Recombinant Human MCP-1/JE Induces Chemotaxis, Calcium Flux, and the Respiratory Burst in Human Monocytes

By Barrett J. Rollins, Alfred Waiz, and Marco Baggioni

The JE gene was first described as a platelet-derived growth factor (PDGF)-inducible gene in mouse 3T3 cells. The human homologue of JE encodes a protein whose predicted amino acid sequence is identical to that of the monocyte chemoattractant MCP-1 (also called MCAF and SMC-CF), which belongs to a recently identified family of small secretory proteins with cytokine properties. We purified recombinant human MCP-1/JE (hMCP-1/JE) produced in COS cells and demonstrated that it is chemotactic for human monocytes with a specific activity similar to natural MCP-1. In addition, pure recombinant hMCP-1/JE stimulates monocytes, inducing an increase in cytosolic free calcium and the respiratory burst, but is completely inactive on human neutrophils. These results help to define functionally a well-known growth factor-inducible gene and a member of a new family of cytokines.

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METHODS

Expression in COS cells and purification of hMCP-1/JE protein. COS cells grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum (BCS) were transfected at 60% confluence with the expression plasmid pXM-hJE34 using diethylaminoethyl (DEAE) dextran and chloroquine. pXM-hJE34 contains the previously described human JE cDNA in the pXM expression vector. Medium was concentrated using a YM-5 membrane (Amicon, Danvers, MA), and material soluble in 45% (NH4)2SO4 was dialyzed extensively against 20 mmol/L Tris-HCl (pH 8.0). The retentate was applied to orange A Sepharose (Amicon) in the same buffer, and the column was developed using a 0 to 800 mmol/L NaCl gradient in 20 mmol/L Tris-HCl (pH 8.0). Material eluting from the column was concentrated, desalted on a 0.125 ng/mL endotoxin because control media were devoid of endotoxin because control media were devoid of biologic effects produced by unfractiated COS cell conditioned media were unlikely to be contaminated with endotoxin because control media were devoid of biologic activity (described in Results).

Peripheral blood mononuclear cell (PBMC) preparation. For chemotaxis experiments, heparinized whole blood from healthy volunteer donors was diluted 1:4 in Hank’s buffered salt solution (HBSS) and underlaid with Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Preparations of pure hMCP-1/JE contained less than 0.125 ng/mL endotoxin. Biologic effects produced by unfractiated COs cell conditioned media were unlikely to be contaminated with endotoxin because control media were devoid of biologic activity (described in Results).

Monocyte chemotaxis. Monocyte chemotaxis was measured using a 48-well chemotaxis chamber housing a polycarbonate filter with 5-μm pores (Neuroprobe, Cabin John, MD). All cells migrating through the polycarbonate filter in response to hMCP-1/JE or murine MCP-1/JE (mMCP-1/JE) protein were shown to be monocytes by NSE staining.
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**Monocytic chemotactic activity (MCA) in medium conditioned by transfected COS cells.** Dilutions of medium from cells transfected with a human JE expression plasmid (■■) or a control plasmid (○○) were tested for MCA as described in the Materials and Methods section. Each point represents the average value for duplicate wells, and error bars represent the range. These results are typical of five similar experiments.

**RESULTS**

Monocytic chemotactic activity (MCA) in medium conditioned by transfected COS cells. Dilutions of medium from cells transfected with a human JE expression plasmid (■■) or a control plasmid (○○) were tested for MCA as described in the Materials and Methods section. Each point represents the average value for duplicate wells, and error bars represent the range. These results are typical of five similar experiments.

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**Purification of hMCP-1/JE.** We then purified recombinant hMCP-1/JE protein expressed in the COS cell system to determine whether its specific activity was similar to that reported for natural MCP-1. Purification was performed in three steps: (1) removal of material insoluble in 45% (NH₄)₂SO₄, (2) dye-ligand chromatography on orange A-Sepharose to which recombinant hMCP-1/JE protein binds, as reported for natural MCP-1, and (3) FPLC cation-exchange chromatography on Mono-S. The last step separates the α and β forms of hMCP-1/JE. Figure 2 shows a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purification procedure and Table 1 shows that the procedure resulted in a 70- to 80-fold purification. The specific activities of recombinant hMCP-1/JE-α and hMCP-1/JE-β are similar to those reported for MCP-1 at this stage of purification. Further purification of hMCP-1/JE-β by gel filtration chromatography on Superose-12 did not produce a significant increase in specific activity [Table 1].

We estimate that pure hMCP-1/JE-α has a molecular weight (mol wt) of 15,000 and hMCP-1/JE-β has a mol wt of control plasmid. COS cells transfected with a murine JE expression plasmid also produced monocyte chemoattractant activity for human monocytes that was effective at similar concentrations (data not shown). Our rabbit antiserum MCP-1/JE heteroimmunized did not neutralize the chemoattractant activity of recombinant human or mouse MCP-1/JE in solution.

**Fig 1**. Monocytic chemotactic activity (MCA) in medium conditioned by transfected COS cells. Dilutions of medium from cells transfected with a human JE expression plasmid (■■) or a control plasmid (○○) were tested for MCA as described in the Materials and Methods section. Each point represents the average value for duplicate wells, and error bars represent the range. These results are typical of five similar experiments.

**Fig 2**. SDS-PAGE analysis of purification of recombinant hMCP-1/JE. Conditioned medium from COS cells transfected with a human JE expression plasmid was concentrated, and hMCP-1/JE was purified as described in the text. Aliquots of material from each purification step were analyzed on a 17% polyacrylamide gel with SDS and 2-mercaptoethanol added. Molecular weight markers are indicated. Lane 1, concentrated conditioned medium; lane 2, material soluble in 45% (NH₄)₂SO₄; lane 3, eluate from orange A-Sepharose; lane 4, hMCP-1/JE-α peak from Mono S column; lane 5, hMCP-1/JE-β peak from Mono S column; M, markers.
Table 1. Purification of Recombinant Human MCP-1/JE*

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (mL)</th>
<th>Protein (mg)</th>
<th>MCA (U)†</th>
<th>Recovery (%)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated medium</td>
<td>14.3</td>
<td>64</td>
<td>365,870</td>
<td>100</td>
<td>5717</td>
</tr>
<tr>
<td>45% (NH₄)₂SO₄</td>
<td>27.0</td>
<td>18.9</td>
<td>600,886</td>
<td>164</td>
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</tr>
<tr>
<td>Orange A</td>
<td>3.0</td>
<td>1.28</td>
<td>349,962</td>
<td>96</td>
<td>273,408</td>
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<tr>
<td>Mono S-α</td>
<td>0.170</td>
<td>0.106</td>
<td>44,737</td>
<td>12</td>
<td>422,046</td>
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<tr>
<td>Mono S/S12-β</td>
<td>0.092</td>
<td>0.012</td>
<td>6,133</td>
<td>2</td>
<td>511,083</td>
</tr>
</tbody>
</table>

Abbreviation: MCA, monocyte chemoattractant activity.
*Medium conditioned by COS cells transfected with a JE expression plasmid was concentrated and subjected to the purification steps described in the text.
†An aliquot from each purification step was serially diluted and tested for MCA as described in the Materials and Methods section. MCA concentration was defined as the reciprocal of the dilution showing half-maximal activity.

11,000. Other investigators have demonstrated that the difference between these two proteins is due to O-glycosylation of hMCP-1/JE-α.²⁶ We confirmed this finding in the COS cell expression system and have preliminary evidence that the C-terminal threonine residue is the major site of O-linked carbohydrate (data not shown). During the purification of hMCP-1/JE-α, we occasionally observed a protein of mol wt 15,500 that copurifies with the usual 15,000-mol-wt protein. This protein was variably expressed in independent COS cell transfections, but it represents a JE-encoded protein because: (1) it was never observed in medium conditioned by COS cells transfected using a control plasmid, and (2) it was recognized by an anti-mMCP-1/JE rabbit heterosera.

Stimulation of monocytes by recombinant hMCP-1/JE. We next wished to determine whether recombinant MCP-1/JE, like other chemotactic factors, could stimulate monocytes. Figure 3A shows that medium conditioned by COS cells transfected with the human JE expression plasmid induced a [Ca²⁺] increase in human monocytes, whereas medium conditioned by COS cells transfected with a control plasmid was inactive. The rate of the [Ca²⁺] increase ascended toward a plateau in proportion to the amount of conditioned medium added. Under the same conditions, the medium from the JE transfectants had no effect on [Ca²⁺], of Fura-2-loaded neutrophils even at the highest concentrations tested, indicating that hMCP-1/JE does not stimulate neutrophils. As shown in Fig 3B, both purified hMCP-1/JE-α and f-MLP induced a concentration-dependent increase in the rate of [Ca²⁺] increase. hMCP-1/JE-α induced a measurable increase in [Ca²⁺], at concentrations as low as 4 × 10⁻⁶ mol/L and was about 50% as potent as f-MLP.

hMCP-1/JE-α also elicited the respiratory burst in monocytes, as shown by the chemiluminescence tracings in Fig 4. H₂O₂ formation started after a lag of about 2 seconds, and the activity of NADPH oxidase increased rapidly to a maximum in about 5 seconds. Activity then rapidly returned to near-resting levels. The yield of H₂O₂ increased with hMCP-1/JE-α concentration, but the time lag and time for reaching the maximum remained constant and were similar to f-MLP. This time course is characteristic of the respiratory burst response induced by chemotactic agonists in human neutrophils.²⁷ As shown in Fig 4, the response to f-MLP lasted longer, which is typical for this ligand as compared with other chemotaxins, eg, C5a.²⁷ In three experiments, the average total yield of H₂O₂, which is proportional to the area under the chemiluminescence curve, was 18 nmol/10⁷ cells after stimulation with 10⁻⁷ mol/L f-MLP, and 0.52 nmol/10⁷ cells after stimulation with 10⁻⁶ mol/L hMCP-1/JE. The response to f-MLP is comparable to that obtained in a previous study by Thelen et al,²⁹ ie, 10 to 12 nmol O₂ corresponding to 5 to 6 nmol H₂O₂/10⁶ cells stimulated with 10⁻⁷ mol/L f-MLP. H₂O₂ production after stimulation with 4 × 10⁻⁴ mol/L hMCP-1/JE-α was comparable to that elicited by 10⁻⁴ mol/L f-MLP.

**DISCUSSION**

Our experiments directly demonstrated that the recombinant protein product of JE has cytokine properties in in vitro assays. As predicted by sequence comparisons, recombinant human MCP-1/JE-α and JE-β proteins are monocyte chemoattractants with specific activities similar to pure natural MCP-1-α and MCP-1-β. The murine MCP-1/JE protein is also a chemoattractant for human monocytes (unpublished observations), although we have not purified the murine material to test its specific activity.

In addition to directed migration, hMCP-1/JE also induces monocyte responses, such as [Ca²⁺], changes and the respiratory burst, that are characteristic of the action of chemotactic agents on phagocytes.²⁹ In these experiments, purified hMCP-1/JE-α was about half as potent as f-MLP in promoting an increase in [Ca²⁺]. The observed effects of recombinant hMCP-1/JE-α on monocytes are analogous to those described for NAP-1/interleukin-8 and its homologues on neutrophils. These stimulatory effects are similar to those reported for human monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line and may be consistent with the reported induction by MCAF of tumor cell cytostatic capacity in monocytes. They are also consistent with our own observations that human and murine MCP-1/JE expression in malignant cells suppresses tumor formation in vivo by attracting and activating monocytes.

Although the JE gene is induced in 3T3 cells by PDGF, we could not demonstrate a stimulatory or inhibitory role for mMCP-1/JE in proliferation of these cells. The present demonstration of the monocyte chemoattractant and stimulant properties of hMCP-1/JE makes it even less likely that 3T3 cells are a target of MCP-1/JE's action. An analogous situation occurred with gro/melanoma growth stimulatory...
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Fig 3. (A) Rate of [Ca\textsuperscript{2+}] increase in human monocytes in response to medium conditioned by transfected COS cells. Fura-2-loaded monocytes were exposed to decreasing dilutions of conditioned medium from COS cells transfected with a human JE expression plasmid (■) or a control plasmid (□). The rate of increase in Fura-2 saturation was measured as described in the Materials and Methods section. The results were replicated in an independent experiment. (B) Rate of [Ca\textsuperscript{2+}] increase in human monocytes in response to pure recombinant hMCP-1/JE-α and f-MLP. Varying concentrations of FPLC-purified hMCP-1/JE-α (●) or f-MLP (○) were added to Fura-2-loaded human monocytes, and the rate of Fura-2 saturation was measured as described in the Materials and Methods section. The results were replicated in an independent experiment.

Fig 4. Respiratory burst in human monocytes. Human monocytes were exposed to varying concentrations of FPLC-purified recombinant hMCP-1/JE-α or f-MLP, and changes in NADPH oxidase activity (rate of H\textsubscript{2}O\textsubscript{2} formation) were measured by chemiluminescence over time: (a) 160 nmol/L hMCP-1/JE-α, (b) 40 nmol/L hMCP-1/JE-α, (c) 16 nmol/L hMCP-1/JE-α, (d) 4 nmol/L hMCP-1/JE-α, (e) 1 nmol/L hMCP-1/JE-α, and (f) 0.4 nmol/L hMCP-1/JE-α; dotted line, 1 nmol/L f-MLP. The results are typical of three independent experiments.

act activity (MGSA), a protein with considerable structural similarity to NAP-1/IL-8. Like the JE gene, the murine homologue of gro/MGSA (the KC gene) was first described as a PDGF-inducible gene in 3T3 cells. Although gro/MGSA was originally reported to induce proliferation of a human melanoma cell line, it has since been shown to be a potent neutrophil-activating agonist. Thus both original PDGF-inducible genes (JE and KC) have been shown to be mediators of inflammation rather than mediators of the growth response to PDGF.

Accumulated evidence suggests that MCP-1/JE is involved in the delayed response of tissues to injury and inflammation by its ability to recruit mononuclear phagocytes. Indeed, we and other investigators recently demonstrated MCP-1/JE protein secretion from activated arterial smooth muscle cells and endothelial cells, indicating a potentially important function for MCP-1/JE in vascular injury, particularly in early atherogenesis. The fibroblast's response to PDGF is one component of tissue reaction to injury or inflammation, and in this context, the ability of PDGF to induce high levels of MCP-1/JE expression is consistent with what we now know about the function of MCP-1/JE.

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BJ Rollins, A Walz and M Baggiolini