ELSEVIER

Atherosclerosis 180 (2005) 283–291

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Analysis of gene and protein expression during monocyte-macrophage differentiation and cholesterol loading—cDNA and protein array study

Tiina T. Tuomisto[a], Mervi S. Riekkinen[a], Helena Viita[a], Anna-Liisa Levonen[a], Seppo Ylä-Herttuala[a,b,c,*]

[a] *Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute, Kuopio University, P.O. Box 1627, FIN-70211 Kuopio, Finland*
[b] *Department of Medicine, Kuopio University, Kuopio, Finland*
[c] *Gene Therapy Unit, Kuopio University Hospital, Kuopio, Finland*

Received 27 October 2004; received in revised form 20 December 2004; accepted 22 December 2004
Available online 2 February 2005

Abstract

Background: To better understand the role of macrophages in atherogenesis and to find new strategies to prevent their harmful effects, more information is needed about their gene and protein expression patterns in atherogenic conditions.
Methods: We analyzed gene and protein expression changes during monocyte-macrophage differentiation and lipid-loading by cDNA arrays and antibody-based protein arrays, respectively.
Results: It was found that early response genes, such as transcription factors, were upregulated early during monocyte-macrophage differentiation, while genes functioning in cell proliferation, migration, inflammation and lipid metabolism were activated later during macrophage differentiation. When comparing results from cDNA and antibody arrays, it become evident that changes at the protein levels were not always predicted by changes at the mRNA level. This discrepancy may be due to the different transcript variants that exist for distinct genes, posttranslational modifications and different turnover rates for mRNAs and proteins of distinct genes. When combining cDNA and protein array results with RT-PCR, it was found that CD36, COX-2, and several factors regulating cell signaling, such as Cdk-1, TFII-I, NEMO-like kinase, Elf-5 and TRADD were strongly upregulated both at the mRNA and protein levels.
Conclusions: Time-dependency of the activation of the early response genes and genes functioning in inflammation, lipid metabolism and cell proliferation and migration, is an important feature of the macrophage differentiation. It was also evident that several novel transcription factors were activated during lipid-loading. It is concluded that cDNA and protein arrays will be useful for the identification of genes that are potential targets for therapeutic interventions.

Keywords: Atherosclerosis; cDNA array analysis; Gene expression; Macrophages; Proteomics

1. Introduction

Macrophages are important for the development of atherosclerotic lesions. Monocytes migrate into the vessel wall and differentiate into macrophages [1]. Macrophages internalize modified LDL and become foam cells. Macrophages also act as antigen presenting cells and produce various chemokines and growth factors, which promote inflammatory changes and lesion development [1]. To better understand gene expression in macrophages we used cDNA and protein array analyses of differentiating and lipid-loaded macrophages.

cDNA array analysis is an effective tool to study genome-wide gene expression changes during disease processes [2]. Time-dependency of the expression changes can be visualized using so called self-organizing maps (SOMs) [3]. Recently, antibody arrays have been developed for large scale protein expression studies [4]. In antibody arrays, antibodies are attached to microscope slides. Two protein extracts

* Corresponding author. Tel.: +358 17 162075; fax: +358 17 163751.
E-mail address: seppo.ylaherttula@uku.fi (S. Ylä-Herttuala).

0021-9150/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2004.12.023

are labeled with different fluorophores and applied onto the slides. As a result one can compare relative protein abundancies between the samples.

Gene expression patterns in monocytes and macrophages have been studied by SAGE technique [5] and foam cell formation by microarrays [6] in vitro. Also, gene expression profiles of macrophages in atherosclerotic lesions in ApoE-deficient mice [7] and in human lesion macrophage-rich shoulder areas [8] have been studied in vivo using laser microdissection. However, further information is clearly needed about gene and protein expression patterns during monocyte-macrophage differentiation and lipid-loading.

In this study we have analyzed gene expression changes during monocyte-macrophage differentiation at several timepoints by cDNA arrays. Additionally, gene and protein expression patterns during lipid-loading were studied by cDNA and protein arrays. When studying dynamic changes during monocyte-macrophage differentiation, time-dependency of the activation of the early response genes and genes functioning in inflammation and lipid metabolism was found. It was also found that several novel transcription factors were activated during lipid-loading. Results indicate that cDNA and protein arrays are useful tools for the identification of potential new therapeutic targets for cardiovascular diseases.

2. Materials and methods

2.1. Cell culture

Human monocytic THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 medium according to ATCCs instructions. Cells were stimulated by 0.1 μM phorbol 12-myristate 13-acetate (PMA) (Sigma) to induce differentiation into macrophages. Cells were collected at 0, 3, 12, 24 and 72 h after addition of PMA. At 72 h timepoint 0.1 mg/ml of copper-oxidized LDL [9] (oxLDL), acetylated LDL [10] (acLDL) or native LDL (naLDL) was added and cells were collected after 72 h. Human peripheral blood monocytes (HPBM) were isolated from buffy coats (Finnish Red Cross, Helsinki, Finland) from healthy volunteers ($n = 6$) as described [11]. Adherent cells were collected at 0 h (control monocytes) and 6 days (monocyte-derived macrophages). At 6 days oxLDL, acLDL and naLDL were added as described above and cells were collected at 72 h.

2.2. RNA isolation

Total RNA was extracted from cultured cells using Trizol reagent (Gibco BRL) [8]. RNA was pooled from two independent cell culture experiments at each time point.

2.3. DNA array analysis

Probes were prepared using 50 μg of the total RNA, oligo-dT primers and Cy3-dCTP or Cy5-dCTP and hybridized as described in the Wellcome Trust Sanger Institute web site (http://www.sanger.ac.uk) to microarrays (Sanger Centre Hver1.2.1) containing approximately 10,000 clones from the Integrated Molecular Analysis of Genomes and their expression (IMAGE) collection, representing approximately 6000 different genes. All analyses were repeated three times including the use of reversed fluorophores.

2.4. Data analysis

Quantification of signals was performed with QuantArray software (GSI Lumonics), and the background was substracted from the signal intensities. The linearity of the signal intensities was checked using GeneSpring software (Silicon Genetics) with *M–A* plot, where *M* represents the log ratio of the test and control samples and *A* is the total log intensity of each spot. Because the data was linear (i.e. blot displayed 45° rotation), normalization to the median of the signals was allowed, according to the protocol of the Finnish Center for Scientific Calculation [12]. Signals were normalized using GeneSpring software by dividing each gene by the median of its measurements in all samples, and intensity ratios were calculated. Gene expression was considered significantly up or downregulated when the intensity ratio test/control was ≥ 2.0 or ≤ 0.5, respectively. Statistical significance of the differences was calculated according to Claverie [13]. To reach 5% significance level $|\mathrm{Int}_{\mathrm{test}} - \mathrm{Int}_{\mathrm{control}}|$ must be $\geq 2.8\sigma_{\mathrm{control}}$ when analyzed in triplicates, where $\mathrm{Int}_{\mathrm{test}}$ and $\mathrm{Int}_{\mathrm{control}}$ are the averages of normalized signal intensities in the test and control samples in repeated experiments and σ is the distribution of signal intensities in the control sample. Images were visually inspected to ensure that signals were not due to artefacts or high background. Cluster analysis was performed using GeneSpring. SOMs [3] were generated using default options of 6 rows, 5 columns and 90,000 iterations. Four well-characterized and biologically interesting clusters were found.

2.5. Antibody array analysis

Macrophages collected before and after the addition of oxLDL were analyzed by Ab Microarrays™ (Clontech) according to manufacturers' instructions (http://www.clontech.com). Briefly, proteins were extracted, labelled covalently with Cy3 and Cy5 and hybridized to arrays containing antibodies for 384 genes. Analysis was repeated with reversed fluorophores. Images were analyzed and quantified as for cDNA arrays. Data analysis was performed as described in the manufacturer's protocol. Ratios were considered significant if they were >1.5 or <0.66.

2.6. RT-PCR

DNAase treatment and cDNA synthesis were performed as described [14]. Primers for CD36 were 5′-CTGTGGGTGAGATCATGTGG-3′ and 5′-GAACTCCAGCA-

GGACTCAG-3′, for COX-25′-TGCTGTGGAGCTGTATCCTG-3′ and 5′-CGGGAAGAACTTGCATTGAT-3′, for CDK-15′-CTGGGGTCAGCTCGTTACTC-3′ and 5′-AAATTCGTTTGGCTGGATCA-3′, for TFII-I 5′-CGTGGAAGGCCCTAATAACA-3′ and 5′-AATGCCAAAAGTCGATGGTC-3′, for NEMO-like kinase 5′-GGGCAACAACAGCCATATTT-3′ and 5′-GACAACACCAAAGGCTCCAT-3′, for Elf-5 5′-GAGCATCAGACAGCCTGTGA-3′ and 5′-AGTCTTTGATGGTGGCCTTG-3′ and for TRADD 5′-CTGCAGATGCTGAAGATCCA-3′ and 5′-CTCCAGCTCAGCCAGTTCTT-3′. The PCR reactions consisted of 35 cycles (33 cycles for CD36) of +94 °C for 1 min, +60/58 °C for 1 min and +72 °C for 90 s. Annealing temperature of 58 °C was used for COX-2 and Cdk-1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RT-PCR was used as a control for RNA loading. Controls without reverse transcriptase were included in every analysis [14].

3. Results

3.1. Gene expression in macrophages compared to monocytes

PMA-stimulated THP-1 cells were used as a model of monocyte-macrophage differentiation that takes place during atherogenesis. Cells were collected at 3, 12, 24 and 72 h timepoints and gene expression profiling analysis was performed to compare gene expression patterns to undifferentiated THP-1 cells. At 72 h, 213 genes were upregulated and 332 genes were downregulated, which means changes in approximately 10% of the genes present on the arrays. Table 1 shows 10 most highly up- and down-regulated genes at the 72 h timepoint, where monocytes were fully differentiated to macrophages. Five out of 10 genes are previously connected to inflammation and are expressed in macrophages, namely interleukin-1 beta, macrophage inflammatory protein 3, matrix metalloproteinase-9, interleukin-2 receptor, and monocyte differentiation antigen CD14 [15]. Additionally, many genes that were upregulated to a lesser extent, were connected to inflammation, for example macrophage inflammatory protein 4 (4.8-fold increase), macrophage colony stimulating factor receptor (3.2-fold) and osteopontin (3.2). It is worth noting that several cathepsins were upregulated, for example cathepsin G (9.2), cathepsin L (3.6) and cathepsin H (3.3). Cathepsins are cysteine proteases that are involved in protein degradation and antigen presentation [16].

Table 1
Ten most highly up- and down-regulated genes in PMA-stimulated THP-1 macrophages compared to undifferentiated THP-1 monocytes

Gene	GenBank ID	Ratio
Upregulated		
Interleukin-1 beta	BC0008678	37.8
Macrophage inflammatory protein 3, alpha	NM_004591	37.4
Matrix metalloproteinase-9	BC0006093	35.8
Fibronectin-1	NM_212482	19.3
Serine-threonine protein kinase 25	NM_006374	16.4
Disintegrin and metalloproteinase ADAM10	NM_001110	14.8
Regulator of G-protein signaling-7	BC022009	14.7
Interleukin-2 receptor gamma	NM_000206	10.4
Monocyte differentiation antigen CD14	BC010507	9.9
Cathepsin G	BC014460	9.2
Downregulated		
Interferon consensus sequence binding protein-1	NM_002163	0.07
Carbonic anhydrase II	BC011949	0.07
Cote1 protein	AF070550	0.07
Myb proto-oncogene protein	NM_002466	0.09
Butyrate response factor 2	NM_006887	0.09
Cyclin-dependent kinase 6 inhibitor	BC000598	0.12
Nidogen	BC045606	0.12
Regulator of Fas-induced apoptosis	BC006401	0.13
High mobility group protein 2	X62534	0.14
Sterol delta-7-reductase	AF067127	0.14

3.2. Clusters of gene expression during monocyte-macrophage differentiation

Genes were divided into clusters according to their expression patterns during monocyte-macrophage differentiation. Cluster analysis was performed by GeneSpring program using SOMs. Four well-defined clusters were found and examples of genes in the clusters are presented in Table 2 (see also Online Supplement). Cluster 1 contained genes whose expression increased steadily. BCL-6 regulates chemokine expression in macrophages [17]. The expression of integrin beta-5 increases during macrophage differentiation [18]. Macrophage colony stimulating factor receptor [19], Apo C1, osteopontin, cathepsin G and myosin have previously been connected to macrophages [5]. In addition to these well-known macrophage-related genes, many genes functioning in cell adhesion, signal transduction, cell proliferation and oxidative stress were upregulated during differentiation. In cluster 2, there are several early response genes, for example somatostatin transcription factor 1 (STF 1), transcription factor JUN-B, and EGF-response factor ERF-1; and mediators of intra-cellular signalling, such as GTP-binding proteins, protein kinases and phosphatases, and nuclear hormone receptors NOR-1 and NURR1. Later in differentiation (cluster 3), several genes functioning in cell adhesion, such as cathepsin B and cytohesin; cell movement, such as coronin-like protein p57 and actin-related proteins; and inflammation, such as interleukin-7 receptor, galectin and interferon regulatory factor 7, were upregulated. Many genes that function in lipid metabolism have increased expression at 12, 24 and 72 h, such as fatty acid binding proteins (clusters 2 and 3), apolipoproteins CI and E, 1-AGP acyltransferase, oxysterol-binding proteins and bile acid receptor (cluster 1). Cluster 4 included genes that were downregulated dur-

Table 2
Examples of genes in different clusters of PMA-stimulated THP-1 monocyte-macrophage differentiation

Gene	GenBank ID	Fold change (h)				
		0	3	12	24	72
Cluster 1: genes with continuously increasing expression, 62 genes						
BCL-6	NM_138931	1.0	1.6	2.3	2.8	4.2
PTPRF interacting protein (liprin)	BC021255	0.7	1.1	1.4	1.8	2.7
Integrin beta-5	BC006541	0.9	1.1	1.8	2.7	3.2
Colony stimulating factor receptor-1	NM_005211	0.6	1.0	1.5	2.0	3.1
ApoC1	BC009698	0.7	0.7	1.6	2.2	2.8
Glutathione transferase, omega-1	NM_004832	0.6	1.2	1.7	1.7	2.4
Osteopontin	BC017387	0.9	1.1	1.4	2.3	3.2
Cathepsin G	BC014460	2.3	2.4	6.1	6.6	9.2
Myosin VI	XM_376516	1.1	1.2	1.5	2.3	2.8
Cluster 2: early responding genes, 29 genes						
Nuclear hormone receptor NOR-1, beta	D85241	1.0	3.0	1.0	1.0	1.0
Transcription factor STF1	NM_000209	1.0	2.8	1.3	1.0	1.1
Lymphocyte cytosolic protein 2	BC016618	0.9	2.3	1.1	0.9	0.7
Transcription factor JUN-B	BC009466	0.7	2.7	1.2	1.0	1.0
ERF-1 (zinc finger protein 36)	BC018340	0.9	2.2	1.1	1.0	0.7
Growth arrest and DNA damage induced GADD45 beta	NM_015675	0.7	1.6	0.8	0.8	0.6
Interleukin 23, alpha	NM_016584	0.8	2.1	0.9	1.0	1.1
NF-kappaB-1	NM_003998	0.8	4.8	1.5	1.4	1.4
Urokinase-type plasminogen activator	NM_002658	1.5	4.7	1.1	1.8	2.3
Cluster 3: late-responding genes, 20 genes						
40S ribosomal protein S6	BC071907	0.8	0.8	2.3	1.0	0.7
Interleukin-7 receptor	BC069999	0.8	1.2	2.4	1.0	1.0
Interferon regulatory factor 7	NM_0001572	1.1	1.0	4.3	1.8	1.2
Neutrophil cytosol factor NCF2	NM_000433	0.7	1.3	2.2	1.2	1.0
Ubiquitin-like protein FUBI	XM_370634	0.8	0.8	2.0	1.2	0.5
Coronin-like protein p57 (CORO1A)	AF495470	1.2	0.9	2.3	1.4	0.6
Fatty-acid binding protein, epidermal	NM_001444	1.8	2.4	5.1	3.5	2.1
Epithelial membrane protein-3	BC009718	0.7	0.8	1.5	1.0	0.7
Subtilisin/kexin-like protease PACE4	NM_138324	1.1	1.2	2.3	1.3	1.5
Cluster 4: genes with continuously decreasing expression, 45 genes						
C-myc binding protein MM-1	D89667	1.5	1.3	1.0	0.7	0.4
Mitogen activated protein kinase 14	NM_001315	2.5	2.2	1.9	1.4	0.9
Glypican-3	BC035972	1.5	1.4	1.1	1.0	0.6
A-kinase anchor protein-1	NM_003488	2.0	1.6	1.4	1.2	0.7
Phosphorylethanolamine transferase	NM_553264	1.6	1.5	1.2	1.0	0.4
Putative mucin core protein 24	D14043	2.5	1.6	1.7	1.4	0.9
28S ribosomal protein S12	NM_033362	1.7	1.3	1.2	0.9	0.7
Protein 1-4	BC187443	1.9	1.3	1.3	1.1	0.8
Ras-related protein RAB1A	BC000905	3.0	1.6	1.4	1.3	0.8

Complete lists of genes in clusters are listed in Online Supplement.

ing monocyte-macrophage-differentiation. These genes included for example several genes functioning in the initiation of translation and transcription, such as translation initiation factors 4E and ELF-2B and cell cycle progression proteins. To summarize these results, clear pattern of time-dependency of the activation of different genes was found during monocyte-macrophage activation. In the first phase, genes functioning in signal transduction, transcription and translation were activated. This was followed by activation of processes typical for macrophages, i.e. inflammation, proliferation and lipid metabolism. On the other hand, some transcription and translation factors and cell-cycle activators were downregulated during the differentiation process. Interestingly, many genes that have been characterized as neuronal genes, such as neuronal cadherin-2, neurexin 1-beta and neuronal-specific septin 3, were strongly upregulated during monocyte-macrophage differentiation, suggesting that neurons, despite of having been considered to have unique gene expression patterns, share similarities to at least some aspects of the gene expression in macrophages.

3.3. Protein expression in lipid-loaded macrophages

Protein expression patterns in the lipid-loaded macrophages were compared to PMA-stimulated macrophages using antibody arrays that contained 384 genes. Twenty of the most prominent changes are presented in Table 3. Surprisingly, the protein expression levels as studied

Table 3
Twenty most highly upregulated proteins in PMA-stimulated THP-1 macrophages as classified in functional classes

Function	Protein	SwissProt ID	Ratio
Intracellular signalling	Leukocyte antigen related protein phosphatase	P10586	1.89
	A-kinase anchor protein 149 (AKAP 149)	Q92667	1.86
	Rabaptin-5	Q15276	1.86
	RanBP1	P43487	1.76
	NM23-H1(nucleoside Di-P Kinase)	P15531	1.73
Cell division/growth	Signal-induced proliferation-associated gene	Q60484	1.88
	Cyclin-dependent kinase-1 (CDK-1)	P06493	1.81
	Cell division cycle 27	P30260	1.74
	CLIP-115	Q9UDT6	1.72
	Proliferation antigen Ki-67	P46013	1.63
Transcription and translation	Rnase HI	O60930	1.79
	TFII-I	Q46533	1.74
Cell adhesion	Kalinin B1	Q13751	1.76
	CD36	Q14016	1.88
Inflammation	Cyclooxygenase-2 (COX-2/PGHS)	P35354	1.85
Oxidative stress	p47phox	O43842	1.78
Cell death factors	Caspase-1	P57730	1.68
	Caspase-6	P55212	1.65
	Caspase-8	Q14790	1.62
	TRADD	Q15628	1.59

by the antibody arrays did not reveal any large changes during the lipid-loading. Most of the genes presented in the protein array were connected to cellular signalling and communication. Proteins that had well-characterized macrophage functions included for example CD36, which is a scavenger receptor responsible for oxLDL uptake [20], inflammatory gene cyclooxygenase-2 (COX-2) [21] and leukocyte antigen-related protein phosphatase (LAR), which functions in cell attachment [22]. Several genes that have a role in cell division or transcription and were not previously connected to macrophages, were upregulated, such as signal-induced proliferation-associated gene, cell division cycle 27, CLIP-115, transcription factor TFII-I and proliferation antigen Ki-67. On the other hand, factors promoting cell death, for example caspase-1, caspase-6, caspase-8 and TRADD were upregulated, suggesting that the balance between death-preventing and death-promoting events in cholesterol-loaded macrophages is strictly regulated.

3.4. Comparison of gene and protein expression patterns in lipid-loaded macrophages

During lipid-loading, 115 genes were upregulated and 189 downregulated, indicating changes in about 5% of the genes on the array. The results were very similar to the results obtained by Shiffman et al. [6]. Many genes connected to lipid metabolism, such as scavenger receptors; inflammation, such as several interleukins; and extracellular matrix production, such as fibronectins, were strongly induced. Fifty-two percent (198) of the targets in the protein arrays were also present in the cDNA-arrays. In Table 4 the expression ratios gained by cDNA arrays are compared to the ratios gained by the protein arrays. Twenty of the most highly upregulated proteins that were also present on the cDNA arrays are listed. The changes were parallel in cDNA and protein arrays. When comparing the ratios between oxLDL and PMA-stimulated macrophages, it can be noticed that oxLDL further stimulates the expression of many listed genes.

3.5. Expression patterns of selected genes in oxLDL, acLDL and naLDL-stimulated THP-1 cells and HPBM

The expression of CD36, COX-2, cyclin-dependent kinase 1 (Cdk1), TFII-I, NEMO-like kinase, Elf-5 and TRADD in control monocytes, macrophages and oxLDL, acLDL and naLDL-stimulated macrophages was studied by RT-PCR (Fig. 1A). Similarly, HPBMs were incubated with acLDL, oxLDL and naLDL, and the expression changes were studied by RT-PCR (Fig. 1B). It was found that all genes studied were induced by oxLDL and acLDL stimulation. Native LDL stimulated the expression of CD36, COX-2, TFII-I, Elf-5 and TRADD. Cdk-1 (p34/cdc2) is a protein kinase that plays an important role in the control of cell cycle and the regulation of the activity of tumor suppressor genes [23]. TFII-I is a general transcription factor capable for multiple DNA-protein interactions [24], but its expression in primary macrophages has not been previously shown. TFII-I has been shown to stimulate upstream stimulatory factor (USF-1), which for example regulates the transcription of COX-2 in macrophages [24,25]. NEMO-like kinase is a part of the TAK1-NLK-MAPK pathway, which has a role in beta-catenin and T-cell factor (TCF) signalling [26]. Elf-5 is a novel member of the ETS-family transcription factors that control cell proliferation [27]. TRADD functions in TNFR-mediated cell death [28]. The induction of Cdk-1, TFII-I,

Table 4
Comparison of gene and protein expression

Gene name	GenBank ID/SwissProt ID	cDNA array		Protein array
		PMA-stimulated THP1-cells	oxLDL-stimulated THP1-cells	oxLDL-stimulated THP1-cells
CD36	NM_005506/Q14016	2.4	3.7	1.9
A-kinase anchor protein 149 (AKAP 149)	BC000729/Q92667	2.3	1.4	1.9
Cyclo-oxygenase 2 (COX-2/PGHS)	AJ634912/P35354	2.7	1.5	1.9
Cyclin dependent kinase 1 (Cdk 1)	NM_033537/P06493	3.3	1.8	1.9
Ran Binding protein 1 (RanBP1)	NM_002882/P43487	1.9	1.8	1.9
Transcription factor TFII-I	BC070484/Q46533	2.1	2.0	1.7
NM23-H1	AF487339/P15531	1.6	1.8	1.7
NEMO-like kinase	BC064663/Q9Y6K9	1.8	2.1	1.7
MMAC1	U92436/O00633	1.4	1.3	1.7
Cytoplasmic linker CLIP-115	NM_032421/Q9UDT6	1.2	3.8	1.7
MLH-1	NM_000249/P40691	1.6	3.4	1.7
Transcription factor Elf-5	NM_198381/P55010	1.4	4.2	1.7
Proteosome subunit P45/SUG1	NM_002805/P04626	2.1	2.0	1.7
CaM kinase 2	NM_006549/Q9Y5N2	2.3	2.1	1.7
Adapting	BC003414/O43747	1.0	2.9	1.7
TRADD	NM_153425/Q15628	2.2	3.6	1.6
Tyrosine protein kinase Yes	NM_005433/P07947	1.4	2.6	1.6
Cadherin 5	NM_001795/P33151	1.9	1.3	1.6
Cyclin A/cdk-associated protein p19	NM_006930/P20248	1.3	2.3	1.6
erbB-2/HER-2	NM_000955/P04626	1.4	1.3	1.6

NEMO-like kinase and Elf-5 during macrophage activation has not been demonstrated before, and they might be important factors mediating cell proliferation and adhesion typical for macrophages in atherosclerotic lesions.

4. Discussion

We have used gene and protein expression studies in cultured macrophages to study molecular events during monocyte-macrophage differentiation and lipid-loading. As far as we know, this is the first study, where the gene expression profiling results in lipid-loaded macrophages are compared to protein arrays.

First we validated the array analysis by searching genes that were previously connected to macrophages. Among the 10 most highly upregulated genes at 72 h, five genes were previously connected to inflammation and macrophages. Also, many other genes having lower ratios were connected to macrophages and their inflammatory or lipid-related properties. The gene expression patterns during monocyte-macrophage differentiation were studied by cluster analysis. It was found that at the early time-points the expression of early response genes, such as many transcription factors were increased, and the genes that have a role in proliferation, migration, inflammation and lipid metabolism were changed later. The expression patterns in monocytes and macrophages have been studied before using SAGE by Hashimoto et al. [5]. Many findings are similar to ours, for example the upregulation of cathepsins and genes involved in lipid metabolism. However, they were not able to study the time-dependency of the changes in gene expression.

Antibody protein array analysis of lipid-loaded macrophages showed changes in the expression of genes that were previously connected to macrophages, such as COX-2. More importantly, arrays also revealed changes in many new genes that were not previously connected to macrophages. These included leukocyte antigen related protein phosphatase, which functions in cell adhesion [22] and rabaptin-5, which is part of the proliferative signalling of PDGF-beta receptor [29]. These genes might become new therapeutic targets for vascular diseases, since monocyte and leukocyte adhesion and cell proliferation are significant hallmarks of atherosclerosis. However, it should be pointed out that 384 antibodies for specific proteins present on the current array are not yet enough to get a comprehensive view about the overall changes in proteome. So far, in large-scale protein expression analysis the most widely used method has been 2D page although direct comparison of the antibody-based protein arrays with 2D gels is not yet available. However, it appears that the data analysis from the antibody-based protein arrays seems to be simpler and faster than that of the 2D gels. Other advantages of using the protein arrays is the versatility of the formats and the possibility to apply the methods for studies from enzyme-substrate, protein-drug and receptor-ligand interactions in addition to the antigen–antibody studies [30].

The results from cDNA arrays and protein arrays were compared to each other. In general, expression changes at the protein level were smaller than changes at the mRNA level. The scale of ratios obtained by protein array analysis was also narrower varying from 0.7 to 1.9 compared to the ratios obtained by cDNA arrays from 0.05 to 37.8. Thus, for several proteins, it may be difficult to fully predict changes

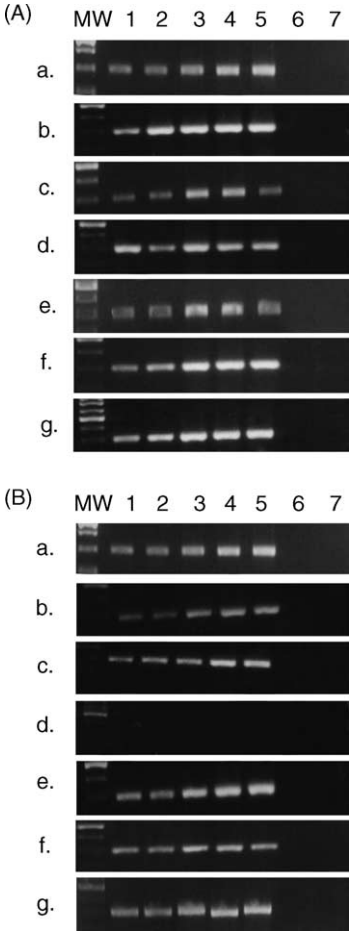

Fig. 1. RT-PCR of CD36, COX-2, Cdk-1, TFII-I, Nemo-like kinase, Elf-5 and TRADD: (A) THP-1-cells; (B) HPBM-cells (a: CD36, b: COX-2, c: Cdk-1, d: TFII-I, e: Nemo-like kinase, f: Elf-5, g: TRADD, MW: molecular weight marker, 1: monocytes, 2: macrophages, 3: oxLDL-stimulated macrophages, 4: acLDL-stimulated macrophages, 5: naLDL-stimulated macrophages, 6: –RT control, 7: blank). The sizes of the bands in a to g are 302, 300, 304, 304, 298, 298 and 304 bp, respectively. GAPDH PCR shows similar loading of RNA in each lane (data not shown).

at the protein level by changes at the RNA level. Gygi et al. [31] have studied correlations between protein and mRNA abundances in yeast by 2D gel electrophoresis and SAGE and were able to draw similar conclusions. There are several reasons for that, for example, there are several transcript variants for active genes, and all of the variants are not necessarily translated. In addition, proteins are often modified posttranslationally, for example by phosphorylation and glycosylation, and the turnover rates for mRNA molecules and proteins may differ. Moreover, the regulation of the gene product can also be mostly at the protein level. Thus, it is very important that findings from gene expression profiling studies are also confirmed at the protein level. This can be done with several methods, including protein arrays.

In atherosclerotic lesions, monocytes migrate into the vessel wall and differentiate into macrophages. Some macrophages become lipid-loaded after uptake of oxLDL. Thus, if one wants to mimic events in atherosclerotic lesions, both monocyte-macrophage differentiation and lipid-loading need to be studied. In this study we used PMA-stimulated human monocytic THP-1 cells as a model of macrophages. During monocyte-macrophage differentiation these cells become adhesive, express differentiation antigens, and functionally resemble macrophages: for example, they can phagocytose, accumulate lipids, and present antigens [32]. However, it remains somewhat unclear how much do these cells resemble in vivo macrophages, and how much does the malignant phenotype affect the results of the gene expression studies, especially those concerning genes related to cell proliferation. Kohro et al. systemically studied gene expression differences in THP-1-monocytes and THP-1-derived macrophages as compared to HPBM-monocytes and HPBM-derived macrophages (induced by M-CSF or GM-CSF) [33]. Although many genes, such as apoE and MMP9 were induced similarly during monocyte-macrophage differentiation in THP-1-derived macrophages and HPBM-derived macrophages, there were also some differences. The correlation coefficient between the expression of individual genes in THP-1-monocytes and HPBM-monocytes was 0.78, and between THP-1-derived macrophages and HPBM-derived macrophages from 0.80 to 0.88 depending on the time-point and CSF used. Therefore, we confirmed the results obtained using THP-1 cells also in HPBM-monocytes and macrophages. The expression patterns of all the selected genes except Elf-5 resembled each other in HPBM-monocytes and macrophages. Elf-5 was not expressed in HPBM-macrophages. Additionally, Cdk-1 was induced in THP-1-macrophages after oxLDL and acLDL stimulation, but on the contrary, oxLDL did not stimulate its expression as much in HPBM-macrophages. The malignant origin of THP-1 cells may explain these discrepancies, since malignant cells may highly express some cell-signalling molecules and transcription factors, and may also have different expression patterns of some cell surface receptors.

We also studied the effects of oxLDL, acLDL and naLDL on selected genes by RT-PCR. There were minor differences in the expression patterns of the selected genes. For example, all the selected genes were activated by oxLDL and acLDL, but native LDL induced the expression of CD36, COX-2, TFII-I, Elf-5 and TRADD. These differences may result from the binding and internalization of the different forms of LDLs via different receptors. Foam cell formation induced by acLDL and oxLDL is to a large extent caused by scavenging LDLs by class A, B (CD36) and D (CD68) scavenger receptors, whereas native LDL is internalized by native LDL receptors and LDL-receptor related protein [34,35]. However, it is evident that the effects of acLDL, oxLDL and naLDL differ in many aspects, and for the complete understanding of the different effects of these lipoproteins large-scale gene expression studies would be needed.

In this study, it was found that genes previously connected to macrophages, such as CD36 and COX-2, and many novel transcription factors, such as Cdk-1, Elf-5 and TFII-I, and genes functioning in cell signalling, such as

NEMO-like kinase and TRADD were upregulated during oxLDL-stimulation both at the mRNA and the protein level. The expression of these genes was upregulated also by acLDL-induced foam cell formation. Native LDL induced the expression of CD36, COX-2, TFII-I, Elf-5 and TRADD. CD36 is a scavenger receptor responsible for lipid uptake in macrophages and thus potentially responsible for the foam cell formation and further development of atherosclerotic lesions [20]. COX-2 is expressed in activated macrophages [21]. The novel transcription factors Cdk-1, TFII-I and Elf-5 have been connected to cell proliferation. Additionally, it has been shown that TFII-I regulates USF-1 expression, and USF-1 in turn regulates COX-2 expression in macrophages [24,25]. This suggests that it would be important to study further the role of TFII-I in macrophages. NEMO-like kinase functions as part of the TAK1-NLK-MAPK pathway, which has a role in cell differentiation via beta-catenin and T-cell factor (TCF)-mediated Wnt-signalling [26]. TRADD functions in TNFR-mediated cell death [28].

In this study we examined two important processes involved in the development of atherosclerotic lesions, i.e. monocyte-macrophage differentiation, and lipid-loading of macrophages. It was found that there is a clear time-dependency of the activation of different gene classes during monocyte-macrophage differentiation: transcription and translation factors are quickly activated followed by the activation of genes functioning in cell proliferation, migration, inflammation and lipid metabolism. Comparison of the results from gene expression and protein expression studies indicates that it is difficult to fully predict changes at the protein level from changes at the mRNA level. This emphasizes the fact that findings at the gene expression level should always be confirmed at the protein level. When combining gene and protein expression studies, we found several genes that could potentially be involved in the development of atherosclerosis and might therefore be useful therapeutic targets. These included leukocyte-antigen-related protein phosphatase, rabaptin-5 and several other signalling molecules, such as transcription factors Cdk-1, TFII-I, NEMO-like kinase, TRADD and Elf-5. It is concluded that cDNA and protein arrays will be useful for the identification of genes and proteins that are potential therapeutic targets for cardiovascular diseases.

Acknowledgements

We thank Riina Kylätie, Anne Martikainen and Marja Poikolainen for technical assistance. This study was supported by grants from Finnish Academy, Sigrid Juselius Foundation, European Vascular Genomics Network (EVGN LSHM-CT-2003-503254), Finnish Foundation for Cardiovascular Research, Finnish Culture Foundation of Northern Savo, Aarne and Alli Turunen Foundation, Helena Vuorenmies Foundation, and Finnish Medical Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.atherosclerosis.2004.12.023.

References

[1] Ross R. Atherosclerosis is an inflammatory disease. Am Heart J 1999;138:S419–20.
[2] Hiltunen MO, Niemi M, Yla-Herttuala S. Functional genomics and DNA array techniques in atherosclerosis research. Curr Opin Lipidol 1999;10:515–9.
[3] Golub TR, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999;286:531–7.
[4] Haab BB, Dunham MJ, Brown PO. Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biol 2001;2(2).
[5] Hashimoto S, Suzuki T, Dong HY, Yamazaki N, Matsushima K. Serial analysis of gene expression in human monocytes and macrophages. Blood 1999;94:837–44.
[6] Shiffman D, et al. Large scale gene expression analysis of cholesterol-loaded macrophages. J Biol Chem 2000;275:37324–32.
[7] Wuttge DM, Sirsjo A, Eriksson P, Stemme S. Gene expression in atherosclerotic lesion of ApoE deficient mice. Mol Med 2001;7:383–92.
[8] Tuomisto TT, et al. Gene expression in macrophage-rich inflammatory cell infiltrates in human atherosclerotic lesions as studied by laser microdissection and DNA array: overexpression of HMG-CoA reductase, colony stimulating factor receptors, CD11A/CD18 integrins, and interleukin receptors. Arterioscler Thromb Vasc Biol 2003;23:2235–40.
[9] Yla-Herttuala S, et al. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J Clin Invest 1989;84:1086–95.
[10] Lehtolainen P, Takeya M, Yla-Herttuala S. Retrovirus-mediated, stable scavenger-receptor gene transfer leads to functional endocytotic receptor expression, foam cell formation, and increased susceptibility to apoptosis in rabbit aortic smooth muscle cells. Arterioscler Thromb Vasc Biol 2000;20:52–60.
[11] Pietarinen-Runtti P, Lakari E, Raivio KO, Kinnula VL. Expression of antioxidant enzymes in human inflammatory cells. Am J Physiol Cell Physiol 2000;278:C118–25.
[12] Laine MM, Tuimala J. In: Tuimala J, Laine MM, editors. DNA microarray data analysis. Helsinki, Finland: CSC-Scientific Computing Ltd.; 2003. p. 34–7.
[13] Claverie JM. Computational methods for the identification of differential and coordinated gene expression. Hum Mol Genet 1999;8:1821–32.
[14] Vajanto I, et al. Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. J Gene Med 2002;4:371–80.
[15] Dobrovolskaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect 2002;4:903–14.
[16] Sohar N, Hammer H, Sohar I. Lysosomal peptidases and glycosidases in rheumatoid arthritis. Biol Chem 2002;383:865–9.
[17] Toney LM, et al. BCL-6 regulates chemokine gene transcription in macrophages. Nat Immunol 2000;1:214–20.
[18] Faull RJ, Wang J, Stavros W. Changes in the expression of adhesion molecules as peripheral blood monocytes differentiate into peritoneal macrophages. Nephrol Dial Transplant 1996;11:2037–44.

[19] Werner K, Bitsch A, Bunkowski S, Hemmerlein B, Bruck W. The relative number of macrophages/microglia expressing macrophage colony-stimulating factor and its receptor decreases in multiple sclerosis lesions. Glia 2002;40:121–9.
[20] Kunjathoor VV, et al. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 2002;277:49982–8.
[21] Wu MH, et al. Distinct mechanisms regulate cyclooxygenase-1 and -2 in peritoneal macrophages of women with and without endometriosis. Mol Hum Reprod 2002;8:1103–10.
[22] Kypta RM, Su H, Reichardt LF. Association between a transmembrane protein tyrosine phosphatase and the cadherin-catenin complex. J Cell Biol 1996;134:1519–29.
[23] Doi T, et al. Induction of instability of p34(cdc2) expression by treatment with cisplatin (CDDP) in mouse teratocarcinoma F9 cells. Cancer Lett 2002;176:75–80.
[24] Roy AL, et al. Cloning of an inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. EMBO J 1997;16:7091–104.
[25] Mestre JR, et al. Overlapping CRE and E-box promoter elements can independently regulate COX-2 gene transcription in macrophages. FEBS Lett 2001;496:147–51.
[26] Ishitani T, et al. The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. Nature 1999;399:798–802.
[27] Zhou J, et al. A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. Oncogene 1998;17:2719–32.
[28] Morgan M, Thorburn J, Pandolfi PP, Thorburn A. Nuclear and cytoplasmic shuttling of TRADD induces apoptosis via different mechanisms. J Cell Biol 2002;157:975–84.
[29] Magnusson MK, et al. Rabaptin-5 is a novel fusion partner to platelet-derived growth factor beta receptor in chronic myelomonocytic leukemia. Blood 2001;98:2518–25.
[30] Zhu H, Snyder M. Protein chip technology. Curr Opin Chem Biol 2003;7:55–63.
[31] Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 1999;19:1720–30.
[32] Verhoeckx KC, et al. A combination of proteomics, principal component analysis and transcriptomics is a powerful tool for the identification of biomarkers for macrophage maturation in the U937 cell line. Proteomics 2004;4:1014–28.
[33] Kohro T, et al. A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. J Atheroscler Thromb 2004;11:88–97.
[34] Luoma J, et al. Expression of alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein and scavenger receptor in human atherosclerotic lesions. J Clin Invest 1994;93:2014–21.
[35] Terpstra V, van Amersfoort ES, van Velzen AG, Kuiper J, van Berkel TJ. Hepatic and extrahepatic scavenger receptors: function in relation to disease. Arterioscler Thromb Vasc Biol 2000;20:1860–72.