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RNA interference for HIF-1α inhibits foam cells formation *in vitro*

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Abstract

Macrophage-derived foam cells in atherosclerotic lesions are generally thought to play a major role in the pathology of the disease. Hypoxia-inducible factor-1α (HIF-1α) was recently found to play an important role in atherosclerosis. Here we applied RNA interference to study the role of HIF-1α in foam cell formation *in vitro*. Transfection of HIF-1α-siRNA reduced HIF-1α synthesis as measured on mRNA and protein level by real-time RT-PCR, Western blot. It was found that RNA interference for HIF-1α with small interfering RNAs (HIF-1α-siRNA) inhibits foam cell formation by the human monoblastic cell line (U937) which was treated with oxidized low-density lipoprotein (ox-LDL) while the majority of atherosclerosis-related genes, such as cyclooxygenase-2 (COX-2), vascular cell adhesion molecule-1 (VCAM-1), interleukin-1β (IL-1β), and so on, were down-regulated, through large scale gene expression analysis using DNA microarrays. These data demonstrate that induction of HIF-1α by atherogenic factors may be a key step in coordinating the cellular events that result in atherosclerotic lesions.

Keywords: Hypoxia-inducible factor-1α; Oxidized low-density lipoprotein; Foam cell

1. Introduction

Oxidatively modified low-density lipoprotein (ox-LDL) emerged as a pathogenetic factor in atherosclerosis. There is accumulating evidence for oxidative stress in vascular dysfunction/atherogenesis and a significant contribution of macrophages in ox-LDL uptake, foam cell formation, and disease progression (Steinberg, 1997). Foam cells are characteristic pathological cells in the lesions of atherosclerosis. During the process of atherosclerosis, monocytes seem to play a central role. Once monocytes adhere to the subendothelial space and enter into the intima of the artery, ox-LDL and other substances associated with atherogenesis may participate in activation of the monocytes into macrophage. Uptake of ox-LDL by the macrophages through scavenger receptors will lead to foam cells formation (Ross, 1993).

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of α and β subunits (Wang and Semenza, 1995). Although HIF-1α is constitutively expressed in many cell types, HIF-1α presents at undetectable amounts under normoxia because of rapid proteasomeal degradation and is stabilized on hypoxia (Huang et al., 1998; Jiang et al., 1997; Salceda and Caro, 1997). HIF-1 adapts cells to low oxygen partial pressure and induces target genes which have influence on energy metabolism (Semenza et al., 1994), cell proliferation (Carmeliet et al., 1998), hematopoiesis (Lacombe and Mayeux, 1998), vascular development (remodeling, angiogenesis) (Liu et al., 1995; Rose et al., 2002), and vasotone (Kourembanas et al., 1998). The regulation of cellular oxygen concentration is disturbed, *e.g.*, in ischemic disease, cancer, and various lung diseases (Semenza, 2000, 2001, 2002a,b). Whereas in ischemic disease (*e.g.*, coronary heart disease, cerebrovascular disease) an up-regulation of HIF-1α is considered to have therapeutical benefit (Warnecke et al., 2003; Willam et al., 2002), the down-regulation of HIF-1α may be beneficial in cancer for the inhibition of tumor angiogenesis or in hypoxic-triggered pulmonary hypertension (Yu et al., 1999).

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Table 1
Primer sequences and PCR conditions for quantitative real-time PCR

mRNA	Oligonucleotide sequence (5′-3′)	Denaturing temperature and time (°C, s)	Annealing temperature and time (°C, s)	Elongation temperature and time (°C, s)	Cycles	Product size (bp)
β-actin	5′CCTGTACGCCAACACAGTGC3′ 5′ ATACTCCTGCTTGCTGATCC3′	94 (20)	58 (20)	72 (20)	35	211
ICAM-1	5′ CTCCTGTGACCAGCCCAAGT 3′ 5′ ACCTGGCAGCGTAGGGTAAG 3′	94 (20)	58 (20)	72 (20)	35	261
VCAM-1	5′GCTGCTCAGATTGGAGACTCA 3′ 5′ CGCTCAGAGGGCTGTCTATC 3′	94 (20)	58 (20)	72 (20)	40	100
IL1-β	5′CAGCTACGAATCTCCGACCAC 3′ 5′GGCAGGGAACCAGCATCTTC 3′	94 (20)	58 (20)	72 (20)	40	100
LDL-R	5′AGTTGGCTGCGTTAATGTGAC 3′ 5′TGATGGGTTCATCTGACCAGT 3′	94 (20)	58 (20)	72 (20)	40	131
COX-2	5′ TTTCGTACATTACTGCCAGCC 3′ 5′ CATGGGGAGGGGGTTTTGA 3′	94 (20)	58 (20)	72 (20)	40	105
LOX-1	5′ GCGACTCTAGGGGTCCTTTG 3′ 5′ GTGAGTTAGGTTTGCTTGCTCT 3′	94 (20)	58 (20)	72 (20)	40	111
HIF1-α	5′GCCCTAACGTGTTATCTGTCG 3′ 5′TTGCTCCATTCCATTCTGTTC 3′	94 (20)	57 (20)	72 (20)	35	245

All templates were initially denatured for 20 s at 94 °C. When using SYBR Green denaturation was done for 5 min. Amplification was done for 45 cycles.

The anoxemia theory of atherosclerosis states that an imbalance between the demand and supply of oxygen in the arterial wall is a key factor for the development of atherosclerotic lesions. Many evidences demonstrate that zones of hypoxia occur at depth in the atherosclerotic plaque that probably resulted from an impaired oxygen diffusion capacity due to the thickness of the lesion, together with high oxygen consumption by the foam cells. Thus, hypoxia actually does exist in the arterial wall *in vivo*, lending support to the anoxemia theory of atherosclerosis (Bjornheden et al., 1999; Martin et al., 1990). Recently, Vladimir et al. showed that ox-LDL provoked HIF-1α accumulation in human Mono-Mac-6 (MM6) macrophages (Shatrov et al., 2003). These results indicate that HIF-1α may play a role in foam cells formation and associate with atherosclerosis.

Suppression of HIF-1α would be a powerful tool for exploring HIF-1α dependent processes and for interfering with hypoxia-induced pathophysiological events. In essence, siRNA for HIF-1α was found to be an appropriate tool for specific interference with HIF-1α related signal transduction and hypoxia-driven downstream events (Hanze et al., 2003). In this study, an siRNA motif for RNA interference for HIF-1α was selected and applied to the human monoblastic cell line (U937). To monitor suppression of HIF-1 pathway by HIF-1α-siRNA, we conducted large scale gene expression analysis of macrophages transfected by HIF-1α-siRNA after exposure to ox-LDL.

2. Materials and methods

2.1. Cell culture

Human monoblastic cell line (U937, provided by Shanghai Institute of Cell Biology, Chinese Academy of Sciences) was grown in RPMI 1640 medium containing 10% fetal bovine serum (v/v), 0.45% glucose (w/v), 10 mM Hepes, 1 mM sodium pyruvate, 1×10^{-5} M β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Medium was replaced every 3 days during the time of culture.

2.2. HIF-1α-siRNA design and plasmid construction

The human HIF-1α cDNA sequence (Genebank accession number: NM_001530) was searched for suitable siRNA target sequences. Three targets (I: AGTTCACCTGAGCCTAATA; II: CAGTTGCCACTTCCACATA; III: TGCCACCACTGATGAATTA) were selected. DNA oligos containing the target sequence, a 9-bp loop sequence, the antisense of the target, an RNA PolyIII termination sequence, the BamH I site at one end and the Hind III site at the other end of the fragment were chemically synthesised, annealed, and inserted into the expression vector by double digestion with BamH I and Hind III and ligation with T4 DNA ligase in accordance with the manufacturer's guidelines. The ligate was transformed into competent *E. coli* DH5a cells. The correct transformant was identified by restriction enzyme analysis and DNA sequencing. GenScript siRNA Expression Vector pRNAT-U6.1/NeoDNA was used. As a control for HIF-1α-siRNA we used a corresponding random siRNA sequence (control-siRNA: TTCTCCGAACGTGTCACGT).

2.3. Cell transfection of HIF-1α-siRNA

Twenty-four hours before siRNA transfections, the U937 cells were seeded onto six-well plates, in 2 ml/well antibiotic-free RPMI 1640 medium, at a density of 2×10^{6} cells/well, corresponding to a density of 70% at the time of transfections. Two hours before transfections, ox-LDL (prepared by $CuSO_4$ oxidation of low-density lipoprotein; Sigma, Taufkirchen, Germany) was added in medium at a concentration of 40 μg/ml. The U937 cells were transfected with siRNAs at a concentration

of 4 μg/well, and were incubated with siRNAs plus Lipofection2000 in Opti-MEM or in Opti-MEM alone (control cells) for 6 h, at which point 1 ml/well fresh RPMI medium with 10% FBS was added. The siRNAs were incubated with the cells for 24 h.

2.4. Real-time quantitative reverse transcriptase polymerase chain polymerase chain reaction (real-time quantitative RT-PCR) for HIF-1α

After the U937 cells transfected, RNA was purified using Trizol and cDNA was reverse transcribed using MMLV (Promega). The expression level of HIF-1α was analyzed by real-time quantitative reverse transcriptase polymerase chain reaction using a LightCycler instrument (Eder et al., 1999; Scherr et al., 2001). The cDNA was used for real-time quantitative RT-PCR using SYBR Green (Sigma) for detection of PCR products. cDNA (1 μl) was used in a 25 μl final volume reaction containing 3U Taq DNA Polymerase, 10 × PCR buffer, 3 μl dNTP, 3 μl $MgCl_2$, 0.5 μl HIF-1α forward (5′-GCCCTA-ACGTGTTATCTGTCG-3′), 0.5 μl HIF-1α reverse (5′-TTGCT-CCATTCCATTCTGTTC-3′), and 1:25,000 dilution of SYBR Green. The HIF-1α values were normalized against human β-actin (fw: 5′-CCTGTACGCCAACACAGTGC-3′; rev: 5′-ATACTCCTGCTTGCTGATCC-3′). The efficacy of the three target sequences was evaluated and the best chosen for subsequent experiments.

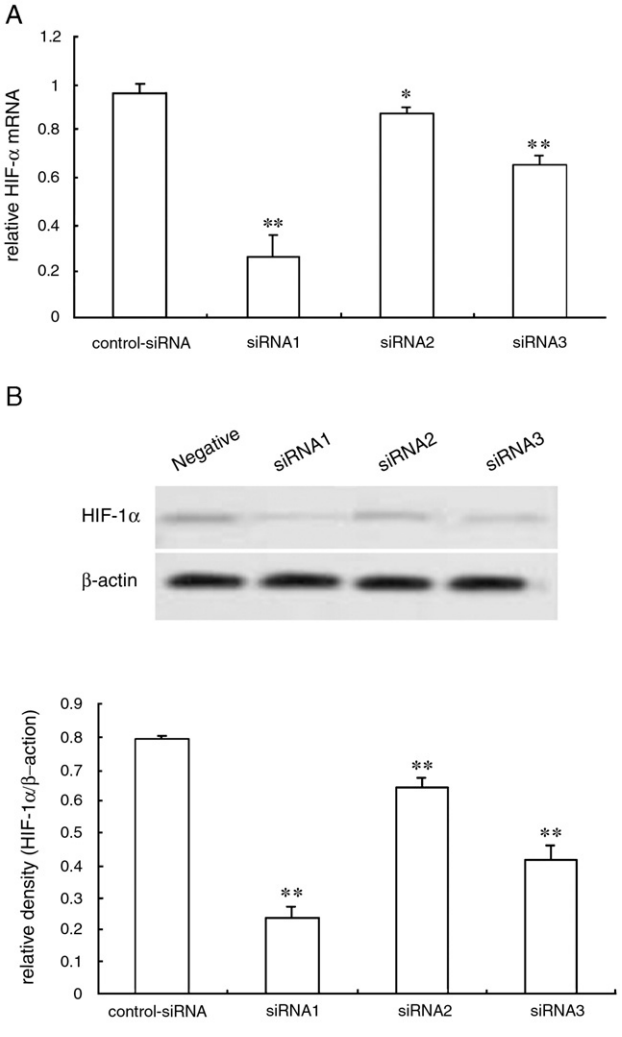

Fig. 1. HIF-1α-siRNA mediated the reduction of HIF-1α mRNA and protein expression in U937 cells in the presence of ox-LDL. (A) Relative HIF-1αmRNA quantification, related to β-actin mRNA by real-time RT-PCR. U937 cells were transfected by control-HIF-1α-siRNA or HIF-1α-siRNA (I, II or III) and cultured with ox-LDL for 24 h. (B) Top: HIF-1αWestern-blot analysis of protein extracts from U937 cells treated by control-siRNA or HIF-1α-siRNA in the presence of ox-LDL for 24 h. Bottom: densitometric quantification of HIF-1α-siRNA Western blot. These results are presented as means±S.D. of these independent experiments. *$P<0.05$ *vs.* negative; **$P<0.01$ *vs.* negative.

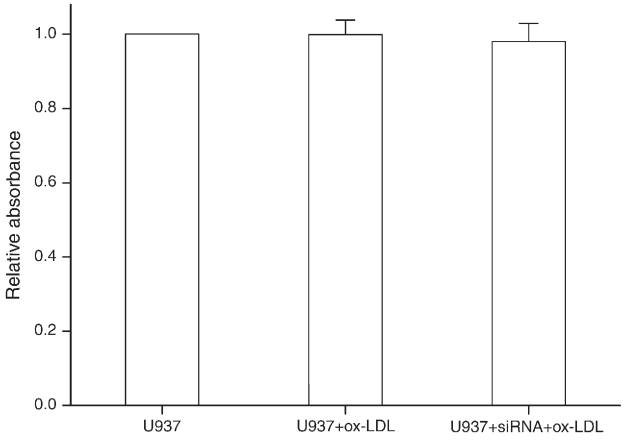

Fig. 2. Cell viability. Cell viability was assessed by the MTT assays. Cultured U937 cells were either not treated, or treated with ox-LDL alone or with ox-LDL in the presence of HIF-1α-siRNA for 24 h. The results are presented as means± S.D. of these independent experiments.

2.5. Western blotting for HIF-1α

After the U937 cells transfected, the cells were rinsed with phosphate-buffered saline (PBS) and were lysed with SDS-PAGE protein loading buffer containing 5% 2-mercaptoethanol. Cell lysates with equal amounts of total protein were then separated on 8% SDS-polyacrylamide gels with a 4% polyacrylamide stacking gel, and the proteins were transferred to nitrocellulose paper. The resulting Western blots were incubated with 5% milk for 1 h and then incubated overnight at 4 °C with a 1:1000 dilution of goat polyclonal anti-human HIF-1α antibody (Santa Cruz Biotechnology). Blots were then washed three times with 0.05% Tween-20 in TBS and incubated with 1:5000 dilution of peroxidase-conjugated rabbit anti-goat immunoglobulin for 2 h at ambient temperature. After further washing, the chemiluminescent substrate stable peroxidase and substrate luminol/enhancer solutions were added. Blots were then applied to Kodak X-Omat film, and immunoreactive proteins were visualized on the developed film.

2.6. Ox-LDL loading experiments

For ox-LDL loading experiments, the U937 cells transfected with HIF-1α-siRNAs were seeded at a density of 1×10^6 cells/ml in serum-free medium with 40 μg/ml ox-LDL for 24 h. At the

same time, the normal U937 cells were incubated with or without ox-LDL as control. After incubation, the cells were split into two parts for foam cell analysis and cDNA microarray analysis.

2.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability

After incubation for 24 h, the cell survival was quantified by the colorimetric MTT (Sigma, St. Louis, MO, USA) assay, which measures mitochondrial activity in viable cells (Hansen et al., 1989). In brief, cells were incubated with 1 mg/ml MTT for 2 h at 37 °C. MTT/formazan was extracted by overnight incubation at 37 °C with 100 μl extraction buffer [20% sodium dodecyl sulfate (SDS), 50% formamide adjusted to pH 4.7 with 0.02% acetic acid and 0.025 N HCl]. Optical densities at 570 nm were measured using extraction buffer as a blank.

2.8. Oil red O-stain analysis for foam cell formation

At defined time points, the U937 cells were fixed in phosphate buffered saline (PBS)-buffered 2% paraformaldehyde solution for 15 min, air dried then kept at −70 °C. 1% Oil red O (in 60% isopropanol) staining was done for 15 min essentially as described (Kalayoglu and Byrne, 1998). In essence, foam cells were defined as macrophages in which the entire cytoplasm was filled with Oil Red O-stainable lipid droplets.

2.9. cDNA microarray analysis for atherosclerosis-related genes

Total RNA was isolated from all the group of the U937 cells by using STAT-60 (TEL-TEST "B", INC., Friendswood, TX) and microarray analysis was carried out using Oligo Atherosclerosis Microarray Systems (SuperArray, USA) according to the instructions from manufacturer.

2.10. Real-time quantitative RT-PCR analysis for atherosclerosis-related genes

The RNA samples used in cDNA microarray analysis were used by real-time quantitative RT-PCR experiments to verify the result of cDNA microarray analysis. cDNA was amplified in 20 μl total volume. For standards curves purified amplicons were used. The sequences of the primers, the experimental conditions and the melting temperature of the products are given in Table 1. The results are analyzed in real-time on the provided program of the LightCycler as described above.

2.11. Western blotting for atherosclerosis-related genes

After the U937 cells exposure to ox-LDL/HIF-1α-siRNA, the cells were rinsed with phosphate-buffered saline (PBS) and were lysed with SDS-PAGE protein loading buffer containing 5% 2-mercaptoethanol. The process of Western-blot analysis of atherosclerosis-related genes was performed as described above. 11% SDS-polyacrylamide gels with a 5% polyacrylamide stacking gel was used. The monoclonal primary antibodies

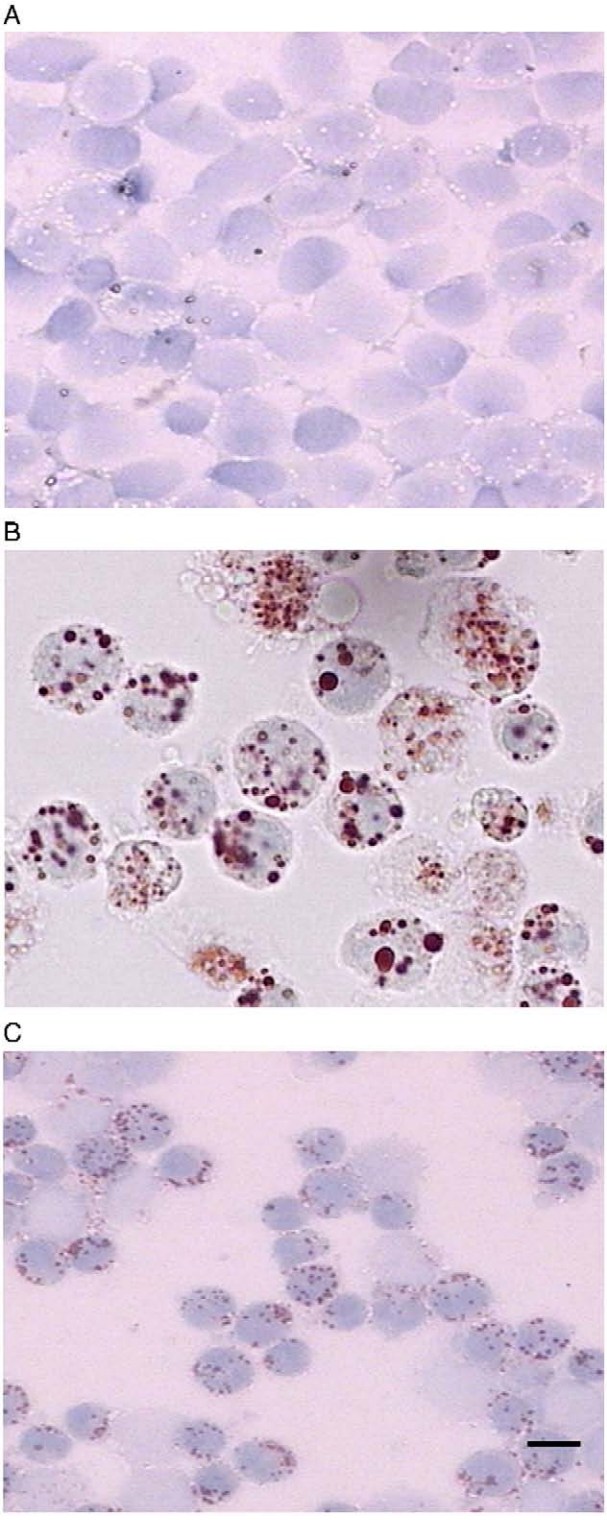

Fig. 3. HIF-1α-siRNAs inhibit foam cell formation by the U937 cells. Cells were stained for lipids with Oil Red O in parallel cultures by incubation with or without ox-LDL (40 μg/ml) at 37 °C for 24 h followed by paraformaldehyde fixation. (A) No foam cells are visible in the normal cells without ox-LDL. (B) The cells incubated with ox-LDL show large amounts of intracellular lipids as cytoplasmic droplets and the transformation of many cells into lipid-laden foam cells. (C) Significant reduction of both the number of foam cells and the number of lipid droplets per cell in HIF-1α-siRNAs-treated macrophages. Scale bar, 100 μm.

Table 2
Differential gene expression analysis identified by Oligo Atherosclerosis Microarrays

GeneBank accession number	Gene name	ox-LDL group/control group	siRNAs and ox-LDL group/ox-LDL group
NM_005502	ABC-1	1.420±0.031	0.801±0.009
NM_001122	ADFP	3.282±0.185	0.599±0.017
NM_004324	Bax	1.529±0.001	0.803±0.004
NM_000633	Bcl-2	3.060±0.147	0.988±0.014
NM_004049	BFL1	3.396±0.359	0.274±0.006
NM_138578	Bcl-x	1.989±0.070	0.648±0.019
NM_001196	Bid	2.145±0.085	0.621±0.023
NM_001165	MIHC/cIAP2	2.702±0.114	0.928±0.009
NM_002986	Eotaxin	4.524±0.223	0.962±0.001
NM_002982	MCP1/ SCYA2	3.542±0.193	0.741±0.007
NM_004591	MIP-3a/ SCYA20	3.547±0.150	0.374±0.021
NM_002985	SCYA5/ RANTES	4.571±0.179	0.939±0.014
NM_001295	MIP1aR	1.248±0.169	0.695±0.205
NM_000648	MCP-1	4.358±0.791	0.239±0.007
NM_000072	CD36	3.972±0.116	0.337±0.014
NM_000610	CD44	3.309±0.135	0.515±0.021
NM_001795	Cadherin 5	2.373±0.085	0.574±0.024
NM_003879	CASPER/ FLIP	2.481±0.149	0.535±0.030
NM_000757	M-CSF	4.857±0.296	0.510±0.011
NM_000758	GM-CSF	2.575±0.113	0.334±0.065
NM_000759	G-CSF	4.005±0.144	0.283±0.017
NM_001901	CTGF	5.461±0.896	0.237±0.017
NM_004414	DSCR1	4.768±0.421	0.928±0.008
NM_001945	HBEGF	3.117±0.644	0.361±0.015
NM_001946	MKP-3	2.280±0.083	0.527±0.034
NM_002006	FGF2	6.635±1.610	0.351±0.001
NM_002026	Fibronectin-1	4.320±0.164	0.229±0.013
NM_024610	HspB	3.089±0.118	0.332±0.024
NM_000201	ICAM-1	2.036±0.075	0.480±0.033
NM_000873	ICAM-2	3.652±0.196	0.360±0.004
NM_000874	IFNAR2	2.234±0.182	0.433±0.027
NM_000619	IFN-r	1.494±0.081	0.579±0.115
NM_002188	IL-13	5.047±0.387	0.256±0.008
NM_000576	IL-1b	4.841±0.966	0.262±0.027
NM_000877	IL1RA	4.602±0.256	0.313±0.011
NM_004633	IL-1R2	3.885±0.836	0.945±0.039
NM_016232	IL1RL1	3.159±0.055	0.416±0.009
NM_000586	IL-2	3.205±0.569	0.367±0.020
NM_000588	IL-3	2.648±0.093	0.502±0.023
NM_000600	IL-6	3.751±0.156	0.924±0.002
NM_000584	IL-8	2.129±0.089	0.457±0.037
NM_002205	Integrin a5	3.407±0.169	0.384±0.002
NM_000887	Integrin aX	2.570±0.067	0.518±0.017
NM_000211	LFA-1/CD18	2.032±0.260	0.594±0.012
NM_000212	CD61/GP3A	2.050±0.045	0.649±0.017
NM_002213	Integrin b5	3.331±0.367	0.861±0.012
NM_000889	Integrin b7	1.466±0.006	0.674±0.020
NM_016270	LKLF	2.713±0.105	0.380±0.020
NM_005559	LAMA1	2.314±0.073	0.578±0.022
NM_002309	LIF	2.273±0.331	0.491±0.010
NM_002445	SR-A	2.796±0.058	0.472±0.011
NM_005693	LXRA	2.207±0.302	0.496±0.003
NM_002543	LOX-1	3.381±0.099	0.389±0.009
NM_002608	PDGF2/SIS	2.592±0.040	0.512±0.010
NM_002609	PDGFR	1.462±0.007	0.868±0.006
NM_005036	PPARA	2.427±0.152	0.539±0.004
NM_006238	PPARD	3.382±1.042	0.316±0.191
NM_015869	PPARG	2.957±0.064	0.371±0.018
NM_000963	Cox-2	2.001±0.049	0.654±0.018
NM_000655	L-Selectin	2.236±0.037	0.584±0.016
NM_003006	PSGL1	1.509±0.007	1.078±0.049
NM_002575	PAI-2	2.883±0.120	0.560±0.022
NM_000602	PAI-1	2.702±0.084	0.785±0.001
NM_000454	Cu/ZnSOD	3.487±0.115	0.552±0.012
NM_000636	IPO-B/ MNSOD	2.828±0.097	0.351±0.021
NM_000582	Osteopontin	2.713±0.036	0.574±0.004
NM_003239	TGF b3	3.416±1.219	0.332±0.119
NM_006290	A20	3.429±0.333	0.461±0.007
NM_000043	Fas/Apo-1/ CD95	2.944±0.153	0.638±0.001
NM_001078	VCAM-1	2.696±0.076	0.592±0.001
NM_003376	VEGF	2.083±0.043	0.639±0.017

Differential gene expression caused by treatment of HIF-1α-siRNAs in the U937 cells pre-incubated with ox-LDL (40 μg/ml) for 24 h identified using the Oligo Atherosclerosis Microarrays, the normal U937 cells were incubated with or without ox-LDL as control. The alterations in mRNA expression for each gene were determined by the ratio of signal intensities of different group. The difference ratio was set as 0.7 and 1.4. The ratio above 1.4 is up-regulation and below 0.7 is down-regulation. The results are presented as means±S.D. of three individual experiments.

against ICAM-1, IL1-β, COX-2,VCAM-1, and lipoxidase-1 (LOX-1) were purchased from Santa Cruz Biotechnology. Each experiment was performed at least 3 times, and representative data are shown. Data are presented as means±S.D. The unpaired Student *t* test was used to evaluate the significance of differences between groups, accepting $P<0.05$ as the level of significance.

3. Results

3.1. Selection of HIF-1α-siRNAs

We first tested and compared the ability of the three prepared siRNAs (see Materials and methods) to reduce the level of HIF-1α mRNA in the U937 cells, which were pretreated with ox-LDL. The cells could be transfected with the three prepared siRNAs up to about 65% (data not shown). Of three target sequences of HIF-1α tested, the most efficient target was found to be I: AGTTCACCTGAGCCTAATA, which reduced HIF-1α mRNA to about 27% and reduced HIF-1α protein to about 30% at 24 h after transfection, compared to control-siRNA treated cells with ox-LDL (Fig. 1A and B).

3.2. Cell viability

Upon exposure of cells to ox-LDL or HIF-1α-siRNAs for 24 h, cell viability was measured by MTT assay (Fig. 2). There was no difference in cell viability between all groups.

3.3. Effect of HIF-1α-siRNAs on foam cells formation

Normal macrophages do not contain high levels of neutral lipids and are not colored with Oil red O, a dye specific for

neutral lipids (Fig. 3A). Incubation of the U937 cells with 40 μg/ml ox-LDL for 24 h, resulted in the occurrence of a number of foam cells filled with large cytoplasmic lipid droplets (Fig. 3B). However, transfection of the U937 cells with HIF-1α-siRNAs led to a remarkable reduction of the number macrophages transformed into foam cell (Fig. 3C). These results show that HIF-1α-siRNAs inhibit the transformation of the macrophages into foam cells.

3.4. Effect of HIF-1α-siRNAs on atherosclerosis-related genes

Next, we studied the mechanism of the HIF-1α-siRNAs inhibition of foam cells formation. For that, we investigated the effects of HIF-1α-siRNAs on atherosclerosis-related genes by the differential gene expression analysis identified by Oligo Atherosclerosis Microarrays. As shown in Table 2, seventy genes of 96 key genes involved in atherosclerosis were up-regulated by ox-LDL treatment and fifty-seven genes [such as cyclooxygenase-2 (COX-2), vascular cell adhesion molecule (VCAM-1), interleukin-1 (IL-1β), and so on] were down-regulated by HIF-1α-siRNAs pretreatment.

3.5. Real-time quantitative RT-PCR analysis for atherosclerosis-related genes

Out of the fifty-seven genes that were down-regulated by HIF-1α-siRNAs pretreatment, the expressions of 7 genes, that are well known to have important role in the development of Atherosclerosis, were analyzed for their respective abundance. As shown in Table 3, the ox-LDL-induced expression profile of intercellular adhesion molecule-1 (ICAM-1), IL1-β, LOX-1, COX-2 and VCAM-1 mRNAs was similar to that obtained after cDNA microarrays hybridization. The combined treatment of HIF-1α-siRNAs and ox-LDL reversed the effect of ox-LDL alone on the expression of most genes analyzed, confirming the results obtained from the microarrays. These findings provide strong evidence that ox-LDL loading in U937 macrophages introduces a significant imbalance in chemokine transcription under inflammatory conditions.

Table 3
Differential gene expression as measured by quantitative real-time RT-PCR

Gene name	Normal	ox-LDL treated	siRNAs and ox-LDL treated
ICAM-1	1.10E-2±1.58E-3	1.91E-2±3.27E-3[a]	1.09E-2±1.04E-3[c]
IL1-β	9.30E-3±3.11E-4	1.87E-2±1.11E-3[b]	9.98E-3±1.08E-3[d]
LOX-1	9.09E-3±3.46E-4	2.48E-2±1.75E-3[b]	1.09E-2±1.80E-3[d]
COX-2	4.51E-2±1.84E-3	9.48E-2±2.25E-3[b]	4.68E-2±1.56E-3[d]
VCAM-1	8.74E-2±3.61E-3	1.83E-1±1.47E-2[b]	9.27E-2±2.07E-3[d]
LDL-R	2.49E-3±3.66E-4	3.56E-3±3.43E-4[a]	2.94E-3±3.74E-4
HIF1-α	2.25E-4±3.10E-5	6.45E-4±2.02E-5[b]	1.03E-4±2.04E-5[d]

The amount of each mRNA was obtained by quantitative real-time RT-PCR. The amount of each product was normalized to the housekeeping gene, β-actin. The results are represented as means±S.D. of three individual experiments. [a]$P<0.05$, [b]$P<0.01$ *vs.* normal U937 cells; [c]$P<0.05$, [d]$P<0.01$ *vs.* ox-LDL treated control.

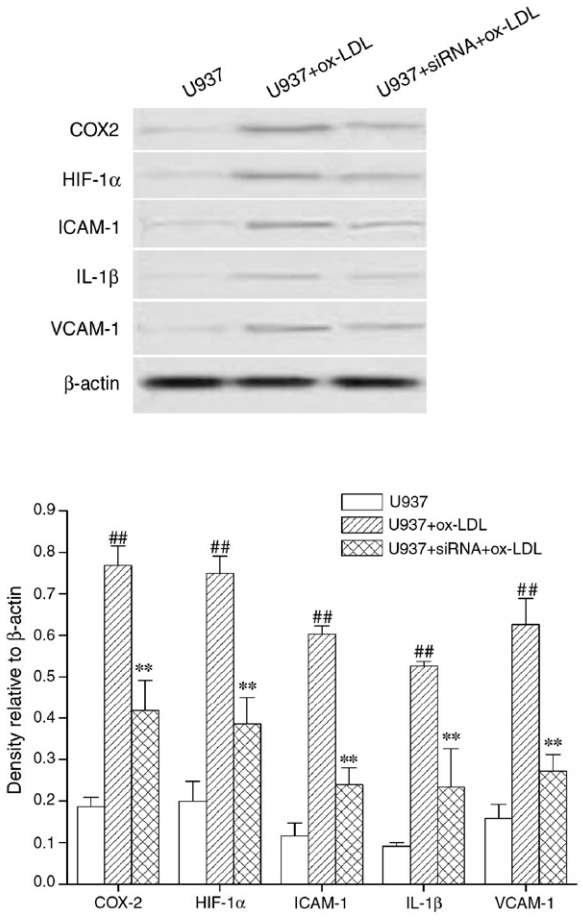

Fig. 4. Protein expression of HIF-1α, COX2, ICAM-1, IL-1β, VCAM-1. Western-blot analysis of protein extracts from U937 cells treated with ox-LDL alone or with ox-LDL in the presence of HIF-1α-siRNA for 24 h. Bottom: densitometric quantification of HIF-1α, COX2, ICAM-1, IL-1β, VCAM-1 Western blot. These results are presented as means±S.D. of these independent experiments. ##$P<0.01$ *vs.* U937; **$P<0.01$ *vs.* U937+ox-LDL.

3.6. West-blot analysis for atherosclerosis-related genes

Exposure of U937 cells to ox-LDL for 24 h resulted in a significant increase in HIF-1α, COX2, ICAM-1, IL-1β, VCAM-1 protein expression ($P<0.01$ U937+ox-LDL *vs.* U937, Fig. 4). The combined treatment of HIF-1α-siRNAs and ox-LDL reversed the effect of ox-LDL alone, decrease the protein expression of HIF-1α, COX2, ICAM-1, IL-1β, VCAM-1 ($P<0.01$U937+HIF-1α-siRNAs+ox-LDL *vs.* U937+ox-LDL, Fig. 4).

4. Discussion

Atherosclerosis is a multifactorial disease, where more than one mechanism, along more than one step, contributes to macrophage cholesterol accumulation and foam cell formation. The appearance of lipid-laden foam cells is one of the hallmarks of fatty streaks and atherosclerotic plaques. Macrophages express several scavenger receptors that are capable of taking up ox-LDL, and mediate the massive accumulation of cholesterol

characteristic of macrophage foam cells (Linton and Fazio, 2001). The hypoxia-inducible factor-1 (HIF-1), the most important factor involved in the cellular response to hypoxia, a local tissue decrease in the oxygen concentration, has been extensively studied during the last decade. HIF-1α accumulations triggered by ox-LDL have been detected in patients with atherosclerosis, but the role of these accumulations in the pathophysiology of atherosclerosis has not been determined. Macrophages are central for both inflammation and lipid deposition during atherogenesis.

We performed a study to investigate the involvement of HIF-1α in macrophage foam cell formation. Our data clearly show that HIF-1α-siRNAs blocks the development of macrophage-derived foam cells with ox-LDL by inhibiting expression of HIF-1α. This strongly suggests that under atherogenic conditions, the high expression of macrophage HIF-1α promotes foam cell formation and atherosclerosis.

The recently developed DNA microarray technology provides a powerful and efficient tool to rapidly compare the differential expression of a large number of genes. Using the DNA microarray approach, we investigated gene expression profiles in cultured human U937 cells transfected by HIF-1α-siRNA in response to 24 h of exposure to ox-LDL. Our results indicate that HIF-1α-siRNAs inhibit the increase of the expression of the majority of atherosclerosis-related genes induced by ox-LDL. It is well established that HIF-1α plays a major role in vascular endothelial growth factor (VEGF) expression and angiogenesis (Richard et al., 2000) with the notion that VEGF mediates important alterations associated with atherogenesis and the angiogenic activity of macrophages (Xiong et al., 1998). In line with these findings, RNA interference for HIF-1α attenuated the VEGF expression in this study. Recent advances in basic science have established a fundamental role for inflammation in mediating all stages of this disease from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis. The general increase in IL-1β, COX-2, ICAM-1 and VCAM-1 induced by ox-LDL in U937 cells, confirms the concept of inflammation in atherosclerosis (Libby et al., 2002).

Taken together, our observations demonstrate that the induction of HIF-1α by atherogenic factors may be a key step in coordinating the cellular events that result in atherosclerotic lesions by linking inflammation and atherosclerosis through the mechanism of induction the expression of the majority of atherosclerosis-related genes.

It is noteworthy that recently a series of molecules and molecular pathways emerged that contribute to the pathogenesis and progression of both atherosclerosis and cancer (Ross et al., 2001; Toi et al., 2002). HIF-1 may be added to that list, considering the important tumor angiogenic role of HIF-1α.

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