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• *BRIEF REPORTS* •

Profiling of differentially expressed chemotactic-related genes in MCP-1 treated macrophage cell line using human cDNA arrays

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Abstract

AIM: To study the global gene expression of chemotactic genes in macrophage line U937 treated with human monocyte chemoattractant protein-1 (MCP-1) through the use of ExpreeChip™HO_2 cDNA array.

METHODS: Total RNA was extracted from MCP-1 treated macrophage line U937 and normal U937 cells, reversely transcribed to cDNA, and then screened in parallel with HO_2 human cDNA array chip. The scanned result was additionally validated using RT-PCR.

RESULTS: The result of cDNA array showed that one chemotactic-related gene was up-regulated more than two-fold (RANTES) and seven chemotactic-related genes were down-regulated more than two-fold (CCR1, CCR5, ccl16, GROβ, GROγ, IL-8 and granulocyte chemotactic protein 2) in MCP-1 treated U937 cells at mRNA level. RT-PCR analysis of four of these differentially expressed genes gave results consistent with cDNA array findings.

CONCLUSION: MCP-1 could influence some chemokine and receptor expressions in macrophages *in vitro*. MCP-1 mainly down-regulates the expression of chemotactic genes influencing neutrophilic granulocyte expression (GROβ, GROγ, IL-8 and granulocyte chemotactic protein 2), and the mRNA level of CCR5, which plays a critical role in many disorders and illnesses.

Key words: MCP-1; ExpreeChip™HO_2

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INTRODUCTION

Chemokines are small secreted proteins that function as potent activators and chemoattractants for leukocyte subpopulations and some nonhemopoietic cells. Most chemokines elicit their effects through interactions with seven-transmembrane-domain, G-protein-coupled receptors. The size of this family has grown considerably and now includes dozens of members[1]. According to the position of conserved cysteine residues in their primary sequence, the chemokine superfamily is divided into four subfamilies (C-X-C, C-C, C, and C-X_3-C) which attract specific subsets of leukocytes[2]. Chemokine expression secondary to stimulation with proinflammatory cytokines has been reported in many types of diseases[3].

Monocyte chemoattractant protein-1 (MCP-1) was first purified from conditioned medium of baboon aortic smooth muscle cells in culture on the basis of its ability to attract monocytes, but not neutrophils, *in vitro*. It is a potent chemoattractant for monocytes *in vitro*, with an ED50 similar to that of IL-8 for neutrophils (500 pmol/L). MCP-1 induces the expression of integrins required for chemotaxis, and has also been reported to attract NK cells as well as T lymphocytes[4-6].

Recently, the function of chemokines has extended far beyond leukocyte physiology. For example, MCP-1 was found to play a pathogenic role in many diseases. Its expression could be detected in human atheromatous plaques and in aortic walls of primates fed with high-cholesterol diets, consistent with a model of atherogenesis in which MCP-1 in the vessel walls attracted monocytes that eventually became foam cells[7]. Similarly, the presence of inflammatory cells in the joints of patients with rheumatoid arthritis has been explained by IL-8 and MCP-1 in synovial fluids[8]. This expression was also documented in glomerulonephritis, asthma, inflammatory bowel disease, and allogeneic transplant rejection[6,9].

The ligand-binding repertoires of different chemokine receptors significantly overlap, so do the sets of receptors expressed by different leukocytes and other target cells, this further adds to the versatility of the chemokine system. There are high complexities among the conditions of chemokine expressions and binding to receptors. For example, among the known CC chemokines, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, macrophage inflammatory protein (MIP)-1α, MIP-1β, I309, and HCC-1, all have monocyte chemoattractant activities in vitro. Furthermore, monocytes express at least three cloned CC chemokine receptors, namely CCR1, CCR2, and CCR5, and even though MCP-1 binds to only CCR2 with a high affinity, CCR2 also binds to

MCP-3 and MCP-5[4,6]. The cDNA array technology has been demonstrated as a very useful tool for identifying differentially expressed genes. In order to study the regulation of MCP-1 on other chemokines and their receptors, we studied the potential regulation function of MCP-1 on the expression of chemotactic-related genes in macrophages.

MATERIALS AND METHODS

Materials

huMCP-1 was purchased from Dingguo Biotech Corp.[10]. Human macrophage line U937 was reserved by our study group. FCS, chloroform, isopropanol, DEPC, TRIzol were purchased from Huashun Corp.

Cell culture and huMCP-1 treatment

Macrophage line U937 was incubated in 10 mL RPMI1640 medium containing 10% FCS. When cell count reached 0.5-1×10^6/mL, cells were centrifuged and the supernates were discarded. The cells were resuspended with the same volume of RPMI1640 medium containing huMCP-1 (10 mg/mL) and incubated overnight.

Human cDNA array, probe, hybridization, and data analysis

Culture cells were washed with cooled PBS (pH 7.2) twice, then lysed by TRIzol, extracted with chloroform, precipitated with isopropanol, and washed with 80% ethanol. The deposits were dehydrated by vacuum, then solubilized by Nes buffer. mRNA was purified by an Oligotex mRNA mini kit, then the control mRNA of cells was labeled with cy5-dUTP and the mRNA of stimulated cells was labeled with cy3-dUTP, deposited by ethanol and solubilized in 20 μL hybridization buffer (5× SSC+0.2% SDS). ExpreeChip™HO_2 was made by MERGEN Corp. The chip and probe were degenerated for 5 min at 95 ℃, then hybridized for 15-17 h at 60 ℃, washed with 2× SSC+0.2% SDS and 0.1%× SSC +0.2% SDS, 0.1%× SSC, and dried at room temperature. The chip was scanned by ScanArray3000, then the result was analyzed by ImaGene3.0. The criteria of gene expression changes were cy3/cy5≥2, or cy3/cy5≤0.5.

Semiquantitative RT-PCR

cDNA was generated using 1 μg of total RNA from the two U937 cell lines (normal and MCP-1 treated) as templates in a 20-μL reaction mixture, and reverse transcription was carried out at 42 ℃ for 1 h followed by at 95 ℃ for 10 min using the preamplification system (GIBCOL). cDNA (2 μL) was amplified in a 25 μL PCR reaction mixture containing 2× PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl), 1.9 or 2.4 mmol/L of $MgCl_2$, 0.5 μmol/L of primers, 0.18 mmol/L of deoxynucleotide triphosphate, and 1 unit Taq DNA polymerase (TaKaRa). The conditions of hot-start PCR reaction were as follows: at 95 ℃ for 10 min followed by 25-35 cycles of denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 1 min (for primers of β-actin, GROβ, GROγ and IL-8) or at 50 ℃ for 1 min (for primers of regulated upon activation, normal T cell expressed and secreted, RANTES), and extension at 72 ℃ for 1 min. The final step of extension was at 72 ℃ for 10 min. PCR reagents were purchased from Takara. All of the primers were synthesized by Genecore Corp., Shanghai. The cycle number was optimized for each gene-specific primer pair to ensure the amplification in a linear range, and the results were semiquantitative. PCR products (5 μL) were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide.

RESULTS

Identification of differentially expressed chemotactic-related genes in U937 cells by the human ExpreeChip™HO_2 chip

ExpreeChip™HO_2 (invoice: 0102-003) used in this study was made by MERGEN Corp. The chip contained 10 ng of each gene-specific cDNA from 1 152 known genes and 9 housekeeping genes. Several plasmid and bacteriophage DNAs and blank spots were also included as negative and blank controls to confirm hybridization specificity. A complete list of the genes with array positions and GenBank accession numbers of the chip used here could be accessed at the website. Genes were considered to be up-regulated when the intensity ratio between expressions in the MCP-1 treated U937 cell lines compared with normal cell lines was two-fold. Genes were labeled as down-regulated when the ratio between normal and MCP-1 treated cell lines was two-fold. The analysis of scatter diagrams is seen in Figure 1, using EC cells as system controls.

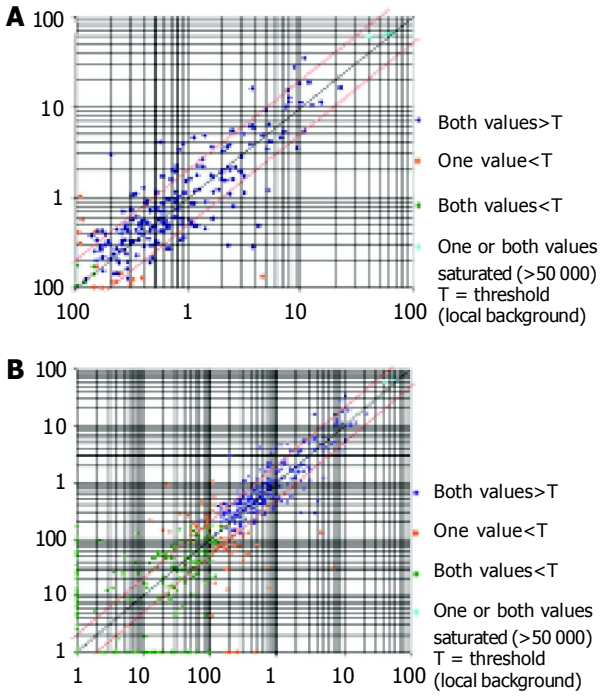

Figure 1 Chip result analysis of EC cells (**A**) and U937 cell line (**B**). The x-axis is the relative intensity of cy3 signal, and y-axis is the relative intensity of cy5 signal. The ratio x/y≥R2 shows that the gene expression was up-regulated, and x/y≤0.5 shows that the gene was down-regulated.

Results of chip detection

The membranes carrying 1 152 cDNA probes of defined human genes, and their accession numbers, names, and the scanned data were given below. Among the 1 152 genes,

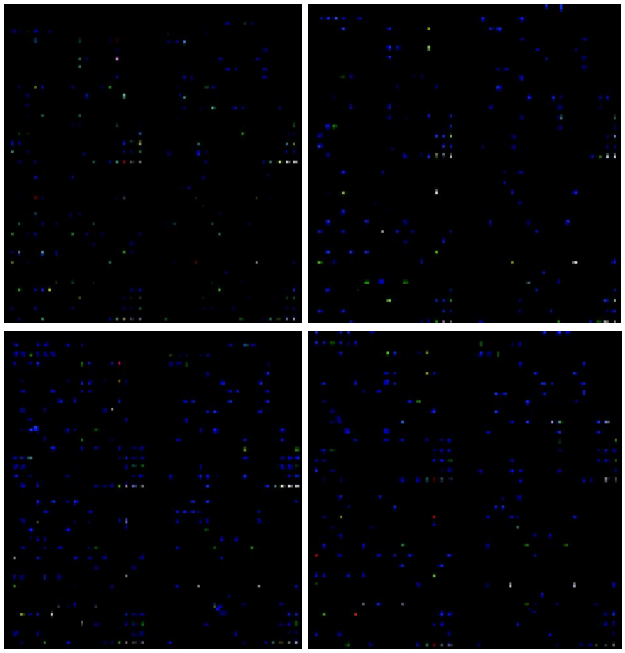

Figure 2 U937 mRNA expression analysis using Mergen cDNA arrays. These maps revealed a number of genes that were significantly expressed in controls and MCP-1 treated cells, with an expression ratio above 2 (the red color dot, more red color dots indicate more highly expressed genes) or below 0.5 (the blue color dot, more blue color dots indicate meagerly expressed genes). Results were the mean from two separate experiments and were arranged in order of decreasing relative expression after treated with MCP-1 compared with untreated controls. A correlation analysis of the results from the two separate experiments showed that the findings were highly reproducible, because r = 0.897 for the 25 genes with the highest relative mRNA expression after MCP-1 treatment.

110 were up-regulated, 91 were down-regulated (Figure 2). We searched for chemokine genes and chemokine receptor genes to study the gene expression changes in chemokine superfamily (Tables 1 and 2). Gene names shown in bold designate that genes with mRNA expression were also analyzed by RT-PCR (Figure 3).

Confirmation of differentially expressed chemotactic-related genes by semiquantitative RT-PCR

The semiquantitative RT-PCR results showed that RANTES genes were up-regulated, whereas GROβ, GROγ and IL-8 were down-regulated in MCP-1 treated cell lines (Figure 3) .These results were similar to those detected by Human ExpreeChip^{TM}HO$_2$ chip (Tables 1 and 2).

Table 1 Expression of chemokine receptor genes in U937 cell line

Sample/ Control	Control/ Sample	Unigene symbol	Gene description
0.1	1.0	CCR1	Chemokine (C-C motif) receptor 1
1.0	19.2	CCR2	Chemokine (C-C motif) receptor 2
0.7	1.3	CCR3	Chemokine (C-C motif) receptor 3
0.6	1.6	CCR3	Chemokine (C-C motif) receptor 3
1.0	1.0	CCR4	Chemokine (C-C motif) receptor 4
0.1	7.3	CCR5	Chemokine (C-C motif) receptor 5
1.0	1.0	CCR6	Chemokine (C-C motif) receptor 6
1.0	1.0	CCR7	Chemokine (C-C motif) receptor 7
1.0	1.0	CCR8	Chemokine (C-C motif) receptor 8
1.0	1.0	CCRL2	Chemokine (C-C motif) receptor-like 2
1.4	0.7	CXCR4	Chemokine (C-X-C motif), receptor 4 (fusin)

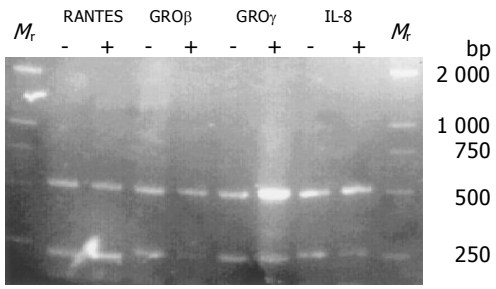

Figure 3 Primers and amplification of RT-PCR analysis. The full names of the genes and their accession numbers are given in bold in Tables 1 and 2. Sense and anti-sense primers were used to detect mRNA expression of the indicated genes by RT-PCR. The last two columns give the quantity of total RNA added to each RT-PCR reaction and the number of PCR cycles for PCR amplification used for RT-PCR analysis in MCP-1 treated U937 cells (+, MCP-1 treated). The same RT-PCR conditions were used for U937 cells (-, controls). β-actin was used as a quantitative control (sense: 5'-GGCATCCTCACCCTGAAGTA-3'; antisense: 5'-CCATCTCTTGCTCGAAGTCC-3'; 60 ℃; 496 bp; 1 μg; 30 cycles), RANTES (sense: 5'-CCCTCACCATCATCCTCACT-3'; antisense: 5'-TCCTTCGAGTGACAAACACG-3'; 50 ℃; 186 bp; 1 μg; 30 cycles), GROβ (sense: 5'-ATTCGGGGCAGAAAGAGAAC-3'; antisense: 5'-ACCCCTTTTATGCATGGTTG-3'; 60 ℃; 207 bp; 1 μg; 35 cycles), GROγ (sense: 5'-GAATTTGGGGCAGAAAATGA-3'; antisense: 5'-CGAACCCTTTTTATGCATGG-3'; 60 ℃; 227 bp; 1 μg; 30 cycles) and IL-8 (sense: 5'-AAGGAACCATCTCACTGTGTGTAAAC-3'; antisense: 5';-TTAGCACTCCTTGGCAAACTG-3'; 60 ℃; 247 bp; 1 μg; 25 cycles).

DISCUSSION

MCP-1 is a CC chemokine that attracts monocytes, memory T lymphocytes, and natural killer cells. The interaction of MCP-1 with its receptor is essential for monocyte activation and induction of chemotaxis during an inflammatory response. Because of its target cell specificity, MCP-1 has been postulated to play a pathogenic role in a variety of diseases characterized by mononuclear cell infiltration, including atherosclerosis, rheumatoid arthritis, and multiple sclerosis[6,11]. MCP-1 may exert these effects by influencing the expression of other chemokines, which is hard to be demonstrated for the complexity of the chemokine network. Genechip is a high-throughput method to evaluate hundreds of genes at one time, so it is the best method to investigate this complex process and the relationship between MCP-1 and other chemokines and receptors.

Although U937 cell line may differ in some aspects from human blood macrophages, it expresses functional chemokines and cytokines as human blood macrophages. The major advantage of using U937 cells is the homogeneity of the cell line, allowing comparison of findings between different experiments. For this reason, we used U937 cells in the present study to examine macrophage responses to MCP-1, even though they were not absolutely identical to human peripheral macrophages.

In the present study, we specifically examined the global gene expression of chemokines and their receptors, and demonstrated that MCP-1 could strongly down-regulate the expression level of the CXC subfamily chemokines: IL-8, GROβ, GROγ and granulocyte chemotactic protein 2. MCP-1 also could up-regulate the expression level of RANTES (CC subfamily), down-regulate the expression level of CCL16 (CC subfamily). It had no effect on the expression of XCL2 (C subfamily) and fractalkine (the only member of CX3C subfamily). In chemokine receptors, it could down-regulate the expression level of CCR2 and CCR5.

Table 2 Expression of chemokine genes in U937 cell line

Sample/Control	Control/Sample	Unigene symbol	Gene description
1.6	0.6	SCYA3	Small inducible cytokine A3 (homologous to mouse Mip-1a)
2.6	0.4	SCYA5	Small inducible cytokine A5 (RANTES)
1.0	1.0	SCYA17	Small inducible cytokine subfamily A (Cys-Cys), member 17
1.0	1.0	SCYA11	Small inducible cytokine subfamily A (Cys-Cys), member 11 (eotaxin)
1.0	1.0	SCYA13	Small inducible cytokine subfamily A (Cys-Cys), member 13
1.0	1.0	SCYA14	Small inducible cytokine subfamily A (Cys-Cys), member 14
0.4	2.8	SCYA16	Small inducible cytokine subfamily A (Cys-Cys), member 16
1.0	1.0	SCYA18	Small inducible cytokine subfamily A (Cys-Cys), member 18, pulmonary and activation-regulated
1.0	1.0	SCYA19	Small inducible cytokine subfamily A (Cys-Cys), member 19
1.0	1.0	SCYA20	Small inducible cytokine subfamily A (Cys-Cys), member 20
1.0	1.0	SCYA21	Small inducible cytokine subfamily A (Cys-Cys), member 21
1.0	1.0	SCYA22	Small inducible cytokine subfamily A (Cys-Cys), member 22
1.0	1.0	SCYA23	Small inducible cytokine subfamily A (Cys-Cys), member 23
1.0	1.0	SCYA25	Small inducible cytokine subfamily A (Cys-Cys), member 25
0.0	469.8	GRO2	GROβ (melanoma growth stimulating activity, beta)
0.0	47.6	GRO3	GROγ (melanoma growth stimulating activity, gamma)
0.0	34.5	IL8	Interleukin 8
1.0	1.0	SCYB11	Small inducible cytokine subfamily B (Cys-X-Cys), member 11
1.0	1.0	SCYB5	Small inducible cytokine subfamily B (Cys-X-Cys), member 5 (epithelial-derived neutrophil-activating peptide 78)
0.0	261.6	SCYB6	Small inducible cytokine subfamily B (Cys-X-Cys), member 6 (granulocyte chemotactic protein 2)
1.0	1.0	SCYC2	Small inducible cytokine subfamily C, member 2
1.0	1.0	SCYD1	Small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (fractalkine, neurotactin)

IL-8, GROβ, GROγ and granulocyte chemoattractant protein-2 are all members of the CXC chemokine family. The GRO proteins are about 90% identical in amino acid sequence. IL-8 and granulocyte chemoattractant protein-2 are about 40-50% identical to each other and to any of the GRO proteins. The CXC subfamily can be further subdivided into ELR^+ and ELR groups, based on the presence or absence of the sequence motif glutamic acid-leucine-arginine (ELR) N-terminal to the first cysteine. They are all ELR^+ CXC chemokines. All ELR^+ CXC chemokines are powerful activators of neutrophils and induce chemotaxis, shape change, a rise in intracellular free calcium levels, exocytosis, and respiratory burst *in vitro* and neutrophil accumulation *in vivo*, whereas the ELR CXC chemokines are not neutrophil chemoattractants[12-14]. Our findings suggest that MCP-1, which activated and chemoattracted monocytes and macrophages, could depress the infiltration of neutrophils in inflammation by rendering macrophages to express less neutrophil chemokines. Many disorders begin as neutrophils infiltrate at the inflammatory location, and further develop as monocytes or macrophages infiltrate, MCP-1 then may be one of the regulating factors of such changes.

RANTES was isolated in a T- *vs* B-lymphocyte differential screen, and found to be inducible by mitogens or antigens in a variety of T-cell lines and circulating lymphocytes. *In vitro*, RANTES was nearly as a potent chemoattractant for monocytes as MCP-1, but was much less effective in stimulating exocytosis. In endothelial cell-free assays, RANTES attracted CD4+, CD45R0+ T lymphocytes, but in transendothelial systems it attracted CD8+ cells as well as CD4+, and was the most potent CC chemokine for CD8+ chemoattraction. The first hint about a connection between chemokines and HIV-1 came from the finding that RANTES could prevent infection by macrophage-tropic, nonsyncytium-inducing strains of HIV-1[15].

In vitro ligand binding experiments suggested that the sole cloned receptor of MCP-1 was CCR2. CCR2 responded to MCP-1, MCP-3 and MCP-5, but maximum responses were only obtained to MCP-1. CCR5 could interact with RANTES, MIP-1, or MCP-2 under physiological conditions[16,17]. CCR5 could also act as a co-receptor in HIV-1-mediated infection of CD4-positive lymphocytes and microglia. In addition, the ligands for CCR5 could inhibit infection with certain strains of HIV-1, and decreased susceptibility to HIV-1 infection has been linked with mutations in CCR5 gene[18,19]. CCR5 was also involved in a diverse array of inflammatory diseases[11,20,21]. Our findings suggested that MCP-1 might influence the process of these diseases, although the mechanism is not clear. Detailed data need to be further explored.

In summary, MCP-1 can influence the expression of some chemokines and receptors in macrophages *in vitro*. MCP-1 can also down-regulate the mRNA level of CCR5, which plays a critical role in many disorders and illnesses. MCP-1 can also greatly change other cytokines of the immune system, such as IL-18, TNF, IFN. Our findings disclose some relationship among MCP-1 and other chemokine-related members, shedding new light on the mechanism of the function of MCP-1 and the pathogenesis of related diseases.

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