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Identification of interleukin-1 and interleukin-6-responsive genes in human monocyte-derived macrophages using microarrays

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ABSTRACT

The transcriptome profile of human monocyte-derived macrophages stimulated *in vitro* by low doses of IL-1 or IL-6 was analyzed by microarrays (Affymetrix, HG-U133A) in 5 independent experiments. Out of 4886 probe sets consistently detected in all 5 array replicates we found approximately 300 genes (FDR<5%) modulated by IL-1 and/or IL-6, among which 34 may be regarded as novel cytokine-responsive macrophage genes of various function. Detailed analysis indicates that cytokine-responsive genes include 125 transcripts significantly up-regulated by IL-1 and only 39 transcripts up-regulated by IL-6, whereas the number of down-regulated transcripts is lower and almost equal for both cytokines. These data indicate that, in comparison to liver cells, IL-1 is more potent than IL-6 in modulating gene expression of human macrophages. Hierarchical clustering analysis of these transcripts yielded 7 separate gene clusters. The most abundant group contains genes strongly activated by IL-1 alone and coding for chemokines, cytokines and their receptors, the components of intracellular signaling as well as transcription factors from NF-kB family. In order to validate the results obtained by microarray analysis the expression of 5 genes from various clusters was determined by quantitative RT-PCR. Moreover, the putative promoter regions of all cytokine-responsive genes were subjected to the *in silico* identification of transcription factor binding sites (TFBS). We found that TFBS corresponding to RelA/NF-kB is the most strongly over-represented group and we demonstrated involvement of NF-kB in the expression of selected genes.

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1. Introduction

Regulation of gene expression in macrophages is of a key importance for understanding the innate and adaptive immunological responses of the host to pathogens and stimulants of various origin. Blood mononuclear cells and tissue macrophages are not only the primary source of these cytokines *in vivo* but also the principal responders, and due to this a vicious circle of inflammatory reactions may be perpetuated. During the last decade, microarray technology has been introduced and widely used to study this subject ([reviewed in [1]). The majority of reports concern the response of human or murine macrophages, or macrophage-like cells, to bacteria-derived pathogens [2–4]. Relatively less is known about internal stimulants such as thymic stromal lymphopoietin [5], activated clotting factors [6], chemokine ligands [7], M-CSF [8], interferons [9] and proinflammatory [10] or anti-inflammatory cytokines [11]. No systematic studies on specific gene expression profile in human monocyte-derived macrophages stimulated with interleukin-1 (IL-1) and/or interleukin-6 (IL-6) have been reported so far.

The innate immunological response involves acute phase reaction of liver cells stimulated by proinflammatory cytokines leading to a drastic alteration of their gene expression profile [12,13]. Recently, we searched for genes responsive to IL-1 and IL-6 using human hepatoma HepG2 cells stimulated with low concentrations of these cytokines [14–16]. By employing differential display for transcriptome analysis, and two-dimensional electrophoresis and mass spectrometry for identification of proteome components, we found several classes of genes coding for intracellular proteins of HepG2 cells either up-regulated or down-regulated by IL-1, IL-6 and a mixture of the two cytokines.

Macrophages are the main effector cells in initiating the acute phase response, and both IL-1 and IL-6 are not only produced by macrophages but also affect the cells in an autocrine manner [17], and therefore these cells are particularly important to evaluate the cytokine-induced change in their transcriptome.

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Using whole-genome Affymetrix microarrays (containing probe sets for over 20 000 transcripts), we characterized the transcriptome profile of cultured human macrophages stimulated for 4 h with IL-1 or IL-6. Our results indicate that over 300 macrophage genes are controlled by these cytokines, IL-1 being much more potent than IL-6, although we observed considerable heterogeneity in response of macrophages deriving from individual blood donors.

2. Materials and methods

2.1. Cell culture

Human monocyte-derived macrophages (hMDMs) were obtained from peripheral blood mononuclear cells (PBMCs) as described [18]. Briefly, PBMCs were isolated from human blood of 7 healthy donors using a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient. Cells from each donor were split into three aliquots to stimulate them later with either a mock stimulant (control), or with IL-1 and IL-6. They were plated at 4×10^7 cells in Primaria™ 25 cm^2 cell culture flasks with vented caps (Becton Dickinson, Franklin Lakes, NJ, USA) in RPMI1640 (Invitrogen) supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin (Sigma), and 10% pooled heat-inactivated human AB serum. After 24 h, non-adherent PBMCs were removed by washing with complete medium, and adherent cells were cultured in this medium for 7 days with fresh medium changed every 2 days. The MDM phenotype was controlled, after non-enzymatic detachment of cells, by immunofluorescent staining of CD14 (clone: TÜK4, DakoCytomation Denmark A/S, Glostrup, Denmark), CD16 (clone: DJ130c, DakoCytomation), CD11b (clone: ICRF44, Becton Dickinson and Co., Franklin Lakes, USA), and CD209 (clone: DCN46, Becton Dickinson) and subsequent flow cytometry analysis. The routine procedure used in our laboratory yields cells positive in at least 90% for the first three markers and less than 1% for CD209. It should be added that the adherent cells acquire typical macrophage morphology and show extensive phagocytic activity against live *Staphylococcus aureus* and apoptotic neutrophils. Resting (non-stimulated) cells do not produce proinflammatory cytokines: IL-1, TNF-alpha or IL-6 (data not shown).

Just before the experiment with cytokines the medium was changed to DMEM containing 0.5% of pooled human serum and the cells were stimulated with IL-1 (15 ng/ml), or IL-6 (25 ng/ml) for the period of 4 h. These rather low concentrations of cytokines that are known to occur *in vivo* were selected in preliminary trials as sufficient to stimulate the expression of manganese superoxide dismutase (MnSOD) (see Węgrzyn et al. [15]). Total cellular RNA was isolated from unstimulated (control) and cytokine-stimulated cells by Chomczynski and Sacchi procedure [19].

In some experiments we used HepG2 cells stably transfected with retroviral vector pCFG5-IEG2, containing a nondegradable mutant form of IκBα, and cells with an empty vector (control). The transfected cells were kindly provided by Professor Stephan Ludwig (Heinrich-Heine University, Duesseldorf, Germany).

2.2. Real time PCR (Q-RT-PCR)

cDNA was synthesized from 2 μg of total RNA in 20 μl using SuperScript $RNaseH^-$ reverse transcriptase (Promega). Real time PCR was carried out using the SYBR Green PCR Master Mix (DyNAmo™ HS SYBR Green qPCR (Finnzyme), 1 μl of 5× diluted cDNA, and 2 ng of each primer. For *IER3* transcript the forward primer was 5′-ccctcgagtggtgagtatcgc-3′ and reverse primer was 5′-cagaagacgatggtgagcagcag-3′; for *GADD45beta* transcript the forward primer was 5′-catgcagcgcctggcgcagc-3′ and reverse primer was 5′-ggcctcagcgttcctgaagag-3′; for *MCPIP* transcript the forward primer was 5′-ggcagcgacctgagaccagtg-3′ and reverse primer was 5′-ggtctgtgatgggcacgtcgg-3′; for *PTX3* transcript the forward primer was 5′-gcagtgttggccgagaactc-3′ and reverse primer was 5′-gtggcttgcagcagcatgcg-3′; and for *PLAUR* transcript the forward primer was 5′-tgcggtgcatgcagtgtaagac-3′ and reverse primer was 5′-tcaagccagtccgatagctcag-3′. Reaction was carried out in 15 μl using the following conditions: 95 °C — 30 s, 63 °C — 30 s, and 72 °C — 30 s with a few exceptions, where annealing temperature was 62 °C for *GADD45beta* and 59 °C for *MCPIP*. The amount of each transcript, expressed as -fold change over control (untreated cells), was calculated after determination of the difference between C_T of studied transcript and the calibrator transcript: *GAPDH*. C_T values are means of triplicate measurements.

2.3. Preparation of cRNA and oligonucleotide array

First strand of cDNA was generated from 8 μg of total RNA using a T7-oligo (dT)24 primer and the Superscript II reverse transcriptase (Invitrogen). As a rule, cDNA deriving from cultured macrophages of a single blood donor was used for further analysis, except experimental sets 1 and 2 which were obtained at this stage by pooling cDNA from 2 donors in each set. Thus finally, we had 5 experimental sets, each of them consisting of IL-1, IL-6 and mock stimulant treated cells (thus total 15 samples to be analyzed in 15 microarrays). Subsequently cDNA was converted to double-stranded cDNA using Polymerase I (Invitrogen), *E. coli* DNA Ligase (TaKaRa), and RNase H (TaKaRa). After 2 h of incubation in 16 °C T4 DNA Polymerase I (TaKaRa) was added. The double-stranded cDNA was extracted with GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) and dissolved in RNase-free water and then expressed as biotin-labeled cRNA by *in vitro* transcription (Enzo RNA Transcript Labeling Kit, Affymetrix, P/N 900182). Biotinylated cRNA was fragmented at 94 °C for 35 min. in fragmentation buffer (30 mM magnesium acetate, 40 mM Tris acetate, pH 8.1/100 mM potassium acetate) and subjected to hybridization to the human HG-U133A array (Affymetrix). After hybridization, each array was washed, and stained with streptavidin–phycoerythrin conjugate (Molecular Probes). Arrays were scanned by the GeneChip Scanner 3000 (Affymetrix).

2.4. Microarray quality control and normalization

The gene expression data was processed using GeneChip Operating Software (GCOS, Affymetrix, Santa Clara, CA) to generate MAS5 CEL files. Chip quality was assessed using R 2.3.0 software with Simpleaffy package [20,21]. Quality control data for all the 15 microarrays run in the experiments were obtained using MAS5 algorithm. Arrays determined to be acceptable were further analyzed to identify genes with altered expression patterns. Data was normalized with MBEI algorithm using dChip 2006 (probe-level quantile normalization and PM/MM difference model) [22].

2.5. Gene filtering and ranking

To remove genes that are regarded as not expressed in the analyzed cell culture, probe sets with hybridization signal close to the background level were filtered out. The following criteria were applied for probe-set detection: present call in at least 33% of PM/MM pairs and signal intensity >7.64 (log2) in at least 33% of arrays, both measured by MBEI algorithm. Statistical analysis was performed on the list of detected probe sets. To assess the contribution of blood donor and treatment effects, multiple regression analysis of expression values with donor and treatment as main effect predictors was performed using the *lm* function in R. Contribution of the effects was determined for each gene separately. Significance levels (*p*-values) of differences between the three groups were calculated for each probe set using an analysis of variance (ANOVA). Correction for multiple testing was applied to *p*-values from ANOVA by controlling percent of false discovery rate (FDR) using R software [23]. To reduce pronounced biological variation between the donors, expression values from each blood donor were standardized to have mean 0 and standard deviation 1. Hierarchical clustering was performed with dChip software using Euclidean distance and average linkage method. Relative expression levels and fold change measures were computed on MBEI PM/MM data.

2.6. Gene ontology analysis

Functional annotation analysis tool DAVID 2006 (http://niaid.abcc.ncifcrf.gov) was used to identify over-represented ontological groups among gene expression profiles and to group genes into functional categories [24]. The list of probe sets detected experimentally (4886) was used as a background list. The over-represented GO terms (GOTERM_ALL level) were defined as having at least three transcripts and $p \le 0.01$ under Fisher exact test. The BioCarta and KEGG databases were used to link changes in the profile of gene expression with the activation of specific cellular pathways.

2.7. In silico analysis of transcription factor binding sites (TFBS)

The oPOSSUM database (www.cisreg.ca/cgi-bin/oPOSSUM/opossum) [25] was used to identify over-represented TFBS in the promoter region of cytokine-responsive genes. TFBS over-representation was determined for each set of genes selected from hierarchical clustering based on one-tailed Fisher exact probabilities and the ranking of *Z*-scores using the following criteria: minimum conservation 70%, matrix match threshold 80%, 2000 bp upstream sequence. Over-representation of TFBS was measured using the strict statistical criteria (*Z*-score>7; Fisher score<0.05).

3. Results and discussion

3.1. Sample preparation and microarray quality

Total RNA from unstimulated macrophages (control), or from cells stimulated for 4 h either with IL-1 or IL-6, was converted to cDNA and later to biotin-labeled cRNA (see Materials and methods). For hybridization to the human HG-U133A array (Affymetrix), 5 experimental sets were prepared. Each set, corresponding to 5 independent biological replicates, consisted of RNA deriving from control, IL-1 and IL-6 stimulated cells. A good quality of obtained microarray data was confirmed in all cases. Mean 3>…<0.76), respectively. Mean percentage of the present call 43.7 (38.8>…<49.1) and the mean intensity of average background (ranged from 33.6 to 65.7) indicate that hybridization of the samples to the microarrays was comparable across the dataset composed of 15 arrays. Array normalization resulted in a mean scaling factor of 1.17 (±0.08). These results permit for direct comparison of the data obtained from 5 sets of microarrays. The microarray data reported in this manuscript are publicly available at the GEO database under the accession number GEO: GSE8515.

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