·研究论文·

Effects of *Ginkgo biloba* extract on expressions of IL-1β, TNF-α, and IL-10 in U937 foam cells

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Abstract: This study is to investigate the protein and mRNA expressions of pro-inflammatory and anti-inflammatory cytokines in U937 foam cells and effects of *Ginkgo biloba* extract (GbE) on the cytokines. U937 cells were cultured with different concentrations of GbE (0.1, 1, and 10 μg·L^{-1}), and stimulated by 100 mg·L^{-1} oxidized low density lipoprotein (ox-LDL) for 24 h. The expressions of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) in culture solution were detected by enzyme-linked immunosorbant assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR). The results showed that incubated with 100 mg·L^{-1} ox-LDL for 24 h, the U937 cells became foam cells, the protein or mRNA expressions of IL-1β, TNF-α, IL-10, and its receptor IL-10R in U937 foam cells were higher markedly than those in normal U937 cells. When the cells were pretreated with GbE (0.1, 1, and 10 μg·L^{-1}), the increases of IL-1β and TNF-α in U937 foam cells were remarkably inhibited, but IL-10 expression increased greatly. Especially when cells were pretreated with 10 μg·L^{-1} GbE, the protein and mRNA expressions of IL-1β and TNF-α were markedly lower than those in U937 foam cells. The protein expression of IL-10 and mRNA expressions of IL-10 and its receptor IL-10R were markedly higher than those in U937 foam cells. GbE inhibited production of pro-inflammatory cytokines IL-1β and TNF-α, but up-regulated the production of anti-inflammatory cytokine IL-10 and its receptor IL-10R in U937 foam cells, which might be related with its anti-atherosclerotic actions.

Key words: U937 cell; foam cell; cytokine; interleukin-1; tumor necrosis factor-α; interleukin-10; *Ginkgo biloba*

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银杏叶提取物对 U937泡沫细胞 IL-1β、TNF-α及 IL-10表达的影响

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摘要: 本研究考察了氧化低密度脂蛋白(ox-LDL)刺激的 U937细胞致炎细胞因子白细胞介素 1β(IL-1β)、肿瘤坏死因子 α(TNF-α)、抗炎细胞因子白细胞介素 10(IL-10)及其受体(IL-10R)的蛋白及 mRNA的表达,同时观察银杏叶提取物(GbE)对它们的作用。U937细胞用 100 mg·L^{-1} ox-LDL刺激 24 h形成泡沫细胞,同时分别加入不同浓度的 GbE(0.1, 1及 10 μg·L^{-1})共孵育,采用酶联免疫吸附试验(ELISA)及逆转录聚合酶链式反应(RT-PCR)方法检测 IL-1β、TNF-α、IL-10和 IL-10R的蛋白或 mRNA表达。U937泡沫细胞组 IL-1β、TNF-α、IL-10和 IL-10R的蛋白或 mRNA的表达较对照组显著增加($P<0.01$)。GbE组 IL-1β及 TNF-α的蛋白和 mRNA的表达水平明显降低, IL-10的蛋白、IL-10和 IL-10R的 mRNA表达水平明显提高,与 U937泡沫细胞组相比差异显著($P<0.05$, $P<0.01$)。GbE对

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U937泡沫细胞致炎细胞因子 IL-1β及 TNF-α表达的显著抑制作用,对抗炎细胞因子 IL-10及其受体 IL-10R表达的显著上调作用可能是其抗 atherosclerosis(AS)的机制之一。

关键词: U937细胞; 泡沫细胞;细胞因子; 白细胞介素 1; 肿瘤坏死因子 α; 白细胞介素 10; 银杏

Atherosclerosis (AS) is not merely a disease in its own right, but a process that is the principal contributor to the pathogenesis of cardiovascular and cerebrovascular diseases. Macrophage-derived foam cells are characteristic pathological cells in the lesions of AS. During the process of AS, monocytes and oxidized low density lipoprotein (ox-LDL) seem to play the central roles[1]. Circular monocytes adhere to the subendothelial space, enter into the intima of the artery, and transform into macrophage with high expression of scavenger receptor (SR). Uptake of ox-LDL by the macrophages through SR will lead to the formation of foam cells and excretion of numerous cytokines, cell adhesion molecules, chemotactic factor, and growth factors were excreted. All these factors induce the migration and proliferation of smooth muscle cells, this process repeats until the formation of AS plaque. In the injury theory of Ross, AS can be considered to be a modified form of chronic inflammation. The recent years research results indicated that anti-inflammatory cytokine such as interleukin-10 (IL-10) also is concerned in generation and development of AS, and can play an important protective role[2,3].

Ginkgo biloba extract (GbE) has beneficial effects on cardiovascular and cerebrovascular diseases[4-7]. Previous study showed the inhibitory effects of GbE on vascular endothelial growth factor-induced permeability of bovine coronary endothelial cells[8], however, effect of GbE on expressions of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-10, and IL-10R in U937 foam cells has not been evaluated. The aim of present study is to investigate whether the anti-AS effects of GbE was related with the expressions of pro-inflammatory cytokines such as IL-1β and TNF-α, anti-inflammatory cytokines such as IL-10 and IL-10R in the U937 foam cells.

Materials and methods

Reagents GbE (containing 24% flavone glycosides and 6% gingko lactone) was presented by Prof. CHEN Wei-zhou (Shanghai Institute of Material Medica, Chinese Academy of Sciences, Shanghai, China) from Lüyuan Co. (Shanghai, China, batch No. 960705); $CuSO_4$ and ethylenediamine tetraacetic acid disodium salt were purchased from Sigma Chemical Co. (USA); human IL-1β, TNF-α, and IL-10 ELISA kit were purchased from Jingmei Co. (Shenzhen, China); Tissue/Cell Total RNA Isolation Kit was purchased from Watson Co. (Shanghai, China); TaKaRa RNA PCR kit (AMV) ver 2.1 was purchased from TaKaRa Biotechnology Co. (Dalian, China).

ox-LDL preparation Low density lipoprotein (LDL, d = 1.019 to 1.063 kg·L^{-1}, Sigma Co., USA) was sterilized by filtration through 0.45 μm millipore membrane, and stored at 4 ℃ as described previously[9,10]. After ethylenediamine tetraacetic acid disodium salt was removed by dialysis, LDL was oxidized by incubating in 10 μmol·L^{-1} $CuSO_4$ for 16 h at 37 ℃, and then dialyzed in phosphate buffer solution (PBS) containing 0.1 mmol·L^{-1} ethylene-diamine tetraacetic acid disodium salt for 24 h at 4 ℃.

Cell culture The human monocyte line U937 was obtained from the cell bank in Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. U937 cells were cultured in RPMI 1640 containing 10% fetal bovine serum at 37 ℃ in a 5% CO_2 humidified incubator, counted and diluted to 1×10^9 cells·L^{-1}. For experiments, U937 cells at the confluence in the dishes were pretreated in serum-free RPMI1640 for 24 h and the medium was treated with different concentrations of GbE (0.1, 1, and 10 μg·L^{-1}). Then incubated with 100 mg·L^{-1} ox-LDL for 24 h, the U937 cells were became foam cells. The U937 foam cells group was without GbE treatment. The control group was the normal U937 cell without GbE and ox-LDL treatment.

Oil red O dyeing The U937 foam cells were collected and fixed with 4% paraformaldehyde for 12 h. The cells were then treated with fresh 0.3% oil red O for 20 min.

Enzyme-linked immunosorbant assay (ELISA) The supernatants were collected and used for the measurement of cytokines protein quantification by ELISA as described in kit specification after the cells were centrifuged at 1 000 × g for 10 min.

Reverse transcriptase polymerase chain reaction (RT-PCR) for detection of the cytokine mRNA expressions Total RNA was isolated by

Tissue/Cell Total RNA Isolation Kit. Total RNA was quantified with the ratio of absorption values of RNA samples at 260 nm and 280 nm. RT-PCR was performed by RNA PCR kit. GAPDH was used as an internal positive control. Specific primer sequences of human GAPDH, IL-1β[11], TNF-α[12], IL-10[13], and IL-10R[14] were as follows and the size of production were 443, 384, 383, 352, and 440 bp, respectively: GAPDH sense 5′-CATCACCATCTTCCAGGAGCG-3′, antisense 5′-TGACCTTGCCCACAGCCTTG-3′; IL-1β sense 5′-AATGACAAAATACCTGTGGC-3′, antisense 5′-AAACCTTTCTGTTCCCTTTC-3′; TNF-α sense 5′-ACAAGCCTGTAGCCCATGTTGTA-3′, antisense 5′-ATTGATCTCAGCGCTGAGTCGGTCA-3′; IL-10 sense 5′-CTGAGAACCAAGACCCAGACATCAAGG-3′, antisense 5′-CAATAAGGTTTCTCAAGGGGCTGG-3′; IL-10R sense 5′-CCATCTTGCTGACAACTTCC-3′, antisense 5′-GTGTCTGATACTGTCTTGGC-3′.

The temperature profile of the amplification consisted of 45 s denaturation at 94 ℃, 45 s annealing at 50 ℃, and an extension at 72 ℃ for 2 min for 35 cycles. PCR products were sequenced to verify that desired product was amplified.

Statistical analysis Measurement data were presented as $\bar{x} \pm s$. Statistical significance in different groups was assessed by Analysis of Variance test. $P < 0.05$ was considered statistically significant.

Results

1 Morphological form of the U937 foam cells

After incubation with ox-LDL, many red pellets were found in the plasma of the U937 foam cells, which showed that U937 cells internalized ox-LDL through scavenger receptor and became foam cells (Figure 1).

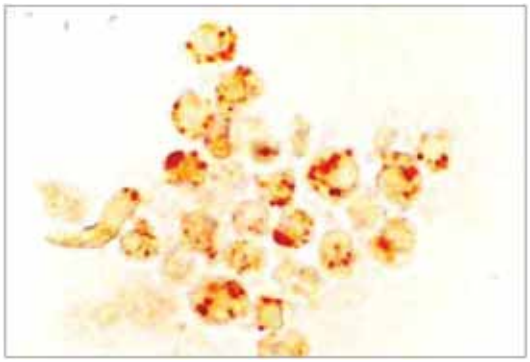

Figure 1 The morphological form of the U937 foam cells dyed with oil red O after incubation with 100 mg·L^{-1} ox-LDL for 24 h (×400)

2 Protein levels of IL-1β, TNF-α, and IL-10 in U937 foam cells

The protein levels of IL-1β, TNF-α, and IL-10 in the U937 foam cells group increased significantly as compared with those in the control group. GbE (0.1, 1, and 10 μg·L^{-1}) inhibited the protein levels of IL-1β and TNF-α, but up-regulated the protein levels of IL-10 of U937 foam cells (Table 1).

3 Expressions of IL-1β, TNF-α, IL-10, and IL-10R mRNA in U937 foam cells

RT-PCR analysis showed that IL-1β, TNF-α, IL-10 and its receptor IL-10R mRNA were all rarely expressed in the control group. The expressions of IL-1β, TNF-α, IL-10 and its receptor IL-10R mRNA were all increased markedly in the U937 foam cells group compared with those in the control group. The expressions of IL-1β and TNF-α mRNA were lower, but the expressions of IL-10 and IL-10R mRNA were higher in the GbE groups than those in the U937 foam cells group (Figure 2, Table 2).

Discussion

The macrophage-derived foam cells not only result in the formation of fatty streaks, which are believed to represent the earliest type of atherosclerotic plaque, but also excrete numerous cytokines such as IL-1β, TNF-α, IL-10, et al.

We observed that the protein or mRNA expressions of IL-1β, TNF-α, IL-10, and IL-10R were markedly higher in the U937 foam cells group than those in control group. The protein and mRNA

Table 1 Effects of GbE on the protein levels of IL-1β, TNF-α, and IL-10 in U937 foam cells

Group	IL-1β/pg·mL^{-1}	TNF-α/pg·mL^{-1}	IL-10/pg·mL^{-1}
Control	18.3 ± 0.3	22.0 ± 1.2	18.4 ± 3.1
ox-LDL	30.3 ± 0.3**	60.0 ± 0.7**	136.8 ± 1.8**
ox-LDL + GbE (0.1 μg·L^{-1})	27.3 ± 0.3△△	47.6 ± 1.2△△	179.6 ± 1.8△
ox-LDL + GbE (1 μg·L^{-1})	25.3 ± 0.3△△	39.4 ± 1.2△△	159.2 ± 3.1△△
ox-LDL + GbE (10 μg·L^{-1})	22.7 ± 0.3△△	33.6 ± 1.2△△	205.1 ± 3.1△△

$n = 3$, $\bar{x} \pm s$. ** $P < 0.01$ *vs* control group; △ $P < 0.05$, △△ $P < 0.01$ *vs* ox-LDL group

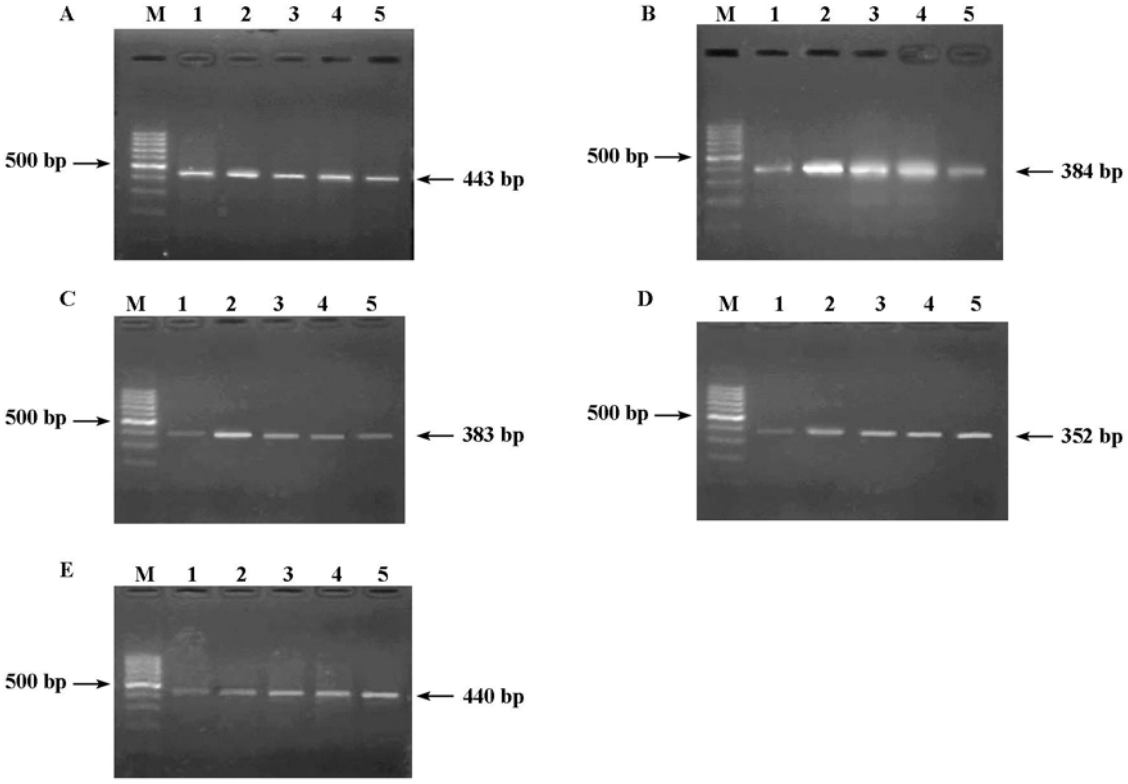

Figure 2 Effects of GbE on expressions of IL-1β, TNF-α, IL-10, and IL-10R mRNA in U937 foam cells. A: GAPDH; B: IL-1β; C: TNF-α; D: IL-10; E: IL-10R. Lane M: DNA marker; Lane 1: Control (normal U937 cells); Lane 2: ox-LDL (U937 foam cells); Lane 3: ox-LDL (U937 foam cells) + GbE (0.1 μg• L^{-1}); Lane 4: ox-LDL (U937 foam cells) + GbE (1 μg• L^{-1}); Lane 5: ox-LDL (U937 foam cells) + GbE (10 μg• L^{-1})

Table 2 Densitometric analysis of GbE on expressions of IL-1β, TNF-α, IL-10, and IL-10R mRNA in U937 foam cells

Group	IL-1β	TNF-α	IL-10	IL-10R
Control	0.53 ±0.04	0.48 ±0.02	0.54 ±0.03	0.46 ±0.03
ox-LDL	1.85 ±0.06**	0.99 ±0.06*	0.81 ±0.06*	0.62 ±0.03*
ox-LDL + GbE(0.1 μg• L^{-1})	1.72 ±0.04	0.70 ±0.04△	1.10 ±0.05△	0.73 ±0.03
ox-LDL + GbE(1 μg• L^{-1})	1.41 ±0.02△	0.55 ±0.05△△	1.05 ±0.05△	0.74 ±0.05
ox-LDL + GbE(10 μg• L^{-1})	0.80 ±0.04△△	0.43 ±0.03△△	1.34 ±0.04△△	1.37 ±0.03△△

The corrected densities with the software were shown as a ratio of GAPDH value. $n=3$, $\bar{x}\pm s$. * $P<0.05$, ** $P<0.01$ *vs* control group; △ $P<0.05$, △△ $P<0.01$ *vs* ox-LDL group

expressions of IL-1β and TNF-α were markedly lower, but the protein expressions of IL-10 and mRNA expressions of IL-10 and IL-10R were markedly higher in the GbE groups than those in the U937 foam cells group. Recent findings indicated that the serum levels of TNF-α and IL-1β in all patients with coronary heart diseases were higher than those in controls[15-17], TNF-α and IL-1β had been considered to be the marker molecules in the inflammatory response of atherosclerosis. Our results were in consistent with them.

IL-10 was an anti-inflammatory cytokine with powerful immunoregulation potency[18,19]. It was excreted from monocyte/macrophage and smooth muscle cells under certain conditions. Serum levels of the anti-inflammatory cytokine and interleukin-10 decreased in patients with unstable angina[20], this important finding also suggests that IL-10 has a protective role in atherosclerosis.

At present, GbE has widely used to treat cardiovascular and cerebrovascular diseases such as coronary heart diseases and stroke[21-24]. Our findings

showed that GbE inhibited protein and mRNA expressions of pro-inflammatory cytokine IL-1β and TNF-α, but up-regulated the protein expression of anti-inflammatory cytokine IL-10 and expressions of IL-10 and IL-10R mRNA in U937 foam cells, which might be related with its anti-AS effects.

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