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Genomic-scale analysis of gene expression profiles in TNF-α treated human umbilical vein endothelial cells

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Abstract. *Objective and design:* TNF-α is a potent proinflammatory cytokine that plays an important role in immunity and inflammation, and in the control of cell proliferation, differentiation and programmed cell death. However, it is known that TNF-α is also the founding member of a still growing family of cytokines with diverse bioregulative functions. Its detailed molecular mechanisms on endothelial activation and injury remain to be elucidated. This study was aimed at determining genomic-scale gene expression profiles in TNF-α treated human endothelial cells.
Materials and methods: In this study cultured human umbilical vein endothelial cells (HUVECs) were stimulated with TNF-α (10 ng/ml) for 2 and 16 h, respectively, and the gene expression pattern was profiled using a cDNA array representing 14000 gene/cDNA clusters.
Results: In total, 72 known human genes were identified the expression levels of which altered over 2-fold in response to TNF-α stimulation. Such alteration was confirmed for IL-8 and MCP-1, with an independent quantitative mRNA assay. It was observed that genes with related biological functions were often temporally co-regulated.
Conclusions: These results indicate the transcriptional pathways mediated by TNF-α inside the HUVECs. Expression profiling in HUVECs responding to TNF-α stimulation should give an understanding of the molecular mechanisms involved in vascular inflammation.

Key words: TNF-α – HUVECs – cDNA array – Gene expression profile

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Introduction

Tumor necrosis factor-alpha (TNF-α) [1], originally defined by its tumoricidal activity, is a multi-functional cytokine and is produced predominantly by activated macrophages, T cells and natural killer cells [2]. It plays a key role in inflammatory and immunological responses [1]. TNF-α belongs to a family of signaling molecules that exist as type II membrane proteins characterized by the C terminus being extracytoplasmic. TNF-α signals as a homotrimer and can exist either as a membrane-bound form or as a truncated soluble form [3]. It binds to two distinct surface receptors, TNFR1 and TNFR2, to activate intracellular signaling pathways and to elicit its biological effects (apoptosis or inflammation mainly via NF-κB and AP-1 transcriptive pathways) [4, 5]. TNF-α and its related signaling pathways and its cytotoxic effects on many cell types have been the subject of intensive investigations [3, 6].

Vascular endothelial cells (ECs) play a central role in maintaining the hemostatic balance. ECs form a lining between vascular wall and circulating immune cells. They constitute the first line of inflammatory response and target of graft rejection. When stimulated by TNF-α, ECs can increase the expression of cellular adhesion molecules on their surface and the elaboration of cytokines and chemokines that, in turn, actively promote the inflammatory responses [7] by the recruitment and activation of leukocytes to the inflammatory site. Of those genes induced, E-Selectin, MCP-1 and IL-8 have been studied in great detail. TNF-α also induces a procoagulant phenotype on ECs by mediating the surface expression of tissue factor and the secretion of plasminogen activator inhibitor. ECs in culture that are induced to undergo apoptosis by TNF-α become procoagulant and also demonstrate increased adhesion to leukocytes [8, 9].

To comprehensively understand the molecular mechanisms of TNF-α in the activation of human endothelial cells,

we sought to use cDNA array to analyze the gene expression profiling in TNF-α treated HUVECs. The cDNA array is based on the robotic deposition of cDNAs, representing 14.000 genes, onto a nylon membrane in an ordered array [10, 11]. Here, we present the profiles of gene changes both at 2 h and 16 h, representing early and late gene response, respectively, in ECs with the stimulation of TNF-α on the genomic scale. These results shed new light on the genomic profile and molecular mechanism of interactive reaction between ECs and TNF-α.

Materials and methods

Cell culture and treatment

Primary HUVECs were prepared from human umbilical cords (obtained from Haidian Women and Children Health Care Center, Beijing) by mild collagenase IV (Gibco, USA) digestion as described [12]. The cells (3×10^5 cells) were plated into a gelatin-coated tissue culture flask (Nunc, Roskilde, Denmark) and grown to subconfluence in M199 (Gibco, USA) supplemented with 20% fetal bovine serum (Hyclone, USA), 60 μg/ml ECGF (Boehinger Mannheim, Germany), 0.03% L-Gln, 100 μg/ml Heparin, 0.6% Hepes, 0.2% $NaHCO_3$, pH 7.2, 10^{-5} M Thymidine (sigma, USA) at 37 °C in a humidified 5% CO_2 atmosphere incubator. The medium was renewed every 3 days. Subcultures were obtained by treating the HUVEC cultures with 0.25% trypsin (Gibco, USA) at 37 °C for 5 min. All cells used were under 10 passages. Origin of the cells was determined by their cobblestone morphology and indirect fluorescent-stained factor-III antibody via flow cytometry as described [13]. The endothelial cells in the assays were treated with TNF-α (R&D, USA) in final concentration of 10 ng/ml for 0, 2 or 16 h, respectively and then were harvested and treated according to experimental procedures detailed below.

Scanning electron microscopy (SEM)

Cells were seeded on the gelatin-coated coverslips and treated with TNF-α as above. Samples were taken after incubation at 37 °C for the indicated time, then prefixed at 4 °C for 1h in a final concentration of 0.5% (v/v) glutaraldehyde and fixed at 4 °C for 1h in the final concentration of 1% OsO_4. After being dehydrated in a graded series of ethanol, the samples were treated in the graded series of mixed solution of ethanol and iso-amyl acetate (2:1, 1:1, 15 min each, then treated twice with pure iso-amyl acetate, 15 min each). The samples were dried in CO_2 at critical point, and then observed and photographed under AMRAY 1910FE scanning electron microscope.

Isolation of mRNA and probe preparation

Total RNA was extracted using standard Trizol (Gibco, USA) protocol and quantified by UV spectrometer and formaldehyde denaturing agarose gel electrophoresis. The poly (A)+ mRNA was purified using the poly (dT) resin (Qiagen, Germany). At least 1.4 μg mRNA was obtained with each of the three samples; approximately 1 μg of mRNA was labeled in reverse transcription using M-MLV reverse transcriptase (Promega, USA) in the presence of 200 μCi [α-^{33}P]-dATP (DuPont NEN, USA).

Hybridization and image procession

The cDNA array nylon membranes (Amersham Pharmacia, Buckinghamshire, UK) were constructed by Shanghai Institute of Cell Biology, Chinese Academy of Sciences (visit http://www.cell.ac.cn/hugenxi.htm or http://www.cell.ac.cn/English.htm and see as described [14, 15] for details). Membranes were prehybridized with 20 ml prehybridization solution (6 × SSC, 0.5% SDS, 5 × Denhardt, 100 μg/ml denatured salmon sperm DNA) at 68 °C for 3 h. Overnight hybridization with the ^{33}P labeled cDNA from the experimental samples in 2 ml hybridization solution (6 × SSC, 0.5% SDS, 100 μg/ml salmon sperm DNA) was followed by stringent washing (0.1 × SSC, 0.5% SDS, 65 °C, 1 h). The cDNA array membranes were quantified using FLA-3000A Imaging Plate/Fluorescent Image Analyzer (Fuji Photo Film, Tokyo, Japan). Images with an intensity density above 10 were considered positive signals, which were then subtracted by the background chosen from the lowest spot located at the same 8 × 8 block. Signals thus obtained were further normalized to the sum of signals on the array as described [16]. We selected those genes as significantly differentially expressed, which signal intensity differences of both identical clone spots are beyond 2 folds between different membranes, while at the same time, the signal intensity differences between two identical clone spots must be less than 2 folds at the identical membranes.

Functional and temporal cluster analysis

The differentially expressed genes of each comparison between control and primary response group, control and activation group, respectively, were clustered according to their biological function and their temporal expression profiles. In order to examine expression profiles, the data set was then clustered according to the method of Eisen et al using gene cluster and then visualized using Treeview v1.32 software [17]. This method allows genes to be grouped together on the basis of the degree of similarity between their temporal expression profiles and allows visualization of the gene expression profiles of a large number of genes simultaneously.

Quantikine mRNA assay

The array results were selectively verified by Quantikine mRNA (R&D, USA) assay via quantitation of IL-8 and MCP-1 mRNA levels according to the manufacturer's instructions. Briefly, cytokine-specific probes and 2 μg calibrator or diluted sample total RNA were mixed to the designated wells of the hybridization plate and incubated in the 65 °C water bath for 60 min. The hybridization mixture was then transfered to the streptavidin plate and incubated for 60 min at RT (20−25 °C) on a shaker set at 500 ± 50 rpm. After washing and adding anti-digoxigenin conjugate to each well, incubation was continued for 60 min on a shaker at RT. Following a further wash substrate was added with amplifier solution to each well and incubated for 60 min and 30 min in turn on a shaker at RT. After adding stop solution, the optical density of each well was determined within 30 min, using a microplate reader set to 490 nm and substraction readings at 655 nm from the readings at 490 nm.

Results

TNF-α induced morphological changes on HUVECs

Scanning microscopy was employed to observe the morphological changes on HUVECs response to TNF-α challenge. As shown in Fig. 1, the stimulated HUVECs show many more vesicle structures on the cell surface than those of the non-stimulated control sample. The sample at 16 h time points is the most significant and displayed numerous hairy protrusions. This phenomenon demonstrated that many HUVECs suffered from apoptosis [18, 19].

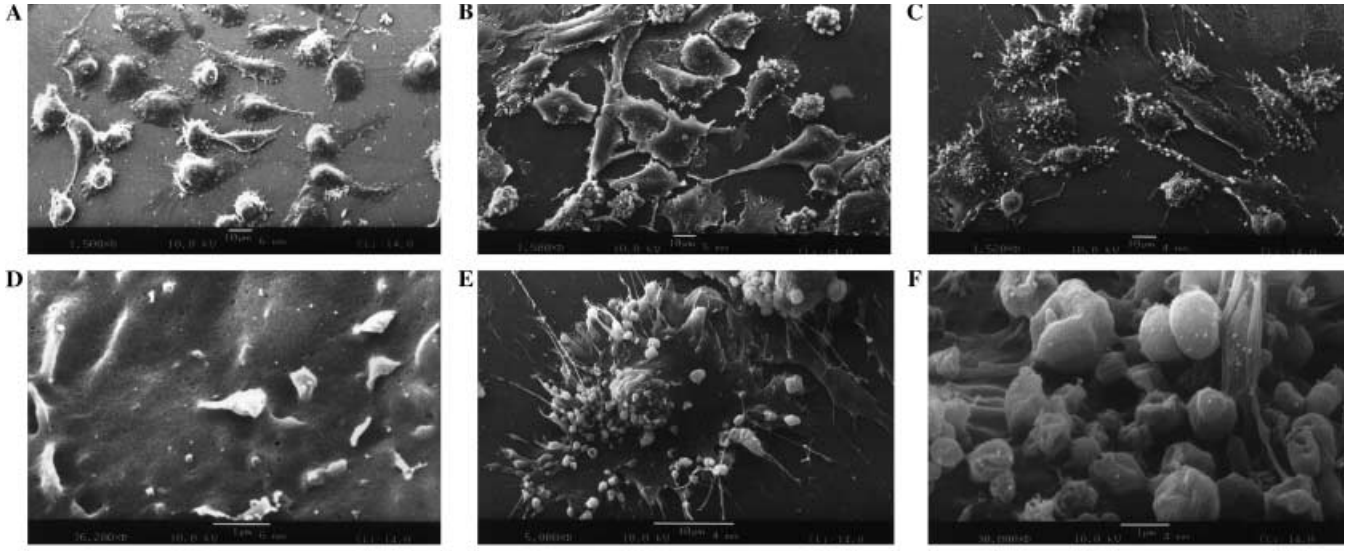

Fig. 1. Morphology of HUVECs at different time points after TNF-α stimulation. Cells on coverslips were incubated with 10 ng/ml TNF-α for 0 (**A**, originally 1500 × D), 2 (**B**, originally 1500 × D) and 16 h (**C**, originally 1520 × D), respectively. (**D**) Surface view of non-stimulated HUVECs (originally 36.200 × D). (**E**) View of single cell after 16 h stimulation (originally 5000 × D). (**F**) Surface vesicles of stimulated HUVECs (originally 30.800 × D).

Identification of TNF-α regulated gene population in HUVECs

To understand the effects of TNF-α on HUVECs at the molecular level, we sought to analyze gene expression patterns at 0 h, 2 h and 16 h following TNF-α treatment. When the selection criteria were applied to each of the 14000 cDNA elements, it was found that, in response to TNF-α stimulation, up to 140 genes/cDNA clusters were differentially expressed at levels more than two fold at 2 h in comparison to untreated cells (of which 37 were up-regulated while 103 were down-regulated), among which 43 were known genes. Up to 129 genes/cDNA clusters (of which 90 were up-regulated while 39 were down-regulated) had intensities that changed at least over two-fold compared to unstimulated HUVECs at 16 h time point (see supplemented data), among which 38 were known genes (Table 1). Further analysis showed that 23 cDNA clusters including 9 known genes were differentially expressed in response to TNF-α stimulation at both time points (Table 2).

Functional cluster analysis

In order to further break down the levels of complexity of the transcriptional response of HUVECs to TNF-α stimulation, the positively selected known genes were grouped according to their biological function and subjected to the clustering process. Those genes whose expression levels varied over two-fold between different samples, late response (16 h) VS control sample and early response (2 h) VS control sample, were selected as positive genes. The functionally associated genes were identified and further classified into eight (Table 1) groups. These groups consisted of genes that were involved in metabolism, cellular signaling, cytoskeleton and trafficking, blood regulation, protein synthesis, transcription regulation, DNA repair and other inflammation-related genes.

Cluster analysis of temporal expression profiles

The temporal gene expression profiles for each of the 72 individual genes are displayed as six cluster images (Fig. 2). A horizontal strip represents an individual gene, with each vertical bar representing a different time point, as indicated. The level of gene expression at the time point show, relative to that in unstimulated control cells (0 h), is represented according to the false-color scale shown in Fig. 2. Using this type of analysis, it is clear that there are a number of grouped genes that share similar expression profiles in response to TNF-α stimulation. Examination of the global clustering of all 71 genes revealed six clusters of genes that shared distinct temporal expression profiles indicated in Fig. 2 by the letters A–F.

Verification of cDNA array by Quantikine mRNA assay

Quantikine mRNA assay was used to further verify the fidelity of the nylon-based cDNA array assays and we selected IL-8 and MCP-1 for the level of their expression. Fig. 3 (A) shows the results of Quantikine mRNA assay. Fig. 3 (B) shows the comparison of expression of IL-8 and MCP-1 by cDNA array and Quantikine mRNA assay, respectively. It is obvious that the temporal expression of these two genes was identical as measured by these two methods, indicating that quantitation of gene expression by nylon-based membrane cDNA array is reliable, at least for these two genes.

Table 1. Classification of known genes differentially expressed in TNF-α treated HUVECs according to their biological function. Unigene database accession numbers are listed under ORF. P/C and T/C refer to the ratio of gene expression level of the early response (2 h) or late response (16 h) versus normal control samples, respectively. The fold increase shown is determined by specific algorithm with normalized preliminary data. The upward arrows indicate those genes whose transcript levels increased more than two-fold in response to TNF-α stimulation. The downward arrows indicate those genes whose gene transcript levels decreased more than two-fold in response to TNF-α stimulation. The blank represents the expression at normal range of the control. Those genes whose expression level were beyond three-fold are highlighted.

ORF	Gene	P/C (fold)	T/C (fold)	Function
Metabolism-related genes				
Hs.76392	Aldehyde dehydrogenase 1, soluble		↓ 2.63	Alcohol metabolism
Hs.77448	Aldehydede hydrogenase 4		↑ 2.08	Amino acid metabolism
Hs.198365	2,3-bisphosphoglycerate mutase		↓ 2.04	Regulating hemoglobin oxygen affinity
Hs.118797	Ubiquitin-conjugating enzyme E2D 3		↑ 2.06	Protein degradation
Hs.279455	Cytochome b5 reductase 1 (B5R.1)		↓ 2.63	Unknown
Hs.62954	**Ferritin, heavy polypeptide 1**		**↑ 3.02**	**Iron metabolism**
Hs.252549	Cathepsin Z	↓ 2.04	↓ 2.08	Major component of the lysosomal proteolytic system
Hs.115945	Mannosidase, beta A, lysosomal		↑ 2.28	Processing of glycoprotein oligosaccharides
Hs.3085	KIAA0054 gene product; Helicase		↓ 2.27	RNA metabolism
Hs.118786	**Metallothionein 2A**	**↑ 3.45**	**↑ 3.47**	**Heavy metals binding**
Hs.274416	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (14 kD, B14)	↓ 2.17		Transfer of electrons from NADH to the respiratory chain
Hs.11817	Nudix (nucleoside diphosphate linked moiety X)-type motif 5	↓ 2.04		Hydrolysis of ADP-sugars
Hs.12492	Cytidine 5-prime-monophosphate n-acetylneuraminic acid synthetase	↓ 2.13		Activation of Neu5Ac to CMP-Neu5Ac to form cell surface glycoproteins and glycolipids
Hs.114366	Pyrroline-5-carboxylate synthase long form	↓ 2.25		Proline biosynthesis
Hs.241535	TNF-inducible protein CG12-1		↑ 2.28	lipoprotein homeostasis
Hs.183583	Protease inhibitor 2 (anti-elastase), monocyte/neutrophil	↓ 2.31		Proteases activity regulation
Cell communication and signal transduction-related genes				
Hs.220689	Ras-GTPase-activating protein SH3-domain-binding protein		↑ 2.43	Ras signaling pathway
Hs.340	**Monocyte chemotactic protein 1(MCP-1)**	**↑ 4.42**	**↑ 4.72**	**Attracts monocytes and basophils but not neutrophils or eosinophils**
Hs.624	**Interleukin 8(IL-8)**	**↑ 9.39**	**↑ 40.68**	**Attracts neutrophils, basophils, and t-cells, but not monocytes**
Hs.25035	Chloride intracellular channel 4 like		↑ 2.23	Unknown
Hs.36975	Follicle stimulating hormone, beta polypeptide		↓ 2.08	Stimulates development of follicle and spermatogenesis
Hs.193470	Purinergic receptor P2X, ligand-gated ion channel, 7		↓ 2.04	Unknown
Hs.130719	NESH protein		↓ 2.5	Unknown
Hs.251415	**Deiodinase, iodothyronine, type I**		**↓ 3.33**	**Thyronine metabolism**
Hs.99948	Bone morphogenetic protein 8		↓ 2.38	Calcium regulation and bone homeostasis
Hs.15589	PPAR binding protein		↓ 2.78	Mammary epithelial differentiation
Hs.75842	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A		↓ 2.04	Cell proliferation
Hs.23016	**Human orphan G protein-coupled receptor (RDC1)**	**↑ 4.50**	**↑ 3.55**	**Receptor of adrenomedullin**
Hs.267288	Dopamine responsive protein DRG-1		↑ 2.27	Unknown
Hs.74471	Gap junction protein, alpha 1, 43 kD (connexin 43)		↑ 2.15	Channel for materials of low MW diffuse
Hs.181366	Major histocompatibility complex, class II, DR beta 5	↑ 2.15		Present processed antigens to T cells.
Hs.2230	Epidermal growth factor (beta-urogastrone)	↓ 2.70		Mitogenic factor
Hs.3439	Stomatin-like protein 2	↓ 2.5		Mechanoreception or lipid domain organization
Hs.227751	**Galectin 1**	**↓ 4.35**		**Binds beta-galactoside, an autocrine negative growth factor that regulates cell proliferation**
Hs.256290	**S100 calcium-binding protein A11 (calgizzarin)**	**↑ 2.52**	**↑ 3.33**	**Calcium binding**
Cytoskeleton transport-related genes				
Hs.166887	Copine		↑ 2.20	Membrane trafficking
Hs.81008	**Filamin B, beta (actin-binding protein-278)**		**↑ 3.32**	**Linking actin and GpIb-α**
Hs.170328	Moesin		↑ 2.86	Connections of major cytoskeletal structures to the plasma membrane
Hs.89546	**Selectin E**	**↑ 8.61**	**↑ 4.96**	**Mediates endothelial cells' binding to leukocytes**
Hs.76716	Pre-alpha (globulin) inhibitor, H3 polypeptide		↑ 2.21	Carrier of hyaluronan

Table 1 (continued)

ORF	Gene	P/C (fold)	T/C (fold)	Function
Hs.35094	Extracellular matrix protein 2, female organ and adipocyte specific	↓ 2.27	↓ 2.17	RGD sequence containing protein
Hs.9615	Myosin regulatory light chain 2, smooth muscle isoform	↓ 2.32		Regulation cell contractile activity
Hs.76293	Thymosin, beta 10	↓ 2.22		Actin-sequestering
Hs.28757	Transmembrane 9 superfamily member 2	↑ 2.39		Endosome ion channel or small molecule transporter
Hs.1447	Glial fibrillary acidic protein	↓ 2.13		Astrocytes specific class-iii intermediate filament
Hs.180941	Vacuolar protein sorting 41 (yeast homolog)	↓ 2.13		Required for vacuolar assembly and vacuolar traffic
Hs.154782	Adaptor-related protein complex 3, sigma 2 subunit	↓ 2.13		Non-clathin-associated adaptor complex
Hs.3059	Coatomer protein complex, subunit beta	↓ 2.94		Intra-golgi transport and transport from the ER to the golgi complex
Hs.288061	**Actin, beta**	**↓ 3.70**		**Nonmuscle, cytoskeletal actins**
Hs.85844	Cytoskeletal tropomyosin TM30 (nm)	↓ 2.73		Unknown
Hs.75442	Albumin	↓ 2.56		Carrier protein
Transcription regulation-related genes				
Hs.78185	MAX-like bHLHZIP protein	↓ 2.17		Transcription factor
Hs.326035	**Early growth response 1**	**↓ 5.26**		**Transcriptional regulator. Required for mitogenesis and differentiation**
Hs.27345	**RNA guanylyltransferase and 5′ phosphatase**	**↓ 3.12**		**Initiating transcription regulation**
Hs.21807	**PR domain containing 4**	**↑ 3.49**		**Transcription factors. NGF signal transduction pathway**
Hs.119640	HBKLF for basic kruppel like factor	↑ 2.31		Transcriptional regulators
Hs.131891	Small nucleSmall ribonucleoprotein polypeptide N	↓ 2.17		Tissue-specific alternative RNA processing events
Hs.82120	Nuclear receptor subfamily 4, group A, member 2	↑ 2.34		General coactivator of gene transcription but not mandatorily associated with cell cycle progression
Hs.51305	V-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein F	↑ 2.06	↑ 2.01	Oxytocin gene up regulation
Protein synthesis and structure-related genes				
Hs.93659	Protein disulfide isomerase related protein		↑ 2.36	Rearrangement of both intrachain & inter chain disulfide bonds in proteins to form the native structures
Hs.38125	Interferon-induced protein 75	↓ 2.86		Ribosome biogenesis
Hs.71819	Eukaryotic translation initiation factor 4E binding protein 1	↓ 2.32		Control of initiation factor 4E Insulin-mediated control pathways
Hs.182426	Homo sapiens ribosomal protein S2 (RPS2)	↓ 2.33		Protein synthesis
Hs.195453	Ribosomal protein S27	↓ 2.04		Protein synthesis
DNA repair-related genes				
Hs.91417	Topoisomerase (DNA) II binding protein	↓ 2.22		Topoisomerase (DNA) II binding
Thombotic system-related genes				
Hs.159509	Alpha-2-plasmin inhibitor		↑ 2.51	Regulation of fibrinolysis
Hs.75572	Carboxypeptidase B2 (plasma)		↑ 2.51	Thombin-activable fibrinolysis inhibitor
Other inflammation related genes				
Hs.20315	**Interferon-induced protein 56**	**↓ 4.16**		**Unknown**
Hs.55777	Fukutin	↓ 2.04		Fukuyama type congenital muscular dystrophy related
Hs.90606	**15 kDa selenoprotein**	**↓ 4.76**		**Implicated in cancer prevention**
Hs.81256	S100 Calcium-binding protein A4		↑ 2.15	Unknown
Hs.119663	CD59 antigen p18–20		↑ 2.42	Inhibiting the complement membrane attack

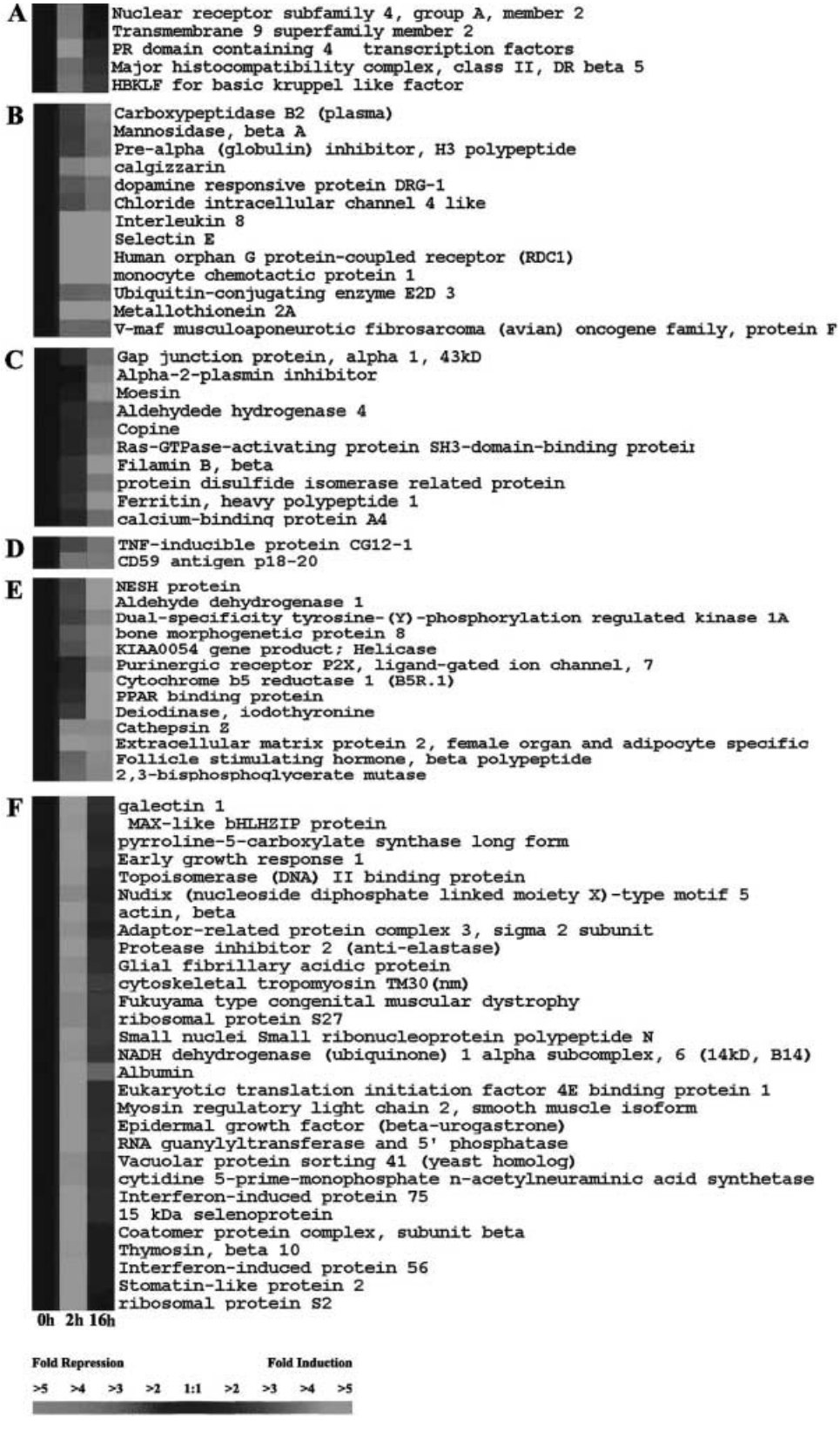

Fig. 2. Cluster image showing the expression profiles for a total of 72 known genes whose mRNA levels altered significantly in response to TNF-α stimulation. Genes were selected as per the criteria stated in the 'Materials and methods' section and clustered hierarchically into groups on the basis of the similarity of their temporal expression profiles. The expression profile of each gene is represented by a horizontal strip. For each gene, the ratio of mRNA levels in HUVECs at the indicated time after stimulation with TNF-α to that of the unstimulated control cells is represented by a color, according to the color scale at the bottom. The genes are clustered into 6 groups with similar temporal expression pattern (indicated by the letters **A**–**F**).

Table 2. Genes whose expression level increases more than two-fold compared to those from unstimulated cells at both 2 h and 16 h in response to TNF-α stimulation. Those genes whose expression level was beyond three-fold are highlighted.

ORF	Gene	P/C (fold)	T/C (fold)	Function
Hs.252549	Cathepsin Z	↓ 2.04	↓ 2.08	Major component of the lysosomal proteolytic system
Hs.118786	**Metallothionein 2A**	**↑ 3.45**	**↑ 3.47**	**Heavy metals binding**
Hs.340	**MCP-1**	**↑ 4.42**	**↑ 4.72**	**Recruitment of monocytes**
Hs.624	**IL-8**	**↑ 9.39**	**↑ 40.68**	**Attract neutrophils, basophiles, and T cells**
Hs.51305	V-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein F	°, 2.06	°, 2.01	Oxytocin gene up regulation
Hs.89546	**Selectin E**	**↑ 8.61**	**↑ 4.96**	**Mediates endothelial cells' binding to leukocytes**
Hs.35094	Extracellular matrix protein 2, female organ and adipocyte specific	↓ 2.27	↓ 2.17	RGD sequence containing protein
Hs.23016	**Human orphan G protein-coupled receptor (RDC1)**	**↑ 4.50**	**↑ 3.55**	**Receptor of adrenomedullin**
Hs.256290	**S100 calcium-binding protein A11 (calgizzarin)**	**↑ 2.52**	**↑ 3.33**	**Calcium-binding protein**

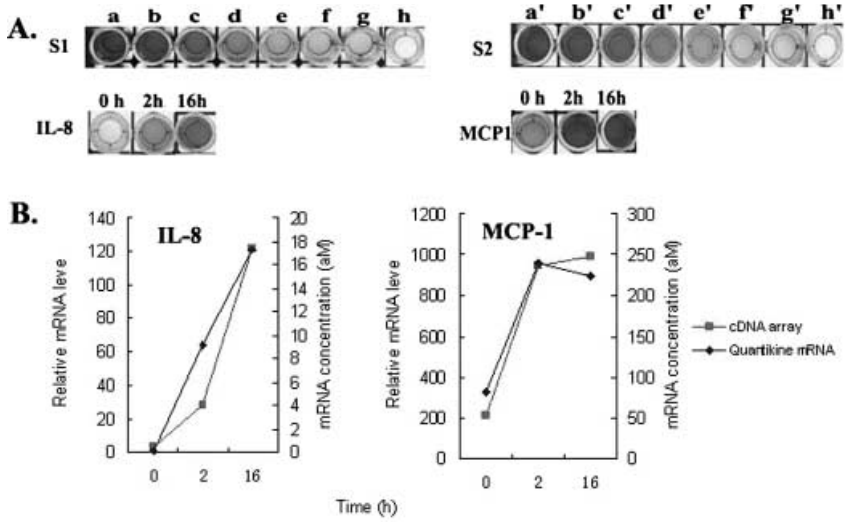

Fig. 3. Quantikine mRNA assay for IL-8 and MCP-1. (**A**) shows the representative result of IL-8 and MCP-1 for Quantikine mRNA assay. S1 and S2 refer to standards target mRNA quantitation; the target standards mRNA concentrations are displayed as: a (70aM), b (35aM), c (17.5aM), d (8.8aM), e (4.4aM), f (2.2aM), g (1.1aM), h (0aM); a' (250aM), b (125aM)',c' (62.5aM), d' (31.2aM), e (15.6aM)', f' (7.8aM), g' (3.9aM), h' (0aM). (**B**). mRNA levels of the indicated genes (IL-8 and MCP-1) were measured using the Quantikine mRNA assay (diamond) in samples taken at the same time as those used to prepare probes for the cDNA array hybridizations (square). Quantitative measurements from both assays produced similar expression profiles for the genes measured.

Discussion

TNF-α is the most prominent cytokine that can exert multi-functional biological effects in response to inflammation, infection, injury and other foreign challenges [4]. Array-based biological technology has provided a very powerful approach to explore the complicated molecular network deep inside various target cells under different conditions [20]. In the present study, we first present a global scan of gene expression profiles of human endothelial cells in response to TNF-α stimulation. We found in total 269 genes/cDNA clusters including 72 known human genes responsive to TNF-α stimulation, of which 23 including 9 known human genes were affected at both 2 h- and 16 h-time points.

It was observed that genes with related biological functions were often temporally co-regulated. We found that the most highly up-regulated genes were inflammation-associated genes, MCP-1, IL-8 and E-selectin, etc. They were regulated both at 2 h- and 16 h-time points. IL-8 and MCP-1 are both chemotactic factors, and together with E-selectin, they play a key role in the inflammation development. Their temporal expression profiles are consistent with a previous report [21, 22], suggesting the reliability and specificity of our cDNA array approach. This approach offers great advantage over methodologies previously employed to detect the differential expression, such as suppression subtraction hybridization (SSH) and DD/RT-PCR in HUVECs upon activation of TNF-α [23, 24]. SSH identified 12 genes [24] and

DD/RT-PCR identified over 100 gene fragments, including 22 known human genes, which were differentially expressed [23].

Among genes induced at both time points, 6 novel genes are induced by TNF-α in HUVECs. V-Maf is known to be a transcription factor with basic leucine zipper [25]. It can bind the promoter region of p53 gene as a homodimer to lead to p53-dependent cell arrest and apoptosis [26]. Cathepsin Z is a newly identified cysteine proteases, which was previously shown to be widely expressed in human tissues (but not in ECs), suggesting that this enzyme could be involved in normal intracellular protein degradation [27]. S100 calcium-binding protein A11 may function as transmitting calcium-dependent cell regulatory signals and involves in foreign stress-induced damage in cardiomyocytes [28]. Metallothioneins (MTs) are cysteine-rich proteins required for heavy metal tolerance both in animals and plants [29]. Then can be induced in response to toxic-level chemical exposures [30]. We hypothesize that Metallothionein 2A may have a protective role against TNF-α challenge. RDC1 was reported by its ability to bind adrenomedullin, which may play a role in the pathophysiology of heart disease [31]. The up-regulation of RDG1 suggests its important potent role in TNF-α mediated inflammation. Extracellular matrix proteins 2 is also a newly found gene and its biological function related to ECs is still obscure. Previous sequence analysis reveals that it contains an RGD sequence, a von Willebrand factor domain (VWFC), and a leucine-rich repeat. Our results support that Extracellular matrix proteins 2 may not participate in protein-protein interactions during TNF-α mediated ECs activation [32].

We found two prothombotic-related genes and they are both up-regulated only after TNF-α stimulation for 16 h. One is Alpha-2-plasmin inhibitor, which is the most rapidly acting and potent plasmin inhibitor and is important in fibrinolysis [33]; another is Carboxypeptidase B2 (plasma), which functions as a thombin-activable fibrinolysis inhibitor [34]. When ECs are activated, the circulating blood tends to be more viscous and forms clots at the inflammatory sites.

We identified 8 transcription regulation-related genes and most of them are only immediately and transiently activated by TNF-α. Their early reaction pattern may be sufficient to promote downstream molecular events and to determine the cell fate. Egr-1, for example, functions as a cancer suppressor gene by binding the promotor of the transforming growth factor beta-1 gene and cooperating with Sp1, Jun-B, p21WAF1/Cip1, and stimulates apoptosis by transactivation of the p53 gene [35, 36].

TNF-α was found to promote migration of endothelial cells, as occurs in wound-healing processes [37], and substantial evidence exists that endothelial cell migration in wound healing is related to changes in cell coupling by means of gap junctions. Gap junctions are clusters of intercellular channels, connecting the cytoplasms of adjacent cells. How the gap junction protein transcription responds to the exposure of TNF-α is controversial. It is reported that, in order to facilitate monocytes migration, the activated endothelial cells could be able to separate, a process requiring disassembly of gap junction [38]. Our data show that the connexin 43 is upregulated more than twice. Connexin 43 is a kind of low molecular mass permeable channel and this property indicates its function in vivo. It is possible that in response to the foreign stress, endothelial cells need to communicate efficiently and rapidly around the stress site. Its physiological implications and ramifications need to be further investigated.

One interesting transcriptional event is that all detectable ribosome-related genes are transiently downregulated as an early response to TNF-α challenge. This may reflect the transient depression of the protein synthesis response to foreign stress. When the stimulation of TNF-α is prolonged, the protein synthesis machinery restores itself to the normal level and may be accelerated. The only protein synthesis-related protein that was found to be up-regulated at a late time point is Protein Disulfide Isomerase Related Protein, which was involved in the rearrangement of both intrachain and interchain disulfide bonds in proteins to prevent protein misfolding [39].

Another interesting finding is the identification of 15 kDa selenoprotein that is suppressed by TNF-α treatment. Selenium behaves both as an antioxidant and anti-inflammatory agent and has been implicated in immunological effects and many other biological processes. 15 kDa selenoprotein was originally found in epithelial cells of ventral prostate and seems to have a GPx4-like redox function, perhaps protecting secretory cells against development of carcinoma [40]. A similar functional protein family is Metallothioneins, but we can not explain the opposite response of Metallothioneins and selenoprotein to TNF-α stimulation.

In summary, we first present a global scan of gene expression profiles of human endothelial cells in response to TNF-α stimulation and have identified a number of novel gene candidates that were induced or down-regulated in HUVEC in response to TNF-α stimulation. Further work is underway to use RACE analysis in order to find the full-length cDNA of the EST. This will facilitate an understanding of EC's biological process and provide targets for diagnosis and drug treatments for related diseases.

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Supplemental data:

Aside from the table displaying the known human genes differentially expressed, we provide EST data as a supplement. These may be retrieved from GeneBank with the indicated clone number for detailed sequence and other background information.

A. Genes that are up or down-regulated more than 3 fold in 2 h TNF-α treated sample compared to control.
B. Genes that up or down-regulated more than 2 fold in 2 h TNF-α treated sample compared to control.
C. Genes that up or down-regulated more than 3 fold in 16 h TNF-α treated sample compared to control.
D. Genes that up or down-regulated more than 2 fold in 16 h TNF-α treated sample compared to control.

A	B	C	D
Clone#	**Clone#**	**Clone#**	**Clone#**
BMFACH04	CBCAGA08	ADBBQA07	ADBCQE09
ADBCKB02	CBCAPB12	NPCBVA06	CBDAAF01
HTCAMB04	ADBBDG02	CBMAUE01	CBLBHC07
GKBABD08	CBCCMG09	cdAASA11	CBLBHD03
CBLBHC08	CBLAFH04	NPCCXB08	NPCBSE11
NPCCXB08	cdAASA11	ADAAEG12	ADBCLG04
cdAAUB12	cdAASF08	cdAAUB12	NPCABF08
MDS3D04	cdAATE07	ADBAXC08	CBMARD06
HTCBIC06	HTCBQC12	MDS3D04	CBLAGH08
BMFADH07	HTBAEH03	DCAACE01	CBLAIG11
HTBBFF10	HTBBZE08	HTCBIC06	CBLALH10
CBNAUD04	HTCAKC03	BMFADH07	CBLANE02
NPCBBF04	MDSEDF04	HTBCEF02	CBLANF09
HTCAUE08	NPAAJC01	NPAACA08	CBDAFD10
GKBAIF09	CDAAHC11	CBFBLH11	HTCBZF04
BMFASC12	CDAAID06	GKCADF01	cdAAVA01
GLCACA06	GKBACF03	GKCADH09	HTBCED03
MDSCJE03	HTCBIC06	MDSCJF03	HTBADB03
ADBATA11	HTCBJE04	CBFBPC04	MDS3G12
cdAAHB03	MDSAJG11	HTCAMB04	MDS4A03
CBCANE01	BMFAKF01	ADBBQH07	TPGACE02
CBNAYE04	HTBCEF02		HTBBPC06
MDSBMB11	ADBABH08		HTCAGD08
MDSCBE05	ADCAPD12		HTCBAH03
MDSCBE06	GKCAAE07		HTCAQF09
MDSCBG10	NPCASA07		MDSDFA01
GKBAKF03	GKBADE12		GKBACF03
DCBACB08	ADBCIE07		CBFAPD06
NPCAXC06	ADBCPC09		CDAADD11
ADBBQH07	HTCAUE12		CBFBRB07
ADBCBD02	GKBAED09		HTCAGA09
ADBBQA07	ADAABE06		CBNAUD04
ADBAFD04	ADCAXF04		NPCCZE11
	NPCBRA08		TPAAEH04
	CBFBLE04		GKCAAA02
	GKCADB08		TPAAGF06
	GKBAKA09		GKCAAE07
	TPGAEF04		TPAARB01
	MDSADH07		GKCACF01
	DCAAAC09		GKCADF02
	DCAACC04		GKCADF03
	TPGAHD08		HTCATH04
	NPCBLA01		ADBCIE07
	NPCBOA06		ADBCLA01
	GLCADF09		ADBCMB06
	GLCADG10		ADBCPC09
	TPGAAC04		HTCAUE12
	CBFAMB05		ADBCYC09
	NPCCLC07		ADCARE10
	HTBBAC02		GKBAGC05
	HTCBMB01		GKBAGF02
	BMFAWG09		CBFBGE10
	MDSDFF03		CBFBNB09
	CDAACF01		MDS3A05
	MDSDHE04		GKCABF05
	TPAAFG07		GKCACA05
	TPAALE05		GKCACB12
	cdAAPH09		GKCACF09
	HTBBQE02		GKCACG02
	HTCBKA12		GKCACG04
	HTBATD02		GKCACH04
	cdAAFF11		GKCADE10
	HTBAVA08		GKCAEH11
	cdAAHA09		DCAAAC09
	BMFAIF02		DCAAAD08

A	B	C	D
Clone#	**Clone#**	**Clone#**	**Clone#**
	MDSBZF06		DCAAAE01
	MDSBHA10		MDSAEG01
	MDSBHC09		BMFARG07
	MDSBZA03		MDSASA03
	MDSCAD02		MDSDMB11
	MDSBJE01		MDSDCF04
	MDSCAF04		CDAACG05
	NPCBGF04		NPCABD01
	CBCBBB06		ADBATA11
	CBCBID02		cdAADH8
	CBNAYE07		cdAAEF08
	CBTABC12		cdAAEG02
	MDSCBE03		HTBATD02
	MDSBOG01		cdAAGD09
	MDSCED01		MDSBZB07
	MDSBRA05		MDSBCA01
	MDSCIA04		MDSBDD11
	GKBALC02		CBCBBA02
	CBFBQF04		NPCBNC06
	CBNAFG01		NPCBNG06
	CBNAIF05		MDSBLE09
	BMFAAA03		MDSCBB04
	BMFACD09		MDSCBB05
	DCBACB09		MDSCHH05
	cdAAEA06		CBFBQF04
	CBCBMD06		ADBAND05
	CBCBMG12		DCBAAG10
	CBCBOC01		BMFADB09
	HTCAPA10		HTCAOB07
	CBCBWF02		CBCCCA04
	GKBACB11		ADBCMA10
	GKBACE11		GKBABA11
	ADBAKG02		ADBAKG02
	ADBCTG03		ADBBTD11
	ADBBPB11		cdABH08
	cdAAVC11		cdABH12
	HTCAIH06		DCAAAA01
	CBDAQG02		HTCANB06
	ADBABA11		NPCCOA01
	ADBABA11		ADBBNC05
	NPCCOD08		ADBBXC05
	ADBBDF01		NPCARF06
	NPCAHG08		NPCAHG08
	NPCAUB04		

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