Gene Expression Profile in Interleukin-4–Stimulated Human Vascular Endothelial Cells

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Interleukin-4 (IL-4)–mediated pro-oxidative and pro-inflammatory vascular environments have been implicated in the pathogenesis of atherosclerosis. The cellular and molecular regulatory mechanisms underlying this process, however, are not fully understood. In the present study, we employed GeneChip microarray analysis to investigate global gene expression patterns in human vascular endothelial cells after treatment with IL-4. Our results showed that mRNA levels of a total of 106 genes were significantly up-regulated and 41 genes significantly down-regulated with more than a 2-fold change. The majority of these genes are critically involved in the regulation of inflammatory responses, apoptosis, signal transduction, transcription factors, and metabolism; functions of the remaining genes are unknown. The changes in gene expression of selected genes related to inflammatory reactions, such as vascular cell adhesion molecule-1 (VCAM-1), E-selectin, monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6), were verified by quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) analyses. IL-4 treatment also significantly increased the adherence of inflammatory cells to endothelial cell monolayers in a dose-dependent manner. These results may help determine the molecular mechanisms of action of IL-4 in human vascular endothelium. In addition, a better understanding of IL-4–induced vascular injury at the level of gene expression could lead to the identification of new therapeutic strategies for atherosclerosis.

INTRODUCTION

Inflammatory responses elicited by a variety of stimuli in the vascular endothelium have been implicated in the development of cardiovascular disease. It is now widely believed that atherosclerosis is an inflammatory disease of the vessel wall, and inflammatory reactions in endothelial cells are primarily regulated through the production of inflammatory mediators and their close interactions (1). In fact, enhanced expressions of adhesion molecules, chemokines, and pro-inflammatory cytokines in vascular endothelial cells facilitate recruiting and adhering of inflammatory cells, such as lymphocytes and monocytes/macrophages, into the vessel wall, and thus stimulate transendothelial migration, which can be considered an early atherogenic process (2–5). These studies strongly support the idea that an inflammatory environment in the vascular endothelium is critical for the initiation and development of atherosclerosis.

Interleukin-4 (IL-4) is a pleiotropic immunomodulatory cytokine secreted by T-helper 2 lymphocytes, eosinophils, and mast cells (6,7). IL-4 is present at high levels in tissues of patients with chronic inflammatory diseases, where it may play a critical role in the disease progression. Indeed, elevated levels of IL-4 were detected in atherosclerotic lesions (8). Additionally, a growing body of evidence indicates that IL-4 may play a role in atherogenesis through induction of inflammatory responses, such as up-regulation of vascular cell adhesion molecule-1 (VCAM-1) (9,10) and monocyte chemoattractant protein-1 (MCP-1) (11,12). IL-4 may also be considered as a pro-oxidative cytokine, which can increase the oxidative potential of target cells (10,13,14).

It has been proposed that the IL-4–mediated overexpression of inflammatory mediators is regulated at the transcriptional level through activation of a variety of redox-responsive transcription factors. For example, we have shown that IL-4–induced oxidative stress up-regulates the expression of VCAM-1 and MCP-1 genes via activation of Sp-1 and signal transducers and activators of transcription, respectively (10,12). Although previous studies have established the potential role of IL-4 in the development of cardiovascular disease, the cellular and molecular regulatory mechanisms underlying this process are not fully understood. In the present study, GeneChip microarray analysis was conducted to investigate the global gene expression changes of IL-4–treated human vascular endothelial cells using the Affymetrix GeneChip® Human Genome U133A Arrays. In addition, we also employed the quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) to confirm changes in the levels of expression of selective genes of interest. We found that IL-4 significantly regulates the expression of genes known to be involved in inflammation, apoptosis, signal transduction, and transcription factors.

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MATERIALS AND METHODS

Cell Cultures

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously (15). HUVEC were cultured in enriched M199 medium supplemented with 20% fetal calf serum, 1% each of penicillin/streptomycin, glutamine, and antibiotic-antimycotic, heparin (300 µg/mL; Gibco BRL, Grand Island, NY, USA), HEPES (6 mg/mL; Sigma Chemical, St. Louis, MO, USA), and endothelial cell growth supplement (40 µg/mL; Collaborative Research, Bedford, MA, USA) in 5% CO_2 at 37 °C. Cells were determined to be endothelial by their cobblestone morphology and uptake of fluorescent-labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate; Molecular Probes Inc., Eugene, OR, USA). HUVEC from passage 2 were used in all experiments. The human monocytic leukemia cells (THP-1) were purchased from American Type Culture Collection (Manassas, VA, USA) and used to study cell adhesion assay. THP-1 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 25 mM glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 50 µM 2-mercaptoethanol, and 1% each of penicillin/ streptomycin in 5% CO_2 at 37 °C.

GeneChip Microarray Analysis

Microarray gene expression analysis was performed using the Affymetrix GeneChip System with Human Genome U133A Arrays (Affymetrix Inc, Santa Clara, CA, USA).

RNA isolation and GeneChip microarray processing. HUVEC were either untreated or treated with 10 ng/mL of IL-4 for 4 h. Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. The labeling of RNA samples, human GeneChip (HG-U133A) hybridization, and array scanning were carried out as described earlier (16,17) and according to the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, an average yield of 40 µg of biotin-labeled cRNA target was obtained from 5 µg of total RNA from each sample, of which 20 µg of cRNA was applied to 1 gene chip. The hybridization was run overnight in a rotating oven (Affymetrix GeneChip Hybridization Oven 640) at 45 °C. The chips were then washed and stained on a fluidics station (Affymetrix GeneChip Fluidics Station 400), and scanned at a resolution of 3 µm in a confocal scanner (Affymetrix GeneArray Scanner).

Microarray data analysis. The gene expression levels of samples were analyzed using the Affymetrix Microarray Suite software according to the manufacturer's recommendation. All data presented in Tables 1 and 2 show the mean fold change in gene expression from 3 independent experiments in IL-4–treated HUVEC compared with untreated control cell cultures. All genes presented were significantly changed ($P < 0.05$) and the mean fold minimum change chosen for presentation was 2.0.

Real-time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Quantitative real-time RT-PCR, also known as fluorescence-based kinetic RT-PCR, was employed to confirm specific gene expression changes detected by the GeneChip analysis. The fluorogenic 5′ nuclease assay technology using TaqMan® probes was used to ensure specificity and sensitivity. Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen) according to the protocol of the manufacturer. Then, 1 µg of total RNA was reverse-transcribed at 25 °C for 15 min, 42 °C for 45 min, and 99 °C for 5 min in 20 µL of 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/µL of recombinant RNasin ribonuclease inhibitor, 15 units/µg of AMV reverse transcriptase, and 0.5 µg of random hexamers. For quantitative PCR, amplifications of individual genes were performed on ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix, gene-specific TaqMan PCR probes and primers, and a standard thermal cycler protocol (50 °C for 2 min before the 1st cycle, 95 °C for 15 s, and 60 °C for 1 min, repeated 45 times). For specific probes and primers of PCR amplifications, Assay-on-Demand™ Products for human VCAM-1 and E-selectin, and TaqMan Pre-Developed Assay Reagents for human MCP-1, IL-6, and β-actin, were obtained from Applied Biosystems. The threshold cycle (C_T), which indicates the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold, from each well was determined using ABI Prism 7000 SDS software. Relative quantification, which represents the change in gene expression from real-time quantitative PCR experiments between IL-4–treated group and untreated control group, was calculated by the comparative C_T method as described earlier (18,19). The data were analyzed using equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = [C_T \text{ of target gene} - C_T \text{ of housekeeping gene}]_{\text{treated group}} - [C_T \text{ of target gene} - C_T \text{ of housekeeping gene}]_{\text{untreated control group}}$. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ represents the fold change in gene expression, normalized to a housekeeping gene (β-actin) and relative to the untreated control.

Enzyme-linked Immunosorbent Assay (ELISA)

Cell surface expression levels of adhesion molecules such as VCAM-1 and E-selectin were quantified by ELISA Development kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's procedure, with modifications. Briefly, HUVEC monolayers were incubated with either anti-human VACM-1 or E-selectin monoclonal antibody (2.5 µg/mL) for 1 h at 37 °C. The cells were then incubated with biotinylated goat antimouse IgG antibody (1:1,000 dilution) for 1 h at 37 °C. After washing the wells thoroughly, the working dilution of Streptavidin-HRP was added to each well and incubated for 20 min at room temperature. The cells were incubated with HRP Substrate Solution for 20 min at room temperature with subsequent addition of Stop Solution. After color development, absorbance from each well was measured by a microtiter plate reader at 450 nm to 570 nm.

The protein levels of human MCP-1 and IL-6 in cell culture supernatants were determined using Human MCP-1 Immunoassay and Human IL-6 Immunoassay kits (R&D Systems) according to the protocol of the manufacturer, respectively. This assay employs the quantitative sandwich enzyme immunoassay technique using a murine monoclonal antibody against human MCP-1 or IL-6, and a polyclonal secondary antibody conjugated

with horseradish peroxidase. The minimum detectable concentration of MCP-1 and IL-6 was less than 5.0 and 0.70 pg/mL, respectively.

Cell Adhesion Assay

Adhesion studies were performed with the human monocytic leukemia cell line, THP-1, as previously described (20) with modifications (21). Briefly, HUVEC were grown to confluence on 24-well plates and exposed to IL-4 for 8 and 24 h. Prior to the cell-cell adhesion assay, the HUVEC monolayers were washed twice with Hank's Balanced Salt Solution (HBSS) and then washed with M199 medium containing 10% fetal bovine serum. Calcein acetoxymethyl ester (calcein AM; Calbiochem, La Jolla, CA, USA) was employed to label THP-1 cells. The fluorescence labeling of THP-1 cells was achieved by incubating cells (2.5×10^5 cells/mL) with 5 μg/mL of calcein AM. After loading of calcein AM for 20 min at 37 °C, the cells were washed 3 times with HBSS, and then washed with M199 medium containing 10% fetal bovine serum. The calcein AM–labeled THP-1 cells were added onto the HUVEC monolayers and incubated for 20 min at 37 °C. The non-adherent THP-1 cells were removed from monolayers by washing each well 3 times with HBSS. The fluorescence intensity was measured by a fluorescence plate reader using excitation of 490 nm and emission of 517 nm.

Statistical Analysis

Routine statistical analysis of data was completed using SigmaStat 2.03 (SPSS, Chicago, IL, USA). Statistical probability of $P < 0.05$ was considered significant.

RESULTS

IL-4 Up-regulates Adhesion of Leukocytes to Human Vascular Endothelial Cell Monolayers

The adherence of human acute monocytic leukemia cells, THP-1, to HUVEC monolayers was determined to verify the functional integrity of inflammatory mediators up-regulated by human vascular endothelial cells after stimulation with IL-4. Following an 8- to 24-h incubation with IL-4 doses ranging from 0.1 to 10 ng/mL, endothelial cell function was significantly and dose-dependently altered as assessed by changes in THP-1 adherence to the HUVEC monolayer (Figure 1). Hence, endothelial cells were exposed to 10 ng/mL of IL-4 for 4 h and changes in gene expression were assessed using microarray analysis.

Identification of Global Gene Expression Changes in IL-4–Treated Human Vascular Endothelial Cells

The gene expression profile of human vascular endothelial cells treated with IL-4 was assessed using microarray technology with the Affymetrix GeneChip Human Genome U133A Arrays, which contain more than 22000 human genes. As shown in Table 1, 106 genes were significantly up-regulated at the mRNA level with more than a 2-fold change in HUVEC after treatment with IL-4 for 4 h. Classification by function revealed that IL-4 treatment up-regulated genes mainly responsible for inflammatory reactions, apoptosis, signal transduction, and transcription factors. Among

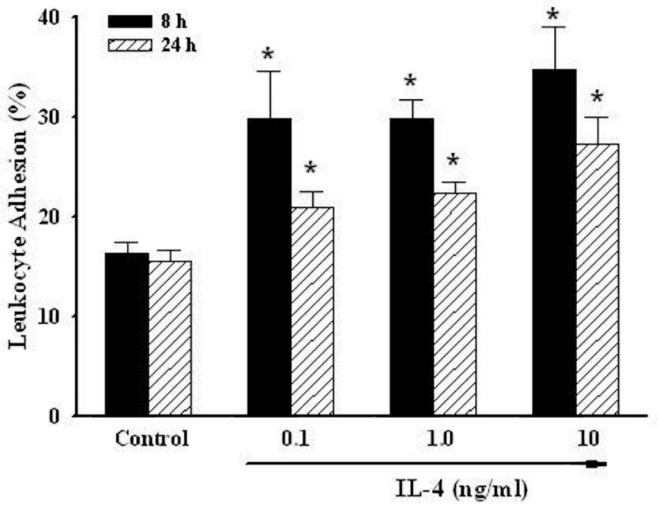

Figure 1. IL-4 up-regulates the adhesion of leukocytes to human vascular endothelial cell monolayers. HUVEC were either untreated or treated with the indicated concentrations of IL-4 (0.1, 1.0, and 10 ng/mL) for up to 24 h. The adherence of calcein AM–labeled THP-1 cells was measured by fluorescent microplate reader using excitation of 490 nm and emission of 517 nm. Data are means ± SD of 4 determinations. *Statistically significant compared with the control group ($P < 0.05$).

these, mRNA levels of the inflammatory mediators, such as adhesion molecules (VCAM-1 and E-selectin), chemokine (MCP-1), and pro-inflammatory cytokine (IL-6), were markedly and significantly induced, suggesting that IL-4 can play a crucial role in the pro-inflammatory pathways in human vascular endothelium. The expression of 2 housekeeping genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase, was not affected with IL-4 treatment. In addition, exposure of HUVEC to IL-4 resulted in a significant down-regulation of 41 genes by at least 2-fold factor, as compared with untreated control cell cultures (Table 2).

Verification of Microarray Analysis Using Real-Time RT-PCR and ELISA

To validate the changes in gene expression of IL-4-treated human vascular endothelial cells observed in microarray analysis, we performed quantitative real-time RT-PCR for several target genes that were up-regulated in HUVEC treated with IL-4. In the present study, we selected 4 inflammatory genes encoding VCAM-1, E-selectin, MCP-1, and IL-6. Real-time RT-PCR showed that increasing concentrations of IL-4 dramatically induced mRNA expression of adhesion molecules, such as VCAM-1 and E-selectin (Figures 2A and 2B). A significant and dose-dependent induction of chemokine MCP-1 gene was also observed in HUVEC treated with IL-4 (see Figure 2C). Additionally, IL-4 treatment markedly up-regulates gene expression of pro-inflammatory cytokine IL-6 (see Figure 2D). These results confirm that up-regulation of selected genes identified by microarray analysis correlates with mRNA expression measured by real-time RT-PCR. In parallel with gene expression analyses, a series of ELISA was conducted to determine whether IL-4–induced increases in mRNA levels could translate to elevated protein expression. Consistent with

Table 1. Up-regulation of specific gene expression in human vascular endothelial cells treated with interleukin-4

Gene symbol	NCBI accession nr	Fold change	*P* value	Description
Adhesion molecules				
VCAM1	NM_001078	21.7	<0.001	Vascular cell adhesion molecule 1
CSPG2	D32039	5.4	0.006	Chondroitin sulfate proteoglycan 2
SELE	NM_000450	4.3	<0.001	E-selectin
AIM1	U83115	4.1	<0.001	Absent in melanoma 1
AGC1	X17406	3.2	0.002	Aggrecan 1
PCDH7	NM_002589	2.7	<0.001	BH-protocadherin
FCN3	NM_003665	2.2	0.023	Ficolin (collage/fibrinogen domain) 3
FAT	NM_005245	2.0	0.007	FAT tumor suppressor homolog 1
Chemokines and cytokines				
CCL2	S69738	8.2	<0.001	Monocyte chemoattractant protein-1
IL6	NM_000600	2.0	0.003	Interleukin-6
Apoptosis				
PAWR	NM_002583	2.6	0.001	PRKC, apoptosis, WT1, regulator
CASP3	NM_004346	2.5	0.008	Caspase 3
CASP2	AF314174	2.0	0.028	Caspase 2
Signal transduction				
PMCH	NM_002674	331.2	<0.001	Promelanin–concentrating hormone
PIK3CG	AF327656	9.9	0.013	Phosphoinositide-3-kinase, catalytic, γ
SOCS1	AB005043	5.8	<0.001	Suppressor of cytokine signaling 1
LIFR	NM_002310	5.7	<0.001	Leukemia inhibitory factor receptor
BMP4	D30751	3.4	<0.001	Bone morphogenetic protein 4
CSF2RB	AV756141	3.2	<0.001	Colony stimulating factor 2 receptor β
KIAA0551	AF172268	3.2	<0.001	Traf2 and NCK interacting kinase
RGS2	NM_002923	3.1	<0.001	Regulator of G-protein signaling 2
INHBA	M13436	3.0	<0.001	Inhibin βA
MET	BG170541	2.7	0.003	Hepatocyte growth factor receptor
RICS	NM_014715	2.3	0.045	Rho GTPase-activating protein
H11	AF133207	2.2	0.002	Protein kinase H11
BMP2	NM_001200	2.2	<0.001	Bone morphogenetic protein 2
GUCY1B3	W93728	2.1	0.010	Guanylate cyclase 1, soluble, β3
EXT1	NM_000127	2.0	<0.001	Exostoses (multiple) 1
ARL7	NM_005737	2.0	0.005	ADP-ribosylation factor-like 7
Transcription factors				
CREM	D14826	8.5	0.004	cAMP responsive element modulator
PKNOX2	AK023792	6.2	0.001	PBX/knotted 1 homeobox 2
MAD	NM_002357	5.4	<0.001	MAX dimerization protein 1
FOXC1	AU145890	4.6	0.011	Forkhead box C1
CITED2	NM_006079	4.2	<0.001	Cbp/p300-interacting transactivator
IRLB	BE268538	4.1	<0.001	c-Myc promoter-binding protein
CEBPB	AL564683	2.9	<0.001	CCAAT/enhancer binding protein β
POU4F1	NM_006237	2.6	0.005	POU domain, class 4, transcription factor 1
GATA6	D87811	2.3	0.018	GATA binding protein 6
KIAA0146	NM_005195	2.3	<0.001	CCAAT enhancer binding protein (CEBP)
SSRP2	NM_012446	2.2	0.003	Single-stranded DNA binding protein 2
ELL2	NM_012081	2.1	0.022	ELL-related RNA polymerase II, elongation factor
KLF4	BF514079	2.1	0.011	Kruppel-like factor 4
TRAP95	NM_005481	2.0	0.038	Thyroid hormone receptor-associated protein, 95-kD subunit
CART1	NM_006982	2.0	0.024	Cartilage paired-class homeoprotein 1
TOX	AI961231	2.0	0.009	Thymus high mobility group box protein

Continued

TABLE 1—*Continued*

Others				
MCHP	S64288	204.2	<0.001	Melanin-concentrating hormone precursor
HS3ST1	BF000296	10.7	0.002	Heparan sulfate 3-O-sulfotransferase 1
CDC45L	NM_003504	7.4	0.003	CDC45 cell division cycle 45-like
SIAT8A	L32867	6.5	<0.001	Sialyltransferase 8A
TMOD1	NM_003275	5.9	0.004	Tropomodulin 1
MTHFR	AJ249275	5.7	0.009	5,10–Methylenetetrahydrofolate reductase
ENPP1	NM_006208	5.2	0.004	Ectonucleotide pyrophosphatase/phosphodiesterase 1
LOX	NM_002317	5.1	<0.001	Lysyl oxidase
AMIGO4	AC004010	4.2	<0.001	Amphoterin induced gene 2
SULF1	AW043713	4.6	<0.001	Sulfatase 1
GJA5	NM_005266	4.3	<0.001	Gap junction protein (Connexin 40)
ARK5	NM_014840	3.9	<0.001	KIAA0537 gene product
FKBP5	NM_004117	3.8	0.002	FK506 binding protein 5
LRRTM2	NM_015564	3.8	0.017	Leucine-rich repeat transmembrane neuronal 2 protein
COVA1	NM_006375	3.6	0.014	Cytosolic ovarian carcinoma antigen 1
DACT1	NM_016651	3.6	<0.001	Dapper homolog 1, antagonist of β-catenin
DMD	NM_004010	3.6	<0.001	Dystrophin
LRRN3	AI221950	3.5	0.006	Leucine rich repeat neuronal 3
PTX3	NM_002852	3.5	<0.001	Pentaxin-related gene
CLCN4	AA071195	3.5	0.006	Chloride channel 4
SLC38A1	NM_030674	3.5	<0.001	Amino acid transporter system A1
FLJ11743	NM_024527	3.5	0.011	Hypothetical protein FLJ11743
OSPBL11	NM_022776	3.4	<0.001	Oxysterol binding protein-like 11
CH25H	NM_003956	3.2	0.026	Cholesterol 25-hydroxylase
FBN1	AI264196	2.8	0.003	Fibrillin 1 (Marfan syndrome)
ELAVL2	NM_004432	2.7	<0.001	ELAV (embryonic lethal, abnormal vision)-like 2
CPT2	M58581	2.7	0.020	Carnitine palmitoyltransferase II
NNMT	NM_006169	2.5	<0.001	Nicotinamide *N*-methyltransferase
UP	NM_003364	2.5	<0.001	Uridine phosphorylase
KCNK3	NM_002246	2.5	0.022	Potassium channel, subfamily K, member 3
DAAM1	AK021890	2.5	<0.001	Dishevelled associated activator of morphogenesis 1
SLC22A4	NM_003059	2.4	<0.001	Solute carrier family 22, member 4
PSCD1	NM_004762	2.4	<0.001	Cytohesin 1
JAG1	NM_000214	2.4	0.024	Jagged 1 (Alagille syndrome)
EGLN3	NM_022073	2.4	0.007	Egl 9 homolog 3
TPK1	NM_022445	2.3	0.005	Thiamin pyrophosphokinase 1
LOC169611	AL050002	2.3	0.004	Hypothetical protein LOC169611
PVRL3	AA129716	2.3	0.004	Poliovirus receptor-related 3
DOK5	AL050069	2.2	0.002	Docking protein 5
MRF2	BG285011	2.2	<0.001	Modulator recognition factor 2
DNAJC3	NM_006260	2.2	0.034	DnaJ (Hsp40) homolog, subfamily C, 3
CPR8	AK022459	2.2	0.005	Cell cycle progression 8 protein
SIAT1	AV695711	2.1	0.002	Sialyltransferase 1
RDX	NM_002906	2.1	0.024	Radixin
CYP1B1	NM_000104	2.0	0.010	Cytochrome P450 1B1
ALDH1A2	NM_003888	2.0	0.002	Aldehyde dehydrogenase1A2
GCNT1	NM_001490	2.0	<0.001	β-1,6-*N*-acetylglucosaminyltransferase
Unknown				
DKK2	NM_014421	26.5	<0.001	Dickkopf homolog 2
T12479	AW029169	8.5	<0.001	Hypothetical protein DKFZp 564N1362.1
FLJ10713	NM_018189	5.0	<0.001	Hypothetical protein FLJ10713
KIAA0977	NM_014900	3.8	<0.001	KIAA0977 protein

Continued

TABLE 1—*Continued*

LOC51334	NM_016644	2.8	0.010	Mesenchymal stem cell protein DSC54
CGI-115	NM_016052	2.6	<0.001	CGI-115 protein
C13orf7	NM_024546	2.4	<0.001	Chromosome 13 open reading frame 7
FLJ20378	AI336206	2.2	<0.001	Hypothetical protein FLJ20378
SCA1	NM_000332	2.2	0.012	Spinocerebellar ataxia 1
FLJ90005	W27419	2.1	<0.001	Hypothetical protein FLJ90005
24739	AF070571	2.1	0.011	Homo sapiens clone 24739
FJX1	NM_014344	2.1	0.007	Four jointed box 1
FLJ10901	NM_018265	2.0	0.020	Hypothetical protein FLJ10901
HRASLS	NM_020386	2.0	0.002	HRAS-like suppressor
Housekeeping genes				
ACTB	Hs.426930	1.0	0.694	β-Actin
GAPDH	M33197	1.0	0.680	Glyceraldehyde-3-phosphate dehydrogenase

the data on gene expression, treatment with IL-4 resulted in a significant and dose-dependent up-regulation of protein expression of VCAM-1, E-selectin, MCP-1, and IL-6 (Figures 3A to 3D).

DISCUSSION

Microarray analysis is one of the most advanced and emerging molecular biological technologies, and it has been widely adopted for analyzing the global gene expression profiles in vivo and in vitro (22,23). Recent studies have demonstrated the potential of this technology for investigating molecular pathophysiological mechanisms involved in a variety of human diseases. In fact, microarray technology has been used as a novel experimental approach to analyze alterations in gene expression in cancer (24), atherosclerosis (25), stroke (26), Alzheimer's disease (27), HIV infection (28), schizophrenia (29), and muscular dystrophy (30).

In the present study, we performed microarray analysis using the Affymetrix GeneChip Human Genome U133A Arrays to further understand transcriptional regulatory mechanisms of action of IL-4 in human vascular endothelium. Our results revealed that mRNA levels of a total of 106 genes were significantly up-regulated and 41 genes significantly down-regulated with more than a 2-fold change in HUVEC treated with IL-4 compared with the control cell cultures (see Tables 1 and 2). Interestingly, many of IL-4–up-regulated genes are involved in inflammatory reactions, which are critical to initiate and promote early stage of atherogenesis. Previous studies from our group and others have demonstrated that IL-4–induced oxidative stress can produce a pro-inflammatory vascular environment through up-regulation of inflammatory genes, such as adhesion molecules, chemokines, and cytokines (9–12,31,32). The present data, showing significant up-regulation of VCAM-1, E-selectin, MCP-1, and IL-6, strongly support that IL-4 is a key mediator to induce pro-oxidative and pro-inflammatory pathways in human vascular endothelium.

To verify the alterations in gene expression observed in microarray analysis, as well as to further explore the potential role of IL-4 in inflammatory pathways in human vascular endothelium, the present study focused on a set of genes related to inflammatory reactions such as VCAM-1, E-selectin, MCP-1, and IL-6. VCAM-1 is expressed primarily on endothelial cells and mediates cell-cell interactions via binding to its integrin counter receptor, very late antigen-4, which may be involved in the recruitment of mononuclear leukocytes to the vascular lesions in early atherosclerosis (33). We and others have shown that IL-4 up-regulates VCAM-1 expression in vascular endothelial cells through antioxidant-sensitive mechanisms (10,31,32). In agreement with previous studies, a marked and significant increase in mRNA and protein expression of VCAM-1 was observed in IL-4–treated HUVEC by real-time RT-PCR and ELISA, respectively (see Figures 2A and 3A).

Another adhesion molecule studied in the present study was E-selectin. E-selectin is present exclusively on the surface of endothelial cells and plays a key role in mediating early leukocyte-endothelial interactions such as initial attachment and rolling during an inflammatory response. It is well documented that E-selectin is up-regulated at the transcriptional level following exposure to a series of pro-inflammatory mediators, such as IL-1β, TNF-α, and lipopolysaccharide (34). In contrast, it has been proposed that treatment of endothelial cells with IL-4 suppresses IL-1β– or TNF-α–stimulated E-selectin gene transcription (35,36). Direct effects of IL-4 on E-selectin expression in human vascular endothelial cells, however, remain unclear. In the present study, we provide new evidence to indicate that IL-4 could directly up-regulate mRNA and protein expression of E-selectin in HUVEC (see Figures 2B and 3B). These results suggest that E-selectin may play an important role in IL-4–mediated inflammatory pathways in vascular endothelium.

Among a variety of chemokines and inflammatory cytokines, MCP-1 and IL-6 are of critical significance in the early stages of atherosclerosis. MCP-1 is secreted by a variety of cell types, including vascular endothelial cells, and promotes the recruitment of inflammatory cells and their migration throughout the vascular endothelium that are thought to be critical early pathological events in atherogenesis (37,38). Consistent with previous experiments (11,12), the present study showed that IL-4 treatment resulted in up-regulation of mRNA and protein expression of MCP-1 in human vascular endothelial cells (see Figures 2C and 3C).

Table 2. Down-regulation of specific gene expression in human vascular endothelial cells treated with interleukin-4

Gene symbol	NCBI accession nr	Fold change	*P* value	Description
Adhesion molecules				
ITGA2	NM_002203	–3.6	<0.001	Integrin α2
Cytokines, chemokines, and receptors				
IL8	NM_000584	–5.6	<0.001	Interleukin-8
IL7R	NM_002185	–3.4	0.005	Interleukin-7 receptor
CXCL2	M57731	–2.2	0.001	Chemokine (C-X-C motif) ligand 2
Growth factors and receptors				
GAS1	NM_002048	–14.0	0.007	Growth arrest-specific 1
NDRG4	AV724216	–2.1	0.011	NDRG family member 4
EGFR	AW157070	–2.1	0.006	Epidermal growth factor receptor
PDGFB	NM_002608	–2.0	0.029	Platelet-derived growth factor β polypeptide
Signal transduction				
KIT	NM_000222	–9.0	<0.001	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
ITPKB	NM_002221	–2.6	0.001	Inositol 1,4,5-triphophate 3-kinase B
RAB11B	AL575337	–2.2	0.016	RAB11B, member RAS oncogene family
TNS	AL046979	–2.0	0.036	Tensin
Transcription factors				
KLF15	NM_014079	–2.7	0.004	Kruppel-like factor 15
SNAPC4	AK023513	–2.2	0.002	Small nuclear RNA activating complex, polypeptide 4, 190 kDa
NPAS2	AW000928	–2.1	<0.001	Neuronal PAS domain protein 2
HOXB6	NM_018952	–2.0	0.011	Homeo box B6
Others				
GJA4	NM_002060	–4.8	0.006	Gap junction protein (connexin 37)
HIP14	AF161412	–2.9	0.019	Huntingtin interacting protein 14
KCNN2	NM_021614	–2.8	<0.001	Potassium intermediate/small conductance calcium-activated channel N2
AMN	NM_030943	–2.6	0.029	Amnionless homolog
NFNG	AI760053	–2.6	0.023	Manic fringe homolog
CLTB	X81637	–2.5	0.032	Clathrin light chain b gene
CHST2	NM_004267	–2.2	<0.001	Carbohydrate (*N*-acetylglucosamine-6-*O*) sulfotransferase 2
FLJ12800	NM_022903	–2.2	0.030	Hypothetical protein FLJ12800
CLDN18	BE551219	–2.2	0.046	Claudin 18
RASGRP3	NM_015376	–2.1	0.003	RAS guanine releasing protein 3
CYP2A6	NM_000762	–2.1	<0.001	Cytochrome P450 2A6
TRPV5	NM_019841	–2.1	0.008	Trasient receptor potential cation channel, subfamily V, member 5
CAV3	NM_001234	–2.0	0.021	Caveolin 3
BPAG1	BG253119	–2.0	0.031	Bullous pemphigoid antigen 1
WIZ	AL390184	–2.0	0.009	Widely interspaced zinc finger motifs
Unknown				
CHI3L1	AJ251847	–4.3	0.005	CHI3L1 gene for cartilage glycoprotein-39
R29124_1	BF110434	–3.3	0.004	Hypothetical protein R29124_1
FLJ11983	AK022045	–2.9	0.004	FLJ11983 fis, clone HEMBA1001337
PRO1598	NM_018503	–2.7	0.022	Hypothetical protein PRO1598
FLJ10002	AK000864	–2.6	0.007	FLJ10002 fis, clone HEMBA1000046
FLJ23497	NM_025089	–2.6	0.044	Hypothetical protein FLJ23497
KIAA0795	NM_025010	–2.5	0.013	KIAA0795 protein
HL14	M14087	–2.4	0.049	HL14 gene encoding β-galactoside-binding lectin
FLJ20378	AI734156	–2.2	0.033	Hypothetical protein FLJ20378
LOC92346	AL035295	–2.0	0.040	PAC 106H8, similar to Dynamin

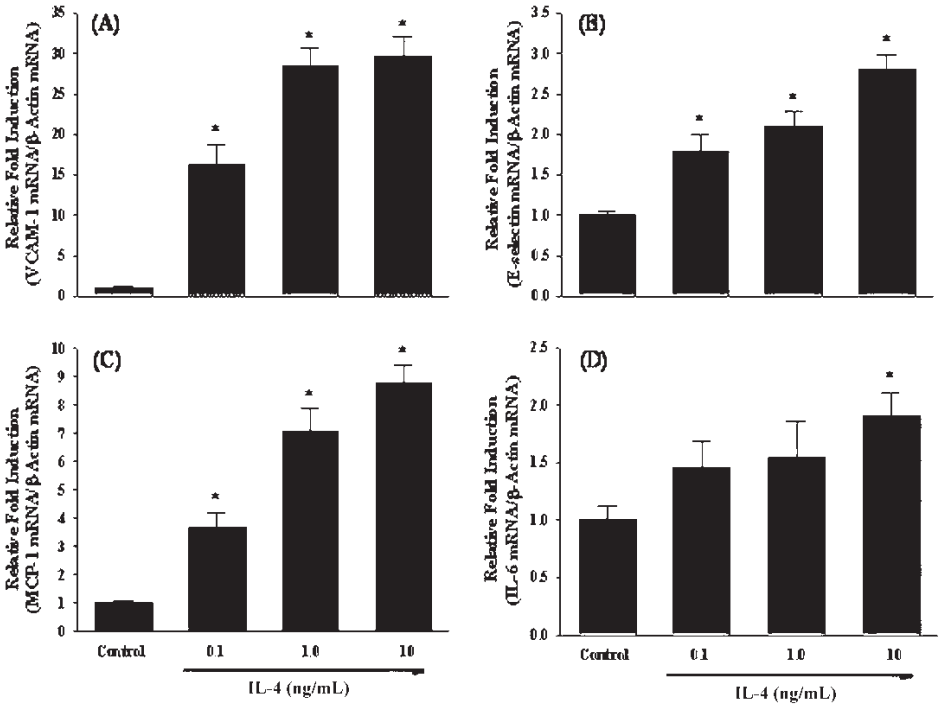

Figure 2. IL-4 up-regulates the mRNA expression of inflammatory mediators in human vascular endothelial cells. HUVEC were either untreated or treated with the indicated concentrations of IL-4 (0.1, 1.0, and 10 ng/mL) for 4 h. The mRNA levels of VCAM-1 (A), E-selectin (B), MCP-1 (C), and IL-6 (D) were determined by real-time RT-PCR as described in Materials and Methods. Data are means ± SE of 4 determinations. *Statistically significant compared with the control group ($P < 0.05$).

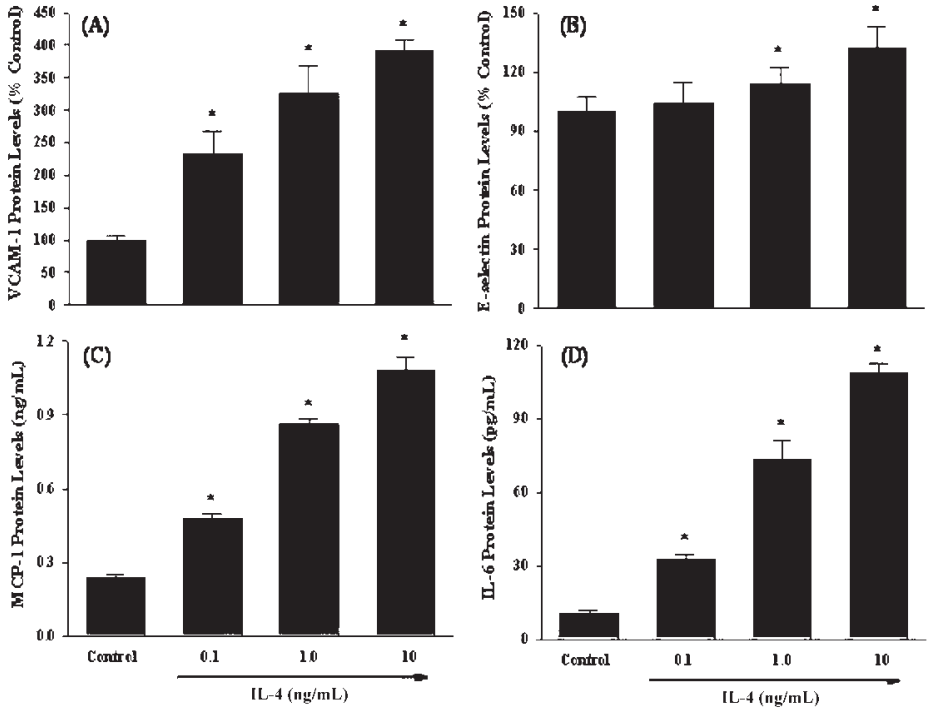

Figure 3. IL-4 up-regulates the protein expression of inflammatory mediators in human vascular endothelial cells. HUVEC were either untreated or treated with the indicated concentrations of IL-4 (0.1, 1.0, and 10 ng/mL) for 12 h (VCAM-1), 6 h (E-selectin), or 16 h (MCP-1 and IL-6). The protein levels of VCAM-1 (A), E-selectin (B), MCP-1 (C), and IL-6 (D) were measured by ELISA as described in Materials and Methods. Data are means ± SD of 4 determinations. *Statistically significant compared with the control group ($P < 0.05$).

IL-6, a multifunctional pro-inflammatory cytokine, plays a major role in inflammatory responses in vascular endothelium and has also been implicated in the pathogenesis of atherosclerosis (1,39). Although recent evidence indicates that IL-4 synergistically amplifies the TNF-α–, IL-1β–, or LPS-induced production of IL-6 protein in HUVEC (40), the molecular basis for the induction of this cytokine by IL-4 has not been elucidated. Therefore, our results, showing that IL-4 significantly induced the expression of IL-6 mRNA and increased IL-6 production in HUVEC (Figures 2D and 3D), appear to be the first to document the stimulatory effect of IL-4 on IL-6 gene expression in human vascular endothelial cells.

In conclusion, the present study provides the first quantitative large-scale gene expression analysis of IL-4–stimulated human vascular endothelial cells. We identified 147 differentially regulated genes that are responsible for the regulation of inflammatory responses, apoptosis, signal transduction, transcription factors, metabolism, and several unknown functions. Because IL-4 is involved in the early stages of atherogenesis, these results could contribute to a deeper understanding of fundamental insights of pathophysiological mechanisms involved in atherosclerosis at the level of gene expression and provide a foundation for development of therapeutic strategies for vascular diseases.

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