

# Monocyte chemoattractant protein-2 can exert its effects through the MCP-1 receptor (CC CKR2B)

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**Abstract** We studied the activities of the monocyte chemoattractant proteins MCP-1, MCP-2 and MCP-3 on human embryonic kidney 293-EBNA cells transfected with the MCP-1 receptor (CC CKR2B). At 4 nM, MCP-2 induced a  $\text{Ca}^{2+}$  influx which was as potent as that with MCP-1 at 4 nM, although the increase by MCP-2 became saturated at higher concentrations. In addition, all three MCPs showed dose-dependent inhibition of adenylyl cyclase activity stimulated by forskolin ( $\text{IC}_{50}$  values: 0.3 nM for MCP-1, 7 nM for MCP-2, and 1.5 nM for MCP-3). In conclusion, our data indicate that MCP-2 can exert its effects through the MCP-1 receptor, CC CKR2B.

**Key words:** Monocyte chemoattractant protein; Chemokine; Chemokine receptor

## 1. Introduction

Chemokines have been identified as structurally and functionally related cytokines which have chemotactic activity for leukocytes. They are structurally divided into two subfamilies, CXC and CC, according to the position of the preserved cysteine residues. It has been postulated that chemokines potentially participate at several points in the cell recruitment process, such as activation of integrins, chemotaxis and stimulation of the effector functions of leukocytes [1–4]. Recently, the importance of chemokines in leukocyte recruitment into inflammatory sites has been confirmed *in vivo* by means of gene targeting techniques [5,6].

Monocyte chemoattractant protein-1 (MCP-1) and two related CC chemokines, i.e. MCP-2 and MCP-3, have been known to have similar activities for monocytes [7–10], T cells [11–14], basophils [15,16] and natural killer cells [17–19], while it has been reported that MCP-2 and MCP-3, but not MCP-1, have chemotactic activity for eosinophils [16]. On the other hand, several chemokine receptors which belong to the seven-transmembrane, G protein-coupled receptor family have been cloned [20,21]. Five types of CC chemokine receptor have been cloned so far: MIP-1 $\alpha$  receptor (CC CKR1) [22,23], two alternatively spliced MCP-1 receptors (CC CKR2A, 2B) [24,25], CC CKR3 [26], CC CKR4 [27] and CC CKR5 [28]. Several studies have been conducted on interactions between the three MCPs and the cloned CC chemokine receptors. Thus, MCP-1 has been considered to be highly selective for CC CKR2B [29], while MCP-3 has been shown to be a func-

tional ligand for both CC CKR1 and CC CKR2B [30,31]. On the other hand, it is still unclear whether MCP-2 can act as a functional ligand for either of these CC CKRs in the receptor transfected systems [30,31].

Here, we report that MCP-2 is also a functional ligand for CC CKR2B based on the results in terms of induction of  $\text{Ca}^{2+}$  influx and inhibition of adenylyl cyclase in CC CKR2B-transfected cells.

## 2. Materials and methods

### 2.1. Chemokines

Recombinant human MCP-1 was obtained using a baculovirus expression system, as described elsewhere [32]. All other recombinant human chemokines, except for MCP-2, were purchased from Peprotech (Rocky Hill, NJ). Recombinant human MCP-2 was purchased from R&D Systems (Minneapolis, MN). The baculovirus-expressed MCP-1 was iodinated by Amersham International plc. (Buckinghamshire, UK). The specific activity was 2000 Ci/mmol.

### 2.2. Cells

A human embryonic kidney 293 cell line expressing EB virus nuclear antigen-1 (EBNA1), designated 293-EBNA cell line, was purchased from Invitrogen (San Diego, CA) and maintained in DMEM (Gibco BRL) containing 10% heat-inactivated FCS, 80  $\mu\text{g}/\text{ml}$  gentamicin and 0.2 mg/ml Geneticin (Gibco BRL).

### 2.3. Establishment of a cell line stably expressing the MCP-1 receptor

The MCP-1 receptor cDNA fragment (identical to CC CKR2B [24]) cut from the plasmid described elsewhere [25] was ligated into the *NotI* site of pCEP-4 (Invitrogen). 293-EBNA cells were transfected with the plasmid purified with QIAGEN (Diagen) by the Lipofectamine (Gibco BRL) method and selected in DMEM containing 10% heat-inactivated FCS, 0.2 mg/ml hygromycin and 0.2 mg/ml Geneticin for 2 weeks. Several clones were obtained, and the clone showing the highest expression in MCP-1 binding experiments was used in this study.

### 2.4. Measurement of intracellular $\text{Ca}^{2+}$ concentration

Changes in the intracellular  $\text{Ca}^{2+}$  concentration were measured with a fluorescent probe, Fura-2, essentially as described elsewhere [25]. The transfected 293-EBNA cells growing in log-phase were harvested, suspended in HEPES-Tyrod buffer (129 mM NaCl, 8.9 mM  $\text{NaHCO}_3$ , 2.8 mM KCl, 0.8 mM  $\text{KH}_2\text{PO}_4$ , 5.6 mM dextrose, 10 mM HEPES, 0.8 mM  $\text{MgCl}_2$ ; pH 7.4) and incubated with 1  $\mu\text{M}$  Fura-2 AM (Dojindo, Japan) at 37°C for 30 min. The cells were washed with HEPES-Tyrod buffer and resuspended (about  $5 \times 10^6$  cells/ml) in the same buffer containing 1 mM  $\text{CaCl}_2$ . After incubation at 37°C for 5 min, 500  $\mu\text{l}$  of the cell suspension was placed in a CAF-100 calcium analyzer (JASCO Corporation, Japan) with continuous stirring at 37°C. Samples were excited at 340 nm and 380 nm, and the emission at 500 nm was monitored. The data were recorded as the relative ratio of fluorescence excited at 340 nm and 380 nm.

### 2.5. Measurements of adenylyl cyclase

The adenylyl cyclase activity of the transfected 293-EBNA cells was measured essentially by the method of Myers et al. [29], but the amount of cyclic AMP was determined by ELISA as described elsewhere [33]. Transfected cells ( $5 \times 10^5$ ) were incubated at 37°C for 20 min in 100  $\mu\text{l}$  of RPMI 1640 medium containing 10% FCS, 1 mM

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**Abbreviations:** MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T expressed and secreted; EBNA, EB virus nuclear antigen;  $\text{IC}_{50}$ , concentration giving 50% inhibition

3-isobutyl-1-methylxanthine, 10  $\mu$ M forskolin and various concentrations of chemokines. The reaction was stopped by placing the tubes in a heat block at 98°C for 10 min. The tubes were then clarified by centrifugation at 10 000  $\times g$  for 15 min. The amount of cyclic AMP in the supernatant was determined with an ELISA kit (Amersham International plc.).

## 2.6. Binding experiments

The transfected 293-EBNA cells growing in log-phase were used for binding experiments. Transfected 293-EBNA cells ( $10^6$ ) in 200  $\mu$ l of binding buffer (RPMI 1640 containing 1 mg/ml BSA and 25 mM HEPES; pH 7.4) were incubated at 18°C for 1 h with 125 pM [ $^{125}$ I]MCP-1 and various concentrations of unlabeled ligands. After the incubation, the cells were collected in a centrifuge, washed in 500  $\mu$ l of PBS containing 1 mg/ml BSA and collected again. The cell-associated radioactivity was counted using a gamma counter.

## 3. Results

### 3.1. $Ca^{2+}$ mobilization by MCPs in the MCP-1 receptor transfectants

The changes in the intracellular  $Ca^{2+}$  concentration induced by the three MCPs were measured in the CC CKR2B-transfected cells (Fig. 1). MCP-1 and MCP-3 each increased the intracellular calcium concentration in a dose-dependent manner, and MCP-1 was more potent than MCP-3. MCP-2 induced as potent a response as MCP-1 at 4 nM, although the increase by MCP-2 became saturated at concentrations higher than 4 nM. It was confirmed that none of the three MCPs induced any change in the intracellular  $Ca^{2+}$  concentration in the parental 293-EBNA cells (data not shown).

### 3.2. Inhibition of adenylyl cyclase by MCPs in the MCP-1 receptor transfectants

Since it has been reported that inhibition of adenylyl cyclase is also characteristic of the intracellular signal transduction by MCPs [30], we examined the effects of the MCPs on the adenylyl cyclase activity induced by forskolin in our transfected

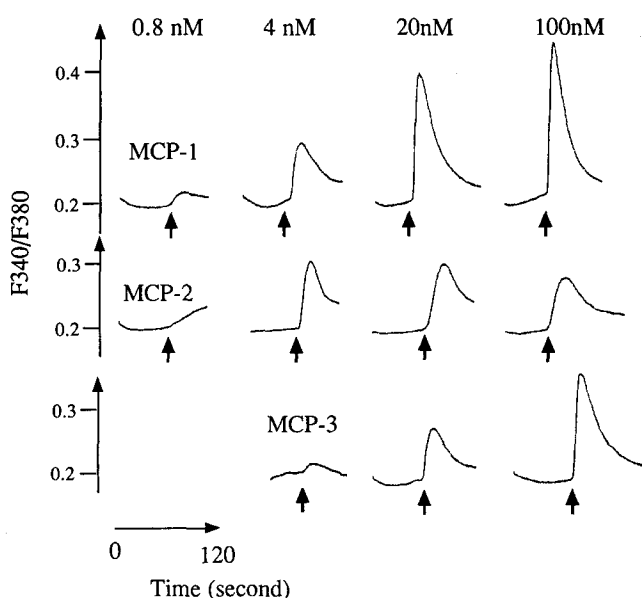


Fig. 1.  $Ca^{2+}$  mobilization by MCPs in the Fura-2-loaded MCP-1 receptor-transfected 293-EBNA cells. The fluorescence ratio (F340/F380) was monitored during addition of various concentrations (0.8–100 nM) of MCP-1, MCP-2 and MCP-3 at the times indicated by the arrows.

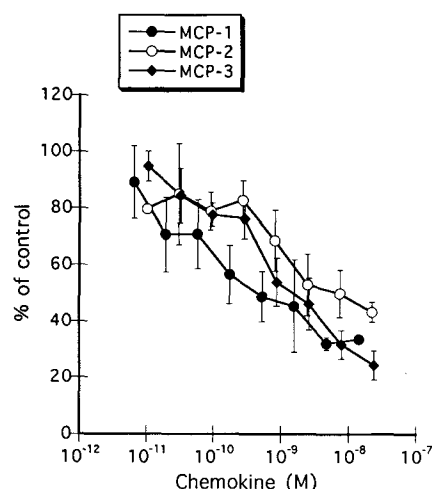


Fig. 2. Inhibition of adenylyl cyclase in the MCP-1 receptor-transfected 293-EBNA cells by MCPs. Cells ( $5 \times 10^6$ ) were stimulated with 10  $\mu$ M forskolin in the presence or absence of various concentrations of MCP-1, MCP-2 and MCP-3. The amount of cyclic AMP accumulated in the cells was determined by ELISA. The amount of cyclic AMP in the absence of MCPs was treated as the 100% control. Each point is the mean  $\pm$  S.E.M. of three separate experiments, each performed in triplicate.

cells (Fig. 2). All three MCPs inhibited adenylyl cyclase activity in a dose-dependent manner. The  $IC_{50}$  values were as follows: 0.3 nM for MCP-1, 7 nM for MCP-2, and 1.5 nM for MCP-3. Taken together with the results for calcium mobilization, these results indicate that MCP-2 can exert its effects through CC CKR2B at a physiological concentration.

### 3.3. Binding studies on MCPs in the MCP-1 receptor transfectants

Fig. 3A shows the inhibition of [ $^{125}$ I]MCP-1 binding to the receptor transfectants by unlabeled chemokines. MCP-1 and MCP-3 inhibited [ $^{125}$ I]MCP-1 binding in a dose-dependent manner ( $IC_{50} = 1.5$  nM for MCP-1, 12 nM for MCP-3), while MIP-1 $\alpha$ , -1 $\beta$ , RANTES and IL-8 did not. On the other hand, [ $^{125}$ I]MCP-1 binding was rather increased as the added concentration of unlabeled MCP-2 became higher. With the parental 293-EBNA cells, as well, a similar increase in [ $^{125}$ I]MCP-1 binding was seen to accompany addition of unlabeled MCP-2, but not other chemokines (Fig. 3B).

## 4. Discussion

In this study we have shown that MCP-2, as well as MCP-1 and MCP-3, acts as a functional ligand for the MCP-1 receptor, CC CKR2B. Thus, MCP-2 induced a  $Ca^{2+}$  influx and inhibited adenylyl cyclase in the CC CKR2B-transfected cells, although it was not necessarily more potent than MCP-1 and MCP-3. It has been controversial whether the intracellular signal of MCP-2 can be also transduced by CC CKR2B, although it is well established that MCP-3, in addition to MCP-1, is a functional ligand for CC CKR2B [30,31]. In terms of the ability to induce chemotaxis of either monocytes or T cells, which are known to express CC CKR2B [34], it has been reported that MCP-2 is as potent as MCP-1 and MCP-3 [9,10,13,14]. On the other hand, the signal transduction mechanism by which MCP-2 induces chemotaxis in monocytes has been reported to be different from that of either MCP-1

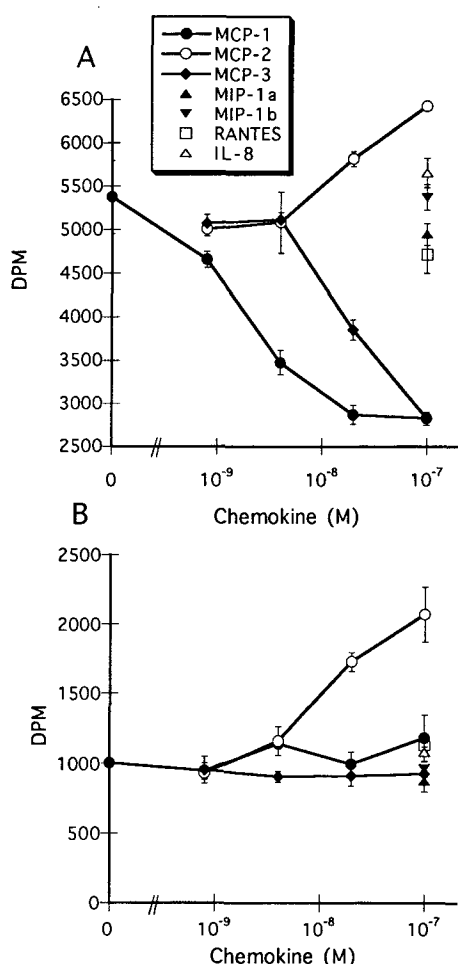


Fig. 3. Competition of [ $^{125}$ I]MCP-1 binding to the MCP-1 receptor-transfected 293-EBNA cells (A) or parental 293-EBNA cells (B) with unlabeled chemokines. Cells ( $1 \times 10^6$  (A) or  $6 \times 10^5$  (B)) were incubated at  $18^\circ\text{C}$  for 1 h with 125 pM [ $^{125}$ I]MCP-1 in the presence of various concentrations of unlabeled MCP-1, MCP-2, MCP-3, MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES and IL-8. The cell-associated radioactivity was measured. Each point represents the mean  $\pm$  S.E.M. of triplicate measurements.

or MCP-3 [8]. In addition, previous reports with CC CKR2B-transfected cells showed that the MCP-1 receptor did not transduce the intracellular signal of MCP-2 in terms of  $\text{Ca}^{2+}$  influx or inhibition of adenylyl cyclase even at 500 nM [30,31]. Therefore, our present findings on MCP-2 responsiveness in our CC CKR2B-transfected cell system contradict those earlier studies. This inconsistency may be partly due to a difference in either the manner of existence or the number of receptors on the transfected cells.

MCP-2 did not inhibit [ $^{125}$ I]MCP-1 binding to the CC CKR2B-transfected cells; rather, it increased the [ $^{125}$ I]MCP-1 binding. There have been several reports showing the anomalous binding nature of CC chemokines in the corresponding receptor-transfected 293 cell system. Thus, Neote et al. reported that [ $^{125}$ I]RANTES binding to the CC CKR1-transfected 293 cells was rather increased by unlabeled RANTES, although [ $^{125}$ I]MIP-1 $\alpha$  binding was inhibited by unlabeled MIP-1 $\alpha$  in a dose-dependent manner [22]. It has been also reported that [ $^{125}$ I]eotaxin failed to show specific binding to the CC CKR3-transfected 293 cells [35]. Therefore, we conclude that MCP-2 acts as a functional ligand for CC CKR2B,

although it did not inhibit [ $^{125}$ I]MCP-1 binding to CC CKR2B-transfected cells. These results also support the concept that either the manner of existence or the number of receptors on the cell surface may affect the ligand binding properties of the receptor.

In conclusion, our present data indicate that MCP-2 can act as a functional ligand for the MCP-1 receptor, CC CKR2B, although it did not inhibit [ $^{125}$ I]MCP-1 binding to the CC CKR2B-transfected cells.

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