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

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INTRODUCTION
Chemokines are small secreted proteins that function as potent activators and chemotactic agents 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. 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-X3-C) which attract specific subsets of leukocytes. Chemokine expression secondary to stimulation with proinflammatory cytokines has been reported in many types of diseases.

Monocyte chemotactic 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.

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 became foam cells. 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. This expression was also documented in glomerulonephritis, asthma, inflammatory bowel disease, and allogeneic transplant rejection.

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 chemotactic 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[4,5]. 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 0.5-1×10⁶/mL, cells were centrifuged and the supernatants 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 Nse 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™HO₂ was made by MERGEN Corp. The chip and probe were degenerated for 5 min at 95 °C, then hybridized for 15-17 h at 60 °C, 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 ImageGene3.0. 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 °C for 1 h followed by at 95 °C 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 MgCl₂, 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 °C for 10 min followed by 25-35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min (for primers of β-actin, GROβ, GROy and IL-8) or at 50 °C for 1 min (for primers of regulated upon activation, normal T cell expressed and secreted, RANTES), and extension at 72 °C for 1 min. The final step of extension was at 72 °C 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 semi quantitative. 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™HO₂ chip**

ExpreeChip™HO₂ (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.

| A | 100 |
|---|-----|
| Both values>T |
| One value<T |
| Both values<T |

| B | 100 |
|---|-----|
| One or both values saturated (>50 000) |
| F = threshold (local background) |

**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,
These results were similar to those detected by Human were down-regulated in MCP-1 treated cell lines (Figure 3). The semiquantitative RT-PCR results showed that RANTES genes by semiquantitative RT-PCR. Confirmation of differentially expressed chemotactic-related genes by RT-PCR (Figure 3). 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™HO2 chip (Tables 1 and 2).

**Table 1** Expression of chemokine receptor genes in U937 cell line

| Sample/Control | Unigene symbol | Gene description |
|----------------|----------------|------------------|
| 0.1            | CCR1           | Chemokine (C-C motif) receptor 1 |
| 1.0            | CCR2           | Chemokine (C-C motif) receptor 2 |
| 0.7            | CCR3           | Chemokine (C-C motif) receptor 3 |
| 0.6            | CCR4           | Chemokine (C-C motif) receptor 4 |
| 1.0            | CCR5           | Chemokine (C-C motif) receptor 5 |
| 0.1            | CCR6           | Chemokine (C-C motif) receptor 6 |
| 1.0            | CCR7           | Chemokine (C-C motif) receptor 7 |
| 1.0            | CCR8           | Chemokine (C-C motif) receptor 8 |
| 1.0            | CCR2L2         | Chemokine (C-C motif) receptor-like 2 |
| 1.4            | CXCR4          | Chemokine (X-C motif), receptor 4 (fusin) |

**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[5,11]. 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-X-Cys), member 17 |
| 1.0            | 1.0            | SCYA11         | Small inducible cytokine subfamily A (Cys-X-Cys), member 11 (etosain) |
| 1.0            | 1.0            | SCYA13         | Small inducible cytokine subfamily A (Cys-X-Cys), member 13 |
| 1.0            | 1.0            | SCYA14         | Small inducible cytokine subfamily A (Cys-X-Cys), member 14 |
| 0.4            | 2.8            | SCYA16         | Small inducible cytokine subfamily A (Cys-X-Cys), member 16 |
| 1.0            | 1.0            | SCYA18         | Small inducible cytokine subfamily A (Cys-X-Cys), member 18, pulmonary and activation-regulated |
| 1.0            | 1.0            | SCYA19         | Small inducible cytokine subfamily A (Cys-X-Cys), member 19 |
| 1.0            | 1.0            | SCYA20         | Small inducible cytokine subfamily A (Cys-X-Cys), member 20 |
| 1.0            | 1.0            | SCYA21         | Small inducible cytokine subfamily A (Cys-X-Cys), member 21 |
| 1.0            | 1.0            | SCYA22         | Small inducible cytokine subfamily A (Cys-X-Cys), member 22 |
| 1.0            | 1.0            | SCYA23         | Small inducible cytokine subfamily A (Cys-X-Cys), member 23 |
| 1.0            | 1.0            | SCYA25         | Small inducible cytokine subfamily A (Cys-X-Cys), member 25 |
| 0.0            | 469.8          | GR02           | GROβ (melanoma growth stimulating activity, beta) |
| 0.0            | 47.6           | GR03           | 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. Our findings suggest that MCP-1, which activated and chemotactically motonecocytes 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 monococytes 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 chemoattraction. The first hint about a connection between chemokines and HIV-1 came from the finding that RANTES could prevent infection by macrophage-tropic, non-synctium-inducing strains of HIV-1[13].

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[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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