCA1) mRNA expression and cholesterol efflux situation. Methods: Human U937 cells were incubated with gradient concentrations of oxLDL (0, 25, 50, 75, 100, 125 mg/L), and then dyed by oil red O to estimate the content of intracelluar lipid and detect the expressing quantity of ABCA1 mRNA by Real-time Fluorescence quantitative PCR simultaneously. Calculating the cholesterol efflux rates by using the scintillation counter to detect the amount of H^3-cholesterol in each well cell culture plate and medium. Results: Real-time Fluorescence quantitative PCR analysis showed that the expression levels of ABCA1 mRNA in monocytes were lower than basal line when not intervened with oxLDL, and increased drastically with oxLDL stimulation, significant difference compared with controls ($P < 0.01$), and reached the highest level at oxLDL 50 mg/L, nevertheless, continuously increasing the concentration of oxLDL above 50 mg/L, the expression decreased. So is the outflowing rate of intracelluar lipid. Oil red O dyeing results also suggested that celluar lipid content was the highest when intervened with 125 mg/L oxLDL, and increased most obviously at 50 mg/L oxLDL. Cholesterol outflow result also demonstrated that cholesterol outflow rate related with the ABCA1 mRNA expressing quantity. Conclusion: With the increase of intervening concentration of oxLDL on U937cells, the exprssion of ABCAl mRNA represented that rising before 50 mg/L oxLDL, and then decreasing, reaching the top point at 50 mg/L oxLDL. So was the change in the outflowing rate of intracelluar lipid.

Keywords: U937 cells, oxidized low-density lipo-protein, ATP-binding cassette transporter 1, cholesterol metabolism, atherosclerosis

Introduction

Cardiovascular and cerebrovascular disease resulted from atherosclerosis (AS) which plays a critical role in human health, has the highest mortality in developed countries. Recently, however, there is also a gradual increasing morbidity with years in China. The main pathological presentation of AS is the formation of plauqe in aterial vessels. Masses of basic and clinical research show that foam cell is ralated to the occurrence and development of AS.

The formation of foam cell, which is as a result of cholesterolipid engulfed and accumulated by mono-/phagocyte and SMS, is the early stage of pathological changes of AS. The latest research demonstrates that there is a close relationship between the formation of foam cells and oxidized low density lipoprotein (oxLDL) which can be engulfed by the external receptor of monocyte and leads to the intracellular accumulation of cholesterol, thus lower the concentration of oxLDL around monocytes can alleviate the lever of AS [1].

In recent years, research found that, Tangier disease [2], a clinical manifestation of serum high density lipoprotein absence of hypercholesterolemia and hypertriglyceridemia early onset of atherosclerotic diseasein the ABCA1 gene mutations, resulting in loss of function of the ABCA1 protein coding, efflux lipid reducing, leading to intracellular lipid accumulation, the formation of atherosclerosis, a typical cell-foam cells. Research [3] also shows some ralationships between ABCA1 the outflowing transporter of intracellular free lipid and phosphorid and the formation of AS, but in foam cell formation process, in order to reverse the foam cells

showed persistent high expression of its main protein mediated lipid outflow is unclear, we conduct this research to discuss the quality-effect relationship between gradient concentration of oxLDL and the expression of ABCA1 mRNA, and analyze the relationship between cholesterol outfow and the expression of ABCA1 mRNA. In such a way, we want to get acquaint with the metabolic features of choleterol in monocyte and provide the mentality to the study of preventive strategies and the mechanism of AS.

Materials and methods

Materials

Human monocyte cell line-U937 were purchased from Shanghai Cell Biology; RPMI1640 medium were purchased from Thermo Fisher Biochemical Products Co., Ltd.; Fetal calf serum were purchased from Shanghai Biological Technology Co., Ltd. Super research; oxidized low-density lipoprotein were purchased from Beijing Association of Health Biosciences; ^{3}H labeled cholesterol were purchased from sigma company; oil red O were purchased from AMRESCO U.S. companies; DEPC reagent, reverse transcription reagents box, fluorescence quantitative kit was purchased from Beijing TransGen company; RNA extraction reagent kit was purchased from Beijing TIANGEN company; PCR primers were synthesized by Beijing three Bo Polygalaceae Biotechnology Co., Ltd. synthesis.

Cell culture

The healthy U937cells were cultured with 10% fetal calf serum RPMI-1640 medium, under the condition 37°C, 5% CO_2, and experimented at the logarithmic growing phase after subculturing.

Intervened with oxLDL

Selecting U937 cells which is growing in good condition. Then replacing the medium and continuous to culture the cells before the day of intervention. So as to make sure they were in the logarithmic phase the next day. The next day again after replacing the medium adjusted to cells. 1×10^6/L back. Dividing the experiment into parallel groups, each 6-hole, seeding the well-adjusted cells into two 6-well plates with each of the volume 500 μl. Adding 1 mg/ml of oxLDL to per Hole in each plate board in

accordance with the order, following a final concentration of 0 mg/L, 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 125 mg/L. Gently blending the two culture plates with the tip after intervention, then placing them into incubator and continuously culturing for 48 hours under the condition 37°C, 50 mL/L CO_2.

Dyed with oil red

Removing the cells intervened for 48 h. Washing the Cell samples with PBS for three times, and then droping on the glass slide with PBS suspension. Fixing with isopropyl alcohol for 1 min, staining with oil red O for 20 minutes, and then hematoxylin for 10 seconds, 10 ml/L HCl separation and re-blue, and at last, mounting with water-based mounting media. Obseving under the ordinary microscope: the intracellular lipids being red, nuclei being blue.

Detection of ABCA1 mRNA expression level

Harvesting the cells and extracting total cellular RNA with RNA extraction kit. Reverse transcriptase synthesis of cDNA after Underwenting its quantitative. reverse transcription conditions were 42°C 30 min, 85°C inactivation for 5 minutes. Again quantitating the reverse transcriptase cDNA and then underwenting quantitative PCR determination, PCR reaction conditions were denaturation at 95°C for 30 seconds; then 95°C for 30 seconds, 55°C for 15 seconds, 72°C for 10 seconds, 40 cycles. PCR primers were: ABCA1 (Primer 1): 5'-GGGTGG-TGTTCTTCCTCATTACTG-3'; ABCA1 (Primer): 5'-CCGCCTCACATCTTCATCTTCATC-3'; βActin (Primer): 5'-ATCGTGCGTGACATTAAGGAGAAG-3'; βActin (Primer 2): 5'-AGGAAGGAAGGCTGGAAGA-GTG-3'.

Ct value of Target gene mRNA expression levels were based on PCR amplification curve, β actin as an internal, using $2^{-\Delta\Delta CT}$ formula to calculate the relative amount.

Determination of cellular cholesterol efflux

1) Adding ^{3}H-cholesterol to 100 ml/L fetal calf serum RPMI-1640 medium to preparation 0.5 μCi/ml concentration.

2) Centrifuging U937 cells to remove the waste, planting the medium after suspension into three 6-hole cell culture plate. And then placing in cell incubator under the condition of 37°C, 50 ml/L CO_2 for 24 hours.
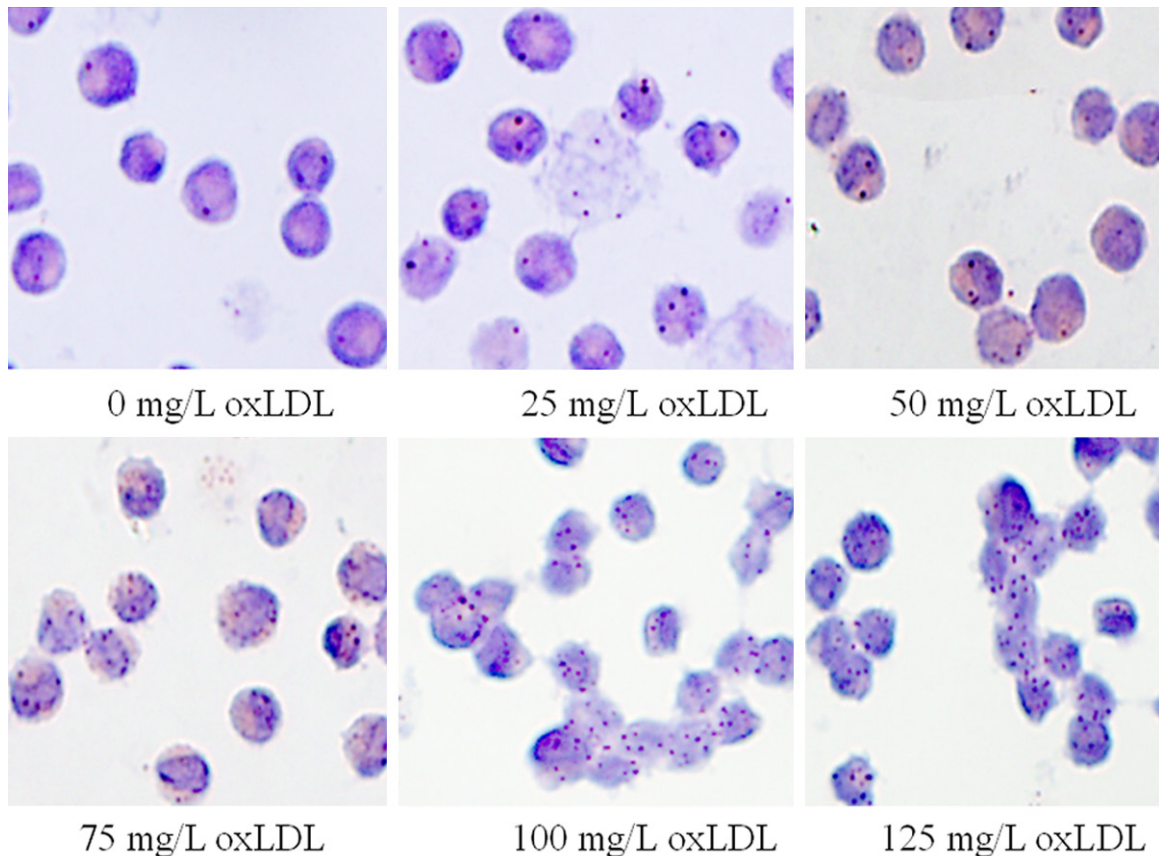
Figure 1. U937 cells were incubated with different concentrations of oxLDL and staining with oil red (10 × 20).

3) Washing the cells with culture medium that Contains 100 ml/L fetal calf serum RPMI-1640, then adding RPMI-1640 medium with 0.2% bovine serum albumin to the hole in each plate board in accordance with the order by adding oxLDL,following a final concentration of 0 mg/L, 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 125 mg/L. Then placing them in 37°C, 50 ml/L CO_2 cell incubator for 48 hours.

4) Using scintillation counter to detect the amount of ^{3}H-cholesterol in each well cell culture plate and medium.

5) Cholesterol efflux rate = CPM/total CPM × 100%.

Statistical analysis

Repeat the experiment three times befor taking the average Ct, after then calculate the target gene expression relative to housekeeping genes by calculating the difference of target gene Ct and Ct housekeeping gene, and use $2^{-\Delta\Delta CT}$ formula to calculate the relative expression of the final result, with $\bar{x} \pm s$, undergo a lin-ear correlation analysis between cholesterol efflux and ABCA1 mRNA expression. Use Statistical software SPSS18.0 to analyze variance and then make a Paired comparison. $P < 0.05$ was considered statistically significant.

Results

Getting out of U937 cells interved with oxLDL for 48 h from the culture fluid, and dyeing the cells washed twice by PBS with oil red, and then observing under the microscope, following is the result (**Figure 1**).

Expression of ABCA1 mRNA after U937 cells intervened with gradient concentration of oxLDL for 48 h

There is a significant difference ($P < 0.01$) of the expression of U937 cells ABCA1 mRNA between the cells with and without intervening with oxLDL. It was at a low lever when without the stimulation of oxLDL. When increasing the dose of oxLDL, we observed that the expression reached the peak at a 50 mg/L, compared

Table 1. Gradient oxLDL 48 h concentration training after ABCA1 mRNA expression cholesterol metabolism (n = 3, $\overline{X}$ ± s)

oxLDL density	ABCA1	β-actin	ABCA1-β-actin	$2^{-\triangle\triangle CT}$
0 (control)	21.53 ± 1.30	22.20 ± 0.31	-0.67 ± 0.92	1.00
25 mg/L	21.84 ± 0.89	22.91 ± 0.08	-1.06 ± 0.63[b]	1.31
50 mg/L	21.30 ± 1.40	23.46 ± 0.70	-2.15 ± 1.45[a,b]	2.81
75 mg/L	21.98 ± 0.45	23.09 ± 1.15	-1.16 ± 0.76[b]	1.35
100 mg/L	21.82 ± 0.54	22.73 ± 0.33	-0.91 ± 0.54[b]	1.18
125 mg/L	22.30 ± 0.64	22.57 ± 3.47	-0.27 ± 0.67	0.76

Notes: contrast with controls, [a]$P < 0.05$; with 125 mg/L [b]$P < 0.05$.

Table 2. Gradient oxLDL 48 h concentration training after cholesterol efflux (n = 3, $\overline{X}$ ± s)

oxLDL density	cholesterol efflux rate
0 (control）	7.08% ± 0.16%[b,c,d,e]
25 mg/L	10.01% ± 0.24%[a,c,d,e]
50 mg/L	16.29% ± 0.31%[a,b,d,e]
75 mg/L	11.20% ± 0.44%[a,b,c,e]
100 mg/L	8.19% ± 0.38%[a,b,c,d]
125 mg/L	6.84% ± 0.28%[b,c,d,e]

Notes: compared with the control, [a]$P < 0.01$; compared with 25 mg/L, [b]$P < 0.01$; compared with50 mg/L, [c]$P < 0.01$; compared with 75 mg/L, [d]$P < 0.01$; compared with 100 mg/L, [e]$P < 0.01$.

with the controls 2.81 times ($P < 0.05$), after then, decreased and got a much lower lever than without intervening at the 125 mg/L (**Table 1**).

Efflux rate of cholesterol

There was a certain outflow of cholesterol in oxLDL stimulated U937 cells, but the outflow rate was low (7.08% ± 0.16%). With the improving of the oxLDL concentration, the flow rate began to increase significantly, and got the peak at 50 mg/L oxLDL concentration, up to (16.29% ± 0.31%), with significant difference compared with control ($P < 0.01$). Then as the concentration of oxLDL increase its flow rate began to reduce, and reached minimum at 125 mg/L oxLDL concentration (**Table 2**).

Relation between ABCA1 mRNA expression and efflux rate of cholesterol

Pearson correlation test between ABCA1 mRNA measurement results and the cholesterol efflux rate showed that cholesterol efflux rate and ABCA1 mRNA expression correlated (r = 0.384, **Figure 2**).

Discussion

The pathological base of AS is the formation of foam cell which is the result of the lipid engufed by mono-phagocyte. The main process of AS is that oxLDL interacts with mono-cyte, then activates and up-regulates the ralated membraneous protein which mediates the phagocytosis of oxLDL. However, the mass it engulfed depends on the concentration of oxLDL encircled the cell. Intervention gradient concentration oxLDL, U937 cells, we also found with the cell the surrounding oxLDL intervention increasing concentration of intracellular lipid particles show gradual increase. That is to say in certain extent when the oxLDL concentration enhances around the cell, the lipid engulfed inside the cell is also increasing, when it accumulates more and more, beyond the tolerance, foam cells is being. We may draw a conculusion that the formation of foam cell not only has correlation with the concentration of oxLDL, but also the involved membraneous protein.

ABCA1 discovered in recent years is a kind of transmembraneous protein ralated with the formation of AS. Both clinical and animal experiment confirm that the mutation or defection of ABCAI gene leads to the decrease of serous anti-AS HDL lever, thus giving rise to the excessively intracellular lipid accumulation, which is vulnerable to AS with the classic cell-foam cell. Human Tangier disease [2, 4], main manifestation such as cardiovascular disease, an extraodinary low lever serum HDL and abnormal precipitation of intracellular cholesterol in peripheral cells, is caused by the defection of ABCAI gene. A research done by Lawn [5] mainly on patients deficient of ABCA1 gene shows that heterozygote's RCT pathway is impaired. Correspondingly some perceptible changes come into being, for instance, decreasing of serum HDL, increasing the risk to CHD, reducing outflow of intracellular cholesterol. They [4] also point out that the expression of ABCA1mRNA increasing among phagocytes in AS plaque, may be an enhanced reaction to the overload of lipid for phagocytes. We also observed in the experiment, the outflow of intracellular cholesterol was positively correlated with expression levels of ABCA1 mRNA, indicating that ABCA1 involved in cellular cholesterol efflux. AS plaque showing high expression
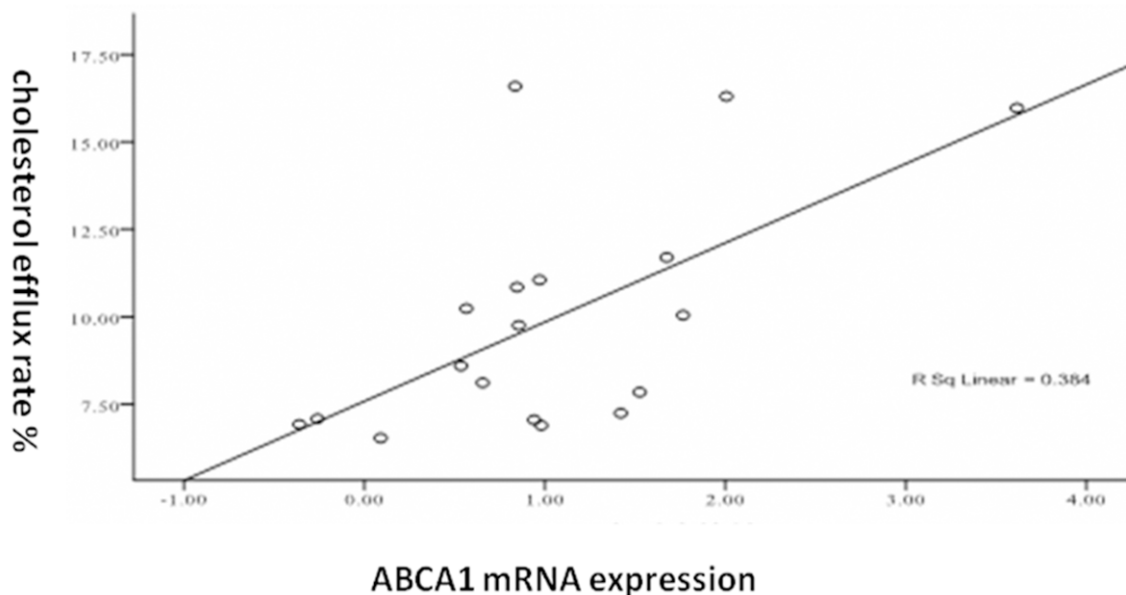
Figure 2. Relationship between ABCA1 mRNA expression and cholesterol efflux rate.

may play the role of anti-AS, but greater than the outflow of lipid within the swallow, and eventually lead to the formation of of AS.

Human ABCA1 gene, whole length 149 kb, including 50 exons and 49 introns locates at 9q31 [6]. The anti-AS function relies on ATP powering for transfering multiple intracellular substrates, especially the redundant intracellular cholesterol. Much more cholesterol could be transferred out of monocots, if gene expression increases, and this process achieved by reverse cholesterol transport RCT [7, 8]. The process [9] is: Surplus intracellular cholesterolipids are hydrolyzed into free cholesterol catalyzed by neutral cholesterolipidase, and then the free cholesterol gets out of the cell and locates on serum HDL mediated by ABCA1. Through Re-esterification, tansport and distribution, HDL-CHOL complex, is finally transported to liveror adenal gland where synthesized into bile and steroids. It is RCT that acts as the protagonist in defrosting, and decides the prognosis of foam cells, which functions as the part of anti-AS. It has been verified by ABCAl transgenosis mice [10] that overexpression of ABCAl could boost the outflow of cholesterol and inhibit the formation of AS plaque, which cuts down 65% aortic AS. Improve human nuclear/macrophage ABCA1 expression can reduce or reverse the artery AS happen?

By investigating the expression of ABCA1 mRNA located on monocytes' membrane which inter-vened by gradient concentration of oxLDL, we discovered that: when stimulation was not more than the dose 50 mg/L, it increased as the dose rised up (up to 2.81 times compared with the control at the 50 mg/L oxLDL stimulation), which was in accordance with articles that reported a peak value at 50 mg/LoxLDL stimulation. Moreover, oil red O staining results suggested that the quality of intracellular lipid is not obviously rising up when cells were at the 50 mg/L oxLDL stimulation. However, it increased significantly at the 75 mg/L oxLDL stimulation. Cholesterol efflux rate measured at the same time also suggested that cholesterol efflux rate related to the expression of ABCA1 mRNA, the highest (16.29% ± 0.31%) outflow rate happened at 50 mg/L concentration of oxLDL. Then as the concentration of oxLDL increased its outflow rate began to reduce, and reached the minimum at 125 mg/L oxLDL concentration (0.76 times the control). The results above suggested that increasing ABCA1 could inhibit formation of foam cell and ABCA1 might be the therapeutic targets for preventing and reversing AS.

Disclosure of conflict of interest

None.

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