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Differential effects of 9-*cis* retinoic acid on expression of CC chemokine receptors in human monocytes

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Abstract

9-*cis* Retinoic acid (9-CRA) is a lipophilic molecule that binds to the retinoid X receptor (RXR). Although retinoic acid (RA) has been known to regulate neutrophil differentiation, a specific role for 9-CRA in chemokine-mediated cellular processes remains obscure. We investigated the effects of 9-CRA on expression of CC chemokine receptors (CCRs) in human monocytic THP-1 cells and peripheral blood monocytes. RNase protection assay was performed to examine the mRNA levels of CCRs in 9-CRA-treated THP-1 cells. mRNA expression of CCR1 and CCR2 was induced in both a dose and time dependent manner. CCR1 and CCR2 mRNA expression began to increase from 6 h after a 100 nM 9-CRA treatment and reached a maximal level at 12 h. Surface expression of CCRs was monitored by flow cytometry. CCR1 and CCR2 surface expression increased in 9-CRA-treated THP-1 cells, but not in untreated cells. Calcium mobilization and chemotactic activity were determined to examine the effect of 9-CRA on cell movement. The intracellular Ca^{2+} concentration and the chemotactic activity increased in 9-CRA-treated cells in response to the CCR1-dependent chemokines Lkn-1, MIP-1α, and RANTES, and the CCR2-specific chemokine MCP-1. Increased surface expression of CCR1 and the Ca^{2+} influx due to 9-CRA were confirmed in peripheral blood monocytes. Taken together, 9-CRA increases the expression levels of mRNA and protein of both CCR1 and CCR2, and the cell migration ability in THP-1 cells and peripheral blood monocytes, indicating that 9-CRA may regulate inflammatory processes through an increased response to CCR1- and CCR2-dependent chemokines.

Keywords: Monocyte; Chemokine; Chemotaxis; Inflammation; Retinoic acid; Chemokine receptor

1. Introduction

Retinoic acid (RA), a metabolite derived from retinol or vitamin A, exists in the two stereoisomers all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-CRA). RA acts through retinoid receptors belonging to the superfamily of steroid/thyroid/retinoid hormone receptors [1]. Retinoid receptors are divided into two groups depending upon the isomers of RA that they bind. The retinoic acid receptor (RAR) binds ATRA and the retinoid X receptor (RXR) binds 9-CRA. RXR can homodimerize with RXR or heterodimerize with a variety of nuclear receptors, including RAR, the thyroid hormone receptor (T3R), the vitamin D3 receptor (VD3R), the peroxisome proliferator activator receptor (PPAR), the liver X receptor (LXR) and several orphan receptors [2–5]. It is widely known that RA is involved in various biological functions, including cellular growth, differentiation, and apoptosis [6]. RA also acts as a potent modulator that regulates immune responses and inflammatory diseases [7].

Chemokines, a superfamily of chemoattractant cytokines, are classified into four major groups based on the positions of the first two cysteines. CXC (α) chemokines with a Glu-Leu-Arg (ELR) motif in front of the first cystein function as neutrophil chemoattractants [8]. CXC chemokines without ELR-motif and CC (β) chemokines act on monocytes, T lymphocytes, basophils, eosinophils, NK

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cells, and dendritic cells [9]. The only known C (γ) chemokine, lymphotactin, plays an important role in T lymphocytes and NK cells [10]. The CX_3C (δ) chemokine fractalkine is effective on T lymphocyte and monocyte migration [11]. Chemokines exert biological effects through chemokine receptors belonging to a large family of the seven transmembrane G protein-coupled receptors. They are classified into two major chemokine receptor subfamilies depending on the chemokines they bind. CXCR1 through CXCR6, which bind CXC chemokines, and CCR1 through CCR11, which bind CC chemokines, have been reported [12–14].

Chemokines and chemokine receptors play essential roles in leukocyte trafficking under inflammatory condition as well as many other immune responses including cell growth and differentiation. Therefore, regulation of the expression levels of chemokine receptors probably results in alteration of immune responses against infection, inflammation, and immune diseases. Chemokine receptors can be regulated by several cytokines in a variety of immune cell types. It has been reported that IL-2 induces expression of CCR1 and CCR2 in T lymphocytes and NK cells [15,16]. Recent studies have demonstrated that IL-10 increases expression of CCR1, CCR2, and CCR5 in human monocytes, while the pro-inflammatory stimulators TNF-α, IL-1 and LPS decrease CCR2 expression in these cells [17,18]. Expression of both CCR1 and CCR3 was elevated by IFN-α and IFN-γ in monocytoid U937 cells [19,20]. Although there have been continuous reports regarding the effects of cytokines on expression of CC chemokine receptors (CCRs), the effect of RA on expression of CCRs is not well understood. In this study, we have investigated the effects of RA on expression of CCRs and the effects of RA on chemotactic activities in human monocytic THP-1 cells and monocytes from human peripheral blood.

2. Materials and methods

2.1. Materials

RPMI 1640, fetal bovine serum (FBS), and Hanks' Balanced Salt Solution (HBSS) were purchased from Life Technologies, Inc. ATRA, 9-CRA, and FITC-conjugated rabbit anti-rat IgG were obtained from Sigma. Normal rabbit and mouse IgG, and FITC-conjugated goat anti-mouse IgG were from Santa Cruz Biotechnology. [α-^{32}P]UTP and Ficoll-Hypaque were from Amersham Pharmacia Biotech. Recombinant human rLkn-1 was the kind gift of Greencross Life Science Corp. Recombinant human MIP-1α, MIP-1β, MCP-1, RANTES, eotaxin, and anti-CCR1, anti-CCR2, anti-CCR3 and anti-CCR5 antibodies were products of R&D Systems. Fluo3-acetoxymethyl (fluo3-AM) ester and pluronic F-127 were purchased from Molecular Probes.

2.2. Cell culture

Human THP-1 cells were obtained from the American Type Culture Collection and were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.3. Isolation of monocytes

Monocytes were obtained from heparinized whole blood of healthy donors. After peripheral blood mononuclear cells were separated by centrifugation over Ficoll-Hypaque density gradient, monocytes were purified using monocyte negative isolation kit as described previously [21]. Monocyte purity was assessed by flow cytometry analysis. Isolated cells were routinely >80% positive for CD14. Viability as assessed by trypan blue stain was >99% for isolated cells. The monocytes were incubated in RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotics in the presence or absence of 9-CRA.

2.4. RNase protection assay

Human THP-1 cells seeded into 150 mm dishes at 1×10^7 cells/dish were cultured in RPMI. Cells were starved for 24 h in 0.5% FBS. After treatment with 9-CRA, the cells were harvested and total RNA was extracted using Trizol reagent (Life Technologies) as described by manufacturer's instruction. The hCR5 template set including DNA templates for CCR1, CCR3, CCR4, CCR5, CCR8, CCR2a + b, L32, and GAPDH was purchased from Pharmingen. [α-^{32}P]UTP-labeled RNA probes were synthesized from the hCR5 template set by T7 RNA polymerase. Probes were hybridized overnight with target RNA. Free probes and other single-stranded RNA were digested with RNase, followed proteinase K treatment and phenol chloroform extraction. After ethanol precipitation with 4 M ammonium acetate, the RNase-protected probes were dissolved in loading buffer. Denaturing polyacrylamide gel (4.75%) was pre-electrophoresed in 0.5 × Tris-borate EDTA (TBE) buffer for 30 min prior to loading and electrophoresis continued at 55 W for 2 h. Dried gel was visualized by autoradiography. Specific bands were identified by their individual migration patterns in comparison with the undigested probes. The bands were normalized with GAPDH and L32 bands.

2.5. FACS analysis

Human THP-1 cells seeded into 6-well plates at 5×10^5 cells/well were cultured in RPMI 1640. After treatment with 9-CRA, the cells were harvested and washed with PBS buffer containing 0.5% BSA. Blocked cells with normal rabbit IgG were separated into new tubes. Each tube was added with PBS buffer containing anti-CCR1, anti-CCR2, anti-CCR3 or anti-CCR5 antibodies. Baseline

fluorescence was obtained by incubation with normal mouse IgG. Following incubation and washings, cells were incubated at 4 °C for 30 min with FITC-conjugated goat anti-mouse IgG or rabbit anti-rat IgG. Finally, cells were washed and analyzed on a FACSort cytofluorimeter (Becton Dickinson). For each experiment, 10,000 events were collected. The mean intensity of untreated cells was considered as 100% and alteration of CCR surface expression by 9-CRA or ATRA treatment was evaluated as mean intensity of 9-CRA or ATRA treated cells/mean intensity of untreated cells × 100.

2.6. Measurement of Ca^{2+} influx

Change of intracellular Ca^{2+} concentration was monitored as previously described [22]. Briefly, cells were harvested, washed, and resuspended in HBSS containing 1 μM fluo3-AM and 0.01% pluronic F-127. After incubation for 30 min at 37 °C, dye-loaded cells were washed three times, resuspended in Ca^{2+} assay buffer (135 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 10 mM glucose, 5 mM HEPES, 0.1% BSA, pH 7.4), and incubated for 30 min at 37 °C. Chemokines (200 ng/ml) were added, then intracellular Ca^{2+} concentration was analyzed at 488 nm of excitation wavelength and 530 nm of emission wavelength on a FACSort cytofluorimeter. Baseline fluorescence was monitored for 40 s before addition of chemokines.

2.7. Chemotaxis assay

Cell migration was performed using a 48-well microchamber (Neuroprobe) as previously described [23]. The lower wells were filled with 28 μl buffer alone or with buffer containing chemokine and a polyvinylpyrrolidone-free filter (Neuroprobe) with 5 μm pores was placed over lower well. Membranes were pre-coated with RPMI 1640 containing rat tail collagen type I at 4 °C overnight. The upper wells were filled with 50 μl of THP-1 cells at 2×10^6 cells/ml in RPMI 1640 containing 1% BSA and 30 mM HEPES. After incubation for 5 h at 37 °C, the filters were removed from the chamber, washed, fixed, and stained with Diff-Quick (Baxter). The cells of four randomly selected fields per well were counted using Axiovert 25 (Carl Zeiss) and Visus Image Analysis System (Foresthill Products). The chemotactic index (CI) was calculated from the number of cells migrating to the test chemokines divided by that migrating to the controls.

2.8. Statistical analysis

Data are expressed as means ± S.E.M. Statistical differences were analyzed by using Mann–Whitney *U* test for a two-group comparison or one-way ANOVA for a three-group comparison. The SPSS statistical software package (Version 10.0) was used for statistical analysis. The significant value is defined as $P < 0.05$.

3. Results

3.1. 9-CRA increases CCR1 and CCR2 mRNA expression in THP-1 cells

To investigate the effect of 9-CRA on mRNA expression of CCRs, we performed an RNase protection assay in THP-1 cells treated with increasing concentrations of 9-CRA (Fig. 1A). CCR1 and CCR2 mRNA expression was detected in cells incubated with the medium without 9-CRA. After addition of various concentrations of 9-CRA for 12 h, the mRNA expression of both CCR1 and CCR2 increased with an effect being apparent at a 9-CRA concentration of 10 nM. A plateau was reached at 100 nM. mRNA expression of the chemokine receptors CCR3, CCR4, CCR5, and CCR8 was not affected by 9-CRA treatment. Exposure of THP-1 cells to 9-CRA for different periods of time resulted in an increased mRNA expression of both CCR1 and CCR2. Increased mRNA expression of both CCR1 and CCR2 was detected within 6 h and reached to a maximum level at 12 h (Fig. 1B). These results indicate that CCR1 and CCR2 mRNA expression is up-regulated by 9-CRA in THP-1 cells.

3.2. Surface expression of CCR1 and CCR2 is induced in 9-CRA-treated THP-1 cells

Since CCR1 and CCR2 mRNA expression was induced by 9-CRA, we examined whether surface protein expression of CCRs is affected by 9-CRA treatment. A strong CCR1 surface expression was detected in 9-CRA-treated THP-1 cells compared with control cells assessed by flow cytometry (Fig. 2). This result correlates with CCR1 mRNA expression due to 9-CRA stimulation. Although CCR2 mRNA expression increased due to 9-CRA treatment, CCR2 surface expression was little affected in response to 9-CRA (Fig. 2). Surface expression of CCR3 and CCR5 was not affected by 9-CRA treatment (Fig. 2).

We next examined dose and time dependency of CCR1 surface protein expression due to 9-CRA in THP-1 cells. CCR1 surface expression began to increase at 10 nM 9-CRA concentration and reached a peak at 100 nM (Fig. 3A). A concentration of 100 nM 9-CRA induced a maximum CCR1 protein expression that was sustained up to 48 h (Fig. 3B). CCR2 surface expression due to 9-CRA also increased in both a dose and time dependent manner, although the effect was minimal (data not shown). Taken together, 9-CRA up-regulated mRNA and protein expression of both CCR1 and CCR2, but did not affect mRNA expression of CCR3, CCR4, CCR5, and CCR8 and protein expression of CCR3 and CCR5.

3.3. 9-CRA induces Ca^{2+} mobilization in response to CCR1- and CCR2-dependent chemokines in THP-1 cells

Most chemokine receptors increase the Ca^{2+} concentration within cells after binding chemokine. We confirmed

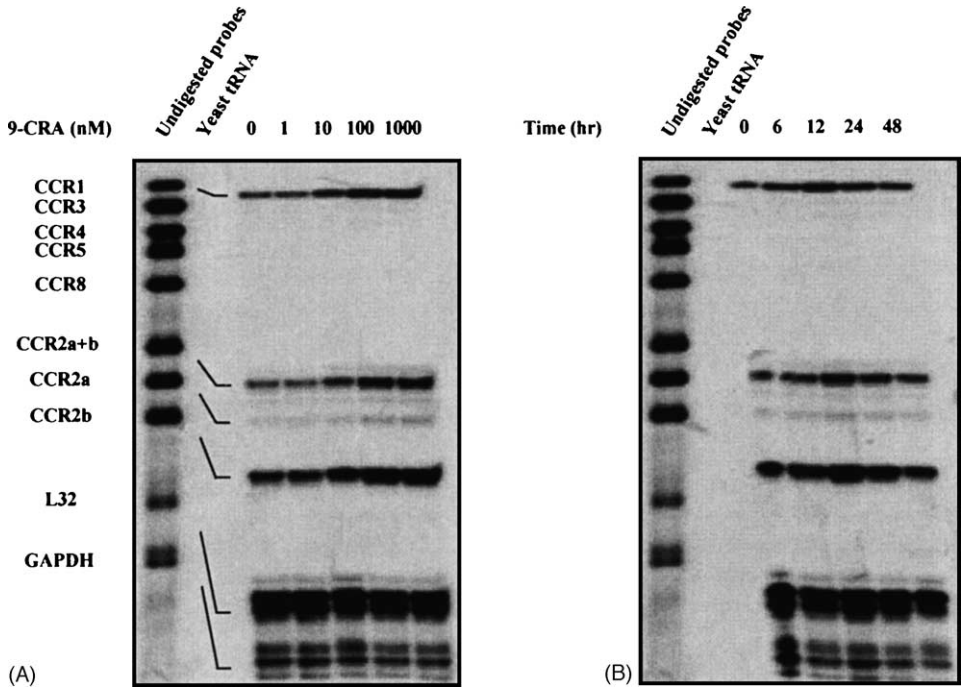

Fig. 1. 9-CRA increases CCR1 and CCR2 mRNA expression in THP-1 cells. THP-1 cells were serum starved with 0.5% serum for 24 h, and were stimulated with the indicated concentrations of 9-CRA for 12 h (A). For time dependence experiments, serum starved THP-1 cells were stimulated with 100 nM 9-CRA for the indicated time (B). Total RNA was extracted from 9-CRA treated THP-1 cells. RNA levels of CCRs were analyzed by RNase protection assay using the hCR5 template set as described in Section 2. Specific bands were detected by comparing with the undigested probes. The bands were normalized with GAPDH and L32 bands. Data are expressed as representative of three individual experiments.

increased CCR1 and CCR2 expression due to 9-CRA using a Ca^{2+} influx assay. THP-1 cells were pre-treated with 9-CRA and stimulation was initiated by addition of 200 ng/ml CCR1- or CCR2-dependent chemokines, followed by measurement of the intracellular Ca^{2+} concentration. Ca^{2+} mobilization into 9-CRA-treated THP-1 cells was apparently enhanced by the CCR1-dependent chemokines Lkn-1, MIP-1α, and RANTES, and by the CCR2 ligand MCP-1 (Fig. 4). Despite a low surface expression of CCR2, 9-CRA markedly increased the Ca^{2+} influx due to MCP-1 to the same degree as for the CCR1-dependent chemokines. These results indicate that CCR2 has a stronger efficiency than CCR1 in THP-1 cells. Neither the CCR3 specific ligand eotaxin nor the CCR5 agonist MIP-1β affected 9-CRA-treated THP-1 cells or untreated cells. Also Ca^{2+} mobilization into 9-CRA-treated THP-1 cells was not affected by fMet-Leu-Phe (fMLP) used as a positive control. These data indicate that 9-CRA increases intracellular Ca^{2+} mobilization in response to CCR1 and CCR2 agonists in THP-1 cells in association with CCR1 and CCR2 up-regulation.

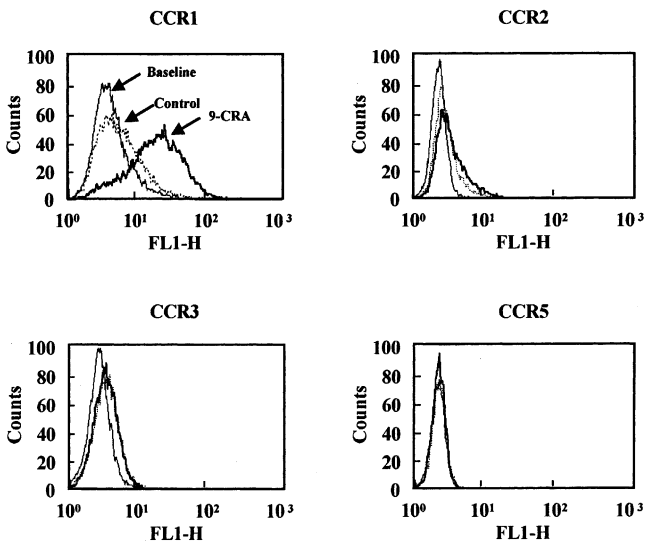

Fig. 2. Surface expression of CCR1 and CCR2 is induced in 9-CRA-treated THP-1 cells. Serum starved THP-1 cells were incubated in the absence (dotted line; control) or presence of 100 nM 9-CRA for 12 h (thick line; 9-CRA). Cells were harvested and analyzed by fluorescence-activated cells sorter using monoclonal anti-CCR1, anti-CCR2, anti-CCR3, or anti-CCR5 antibodies. Baseline fluorescence was obtained by incubating normal mouse IgG (thin line; baseline). Data are expressed as representative of three individual experiments.

3.4. 9-CRA enhances THP-1 cell migration in response to CCR1- and CCR2-dependent chemokines

Since cell migration is the primary cellular event initiated by the chemokine receptor interacting with chemokine, we investigated whether 9-CRA affects the chemotactic activities of CCR1- and CCR2-dependent chemokines in THP-1 cells. To examine the chemotactic activity in 9-CRA-treated THP-1 cells, a cell migration assay was performed in a 48 well microchamber. Fig. 5 shows that THP-1 cell migration in response to Lkn-1,

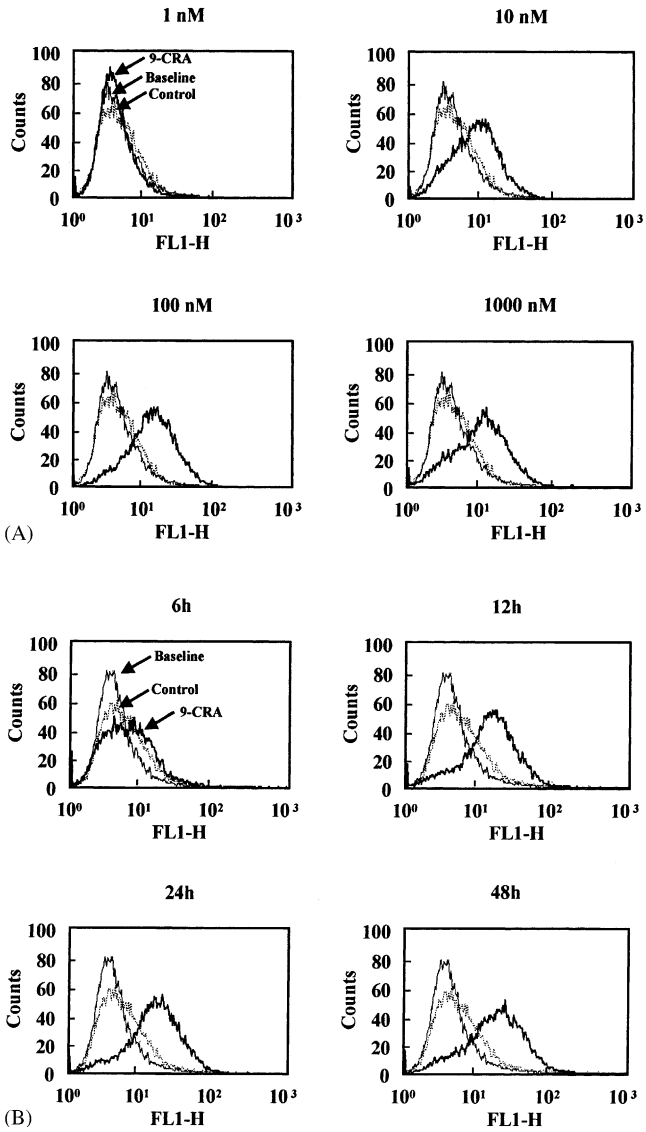

Fig. 3. 9-CRA induces CCR1 expression in both a dose and time dependent manner. (A) Serum starved THP-1 cells were incubated in the absence (dotted line; control) or presence of indicated concentrations of 9-CRA for 12 h (thick line; 9-CRA). (B) For time dependence experiments, serum starved THP-1 cells were incubated in the absence (dotted line; control) or presence of 100 nM 9-CRA for the indicated time (thick line; 9-CRA). Cells were harvested and analyzed by fluorescence-activated cells sorter using monoclonal anti-CCR1 antibody. Baseline fluorescence was obtained by incubating with normal mouse IgG (thin line; baseline). Data are expressed as representative of three individual experiments.

MIP-1α and RANTES increased at least four-fold based on the chemotactic index after 9-CRA stimulation. Although CCR2 surface expression was low in 9-CRA-treated THP-1 cells, the chemotactic effect of MCP-1 was increased seven-fold, consistent with data from the Ca^{2+} influx assay. However, the chemotactic activities of eotaxin and MIP-1α in 9-CRA-treated THP-1 cells were comparable with untreated cells, indicating that CCR3 and CCR5 expression is not affected by 9-CRA treatment. These data demonstrate that 9-CRA up-regulates CCR1 and CCR2 expression at both the mRNA and the protein level in THP-1 cells and enhances chemotactic activities of CCR1- and CCR2-dependent chemokines.

3.5. Effects of 9-CRA and ATRA on CCR1 expression in THP-1 cells

Retinoids have different biological functions and therapeutic effects according to their isoforms [24–27]. We examined whether 9-CRA acts differently than ATRA on expression of chemokine receptors in THP-1 cells. FACS analysis was performed to examine the surface expressions of CCR1 and CCR2 in the presence and absence of two different RA isoforms. 9-CRA increased the CCR1 expression more effectively than ATRA in THP-1 cells (Fig. 6A and B). Statistical analysis showed that there was a significant difference ($P < 0.05$) between the two results. However, there was no difference between 9-CRA and ATRA in CCR2 surface expression (Fig. 6A and B). We next performed a Ca^{2+} influx assay to examine functional differences between 9-CRA and ATRA. 9-CRA increased Ca^{2+} movement into THP-1 cells in response to CCR1-dependent chemokines more effectively than did ATRA. Results from the FACS analysis showed that there was no significant difference between 9-CRA and ATRA in Ca^{2+} influx in response to MCP-1 (Fig. 6C). These data indicate that 9-CRA affects CCR1 expression and the Ca^{2+} influx induced by CCR1 ligands much more effectively than does ATRA.

3.6. 9-CRA increases CCR1 expression and the intracellular Ca^{2+} concentration in response to CCR1-dependent chemokines in human peripheral blood monocytes

Since we examined modification of chemokine receptors and cellular functions due to 9-CRA in THP-1 cells, we investigated whether 9-CRA has the same effect in human peripheral blood monocytes. Isolated monocytes are generally differentiated when they are cultured in vitro, and monocyte differentiation results in modification of specific chemokine receptors [28]. Therefore, we used freshly isolated monocytes and cells that were incubated for 12 h in culture condition as a control. As shown in Fig. 7A and B, CCR1 expression was enhanced in monocytes treated with 9-CRA for 12 h compared with freshly isolated monocytes and cultured monocytes for 12 h with a statistical significance ($P < 0.01$). CCR2 expression decreased in monocytes that were incubated for 12 h compared with freshly isolated monocytes, in agreement with a previous report [28]. However, there was no difference between 9-CRA-treated and untreated monocytes (Fig. 7A and B). Also, 9-CRA had no effect on loss of CCR2 expression during monocyte differentiation (Fig. 7A and B). The intracellular Ca^{2+} concentration in resoponse to the CCR1-dependent chemokines Lkn-1, MIP-1α and

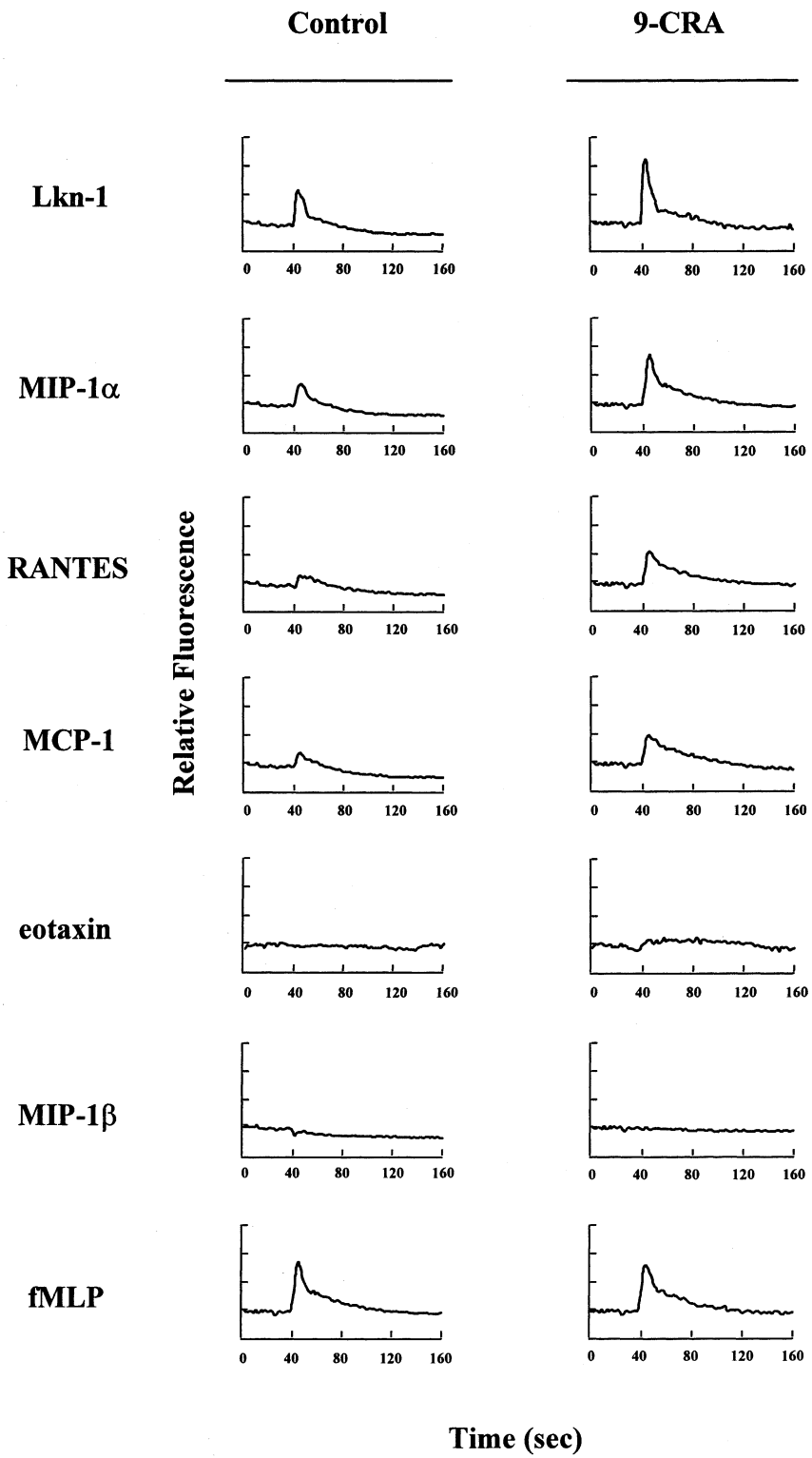

Fig. 4. Ca^{2+} mobilization by CCR1- and CCR2-dependent chemokines is induced in 9-CRA-treated THP-1 cells. Serum starved THP-1 cells were incubated in the absence (control) or presence of 100 nM 9-CRA for 12 h (9-CRA), then loaded with fluo3-AM. Dye-loaded cells were stimulated with various chemokines (200 ng/ml) and fMLP (1 μM), and subsequently monitored for 120 s. Data are expressed as representative of two independent experiments.

RANTES increased in 9-CRA-treated monocytes compared with untreated monocytes (Fig. 7C). CCR3 and CCR5 expression and Ca^{2+} mobilization due to eotaxin and MIP-1β were not altered by treatment of 9-CRA for 12 h (data not shown). Taken together, 9-CRA increases CCR1 expression and the intracellular Ca^{2+} concentration in response to CCR1-dependent chemokines in human peripheral blood monocytes.

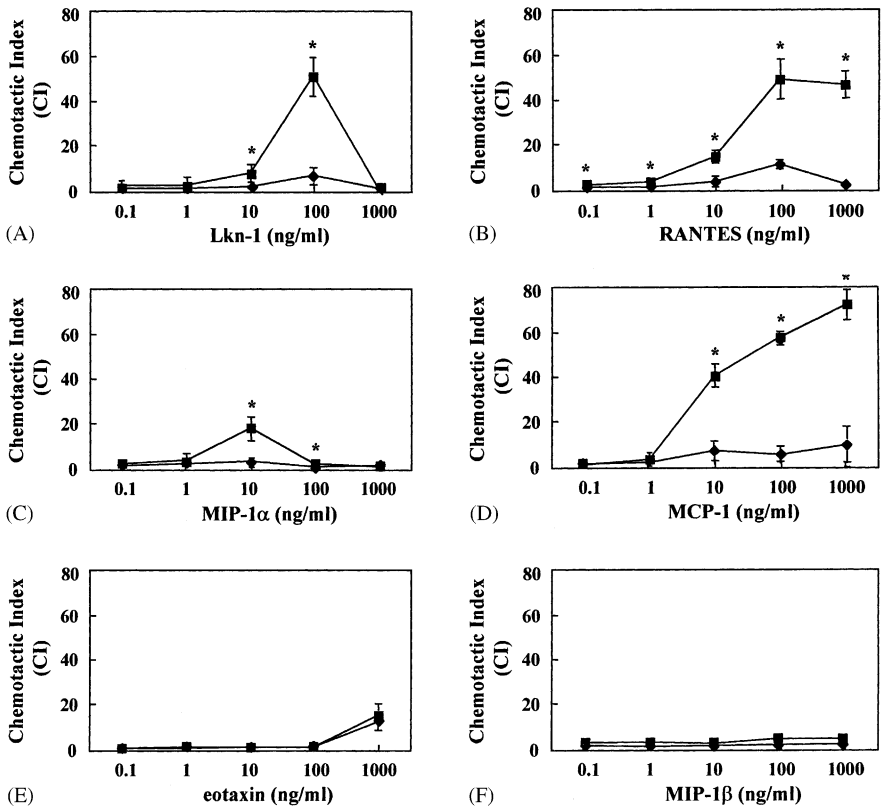

Fig. 5. 9-CRA enhances THP-1 cell migration in response to CCR1 and CCR2 agonists. Serum starved THP-1 cells were incubated in the absence (◆) or presence (■) of 100 nM 9-CRA for 12 h and applied to the indicated concentrations of Lkn-1 (A), RANTES (B), MIP-1α (C), MCP-1 (D), eotaxin (E), or MIP-1β (F). After incubation for 5 h, filters were stained with Diff-Quick. The number of cells that migrated was counted microscopically in four randomly selected fields per well. The chemotactic index (CI) was calculated from the number of cells migrating to the test chemokines divided by that migrating to the controls. Data are expressed as mean CI ± S.E.M. of three independent experiments. $^{*}P < 0.05$ is assessed as critical difference between two groups.

4. Discussion

Although RA plays an important role in regulation of the immune responses, including neutrophil maturation and cytokine release, regulation of CCR expression associated with immunomodulation in response to RA has not been characterized. In this contribution, we examined the effects of 9-CRA on expression of CCRs in human monocytic THP-1 cells and human peripheral blood monocytes. Our results demonstrate that (1) 9-CRA induces CCR1 and CCR2 mRNA expression, (2) 9-CRA increases CCR1 and CCR2 surface protein expression, (3) 9-CRA elevates the transient Ca^{2+} influx and cell migration in response to the CCR1-dependent chemokines Lkn-1, MIP-1α, and RANTES and the CCR2-specific chemokine MCP-1, (4) 9-CRA plays a dominant role in CCR1 expression and the Ca^{2+} influx in response to CCR1-dependent chemokines compared with ATRA, and (5) 9-CRA enhances CCR1 expression and the intracellular Ca^{2+} concentration in response to CCR1-dependent chemokines in human peripheral blood monocytes.

RA is involved in malignant hematopoeisis and is used as a therapeutic drug for acute promyeloblastic, acute myeloblastic and chronic myelogenous leukemia. The side effects of RA, referred to as the RA syndrome, are fever, respiratory distress, edema, and multiorgan failure [29]. The basic mechanism of this syndrome has not been fully understood, but leukocyte activation and extravasation, accumulation of leukemic cells and other leukocytes in tissues, and cytokine release are probably involved. Therefore, RA is apparently involved in an alteration of CCR expression that induces activation of leukocyte migration. It has been reported that RA elevates expression of the pro-inflammatory mediators IL-1β, IL-8 and TNF-α [30–32]. RA also induces adhesion molecules, including ICAM-3 and β2 integrin [24,33]. Our results show that 9-CRA increases THP-1 cell migration via CCR1 and CCR2 induction and that 9-CRA increases the basal level of cell migration in monocytes (data not shown). These results indicate that 9-CRA induces other elements involved in cell migration, such as adhesion molecules. Results from an aggregation assay and microscopic evaluation show that 9-CRA increases homotypic clustering (data not shown), in agreement with a previous report [33]. In addition, cell migration is probably elevated by up-regulation of adhesion molecules induced by RA. Thus, RA apparently supports the cell response to inflammation.

In contrast to pro-inflammatory effects, RA has also been reported to trigger anti-inflammatory effects depending on the cell type [34–38]. RA inhibits survival of monocytes and expression of adhesion molecules in

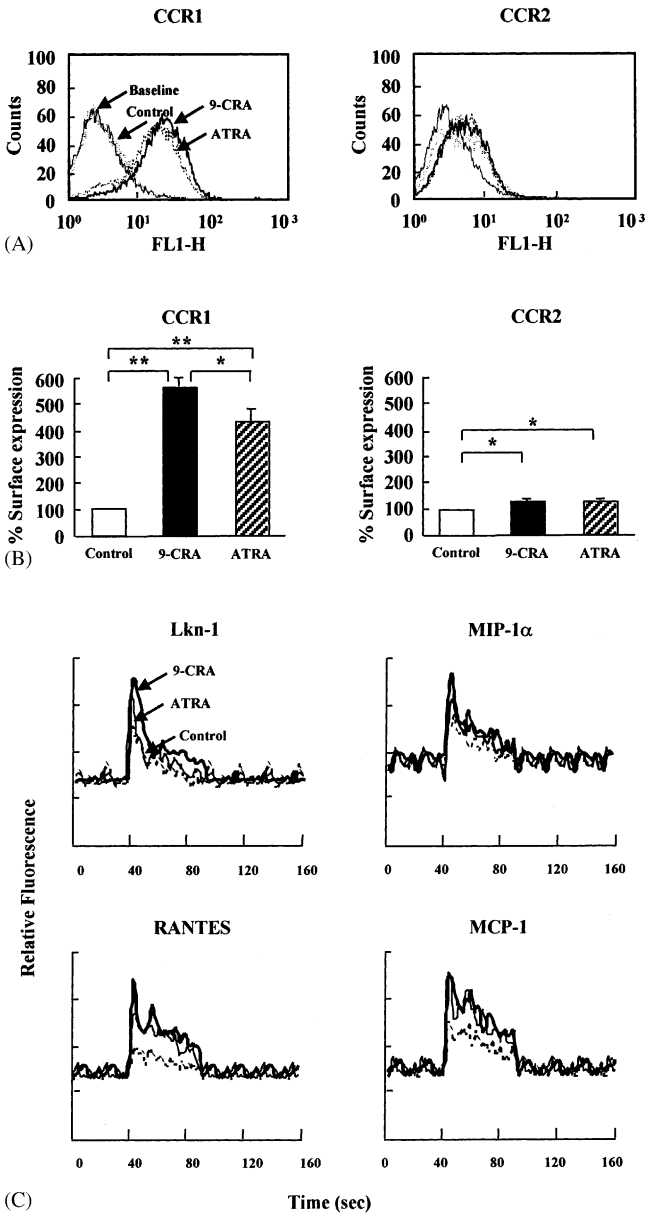

Fig. 6. Effects of 9-CRA and ATRA on CCR1 expression. (A) Serum starved THP-1 cells were incubated in the absence (short dotted line; control) or presence of 9-CRA (thick line; 9-CRA) or ATRA (long dotted line; ATRA) for 12 h. Baseline fluorescence was obtained by incubating with normal mouse IgG (thin line; baseline) Data are expressed as representative of six individual experiments. (B) Data are expressed as mean percentages [mean intensity of 9-CRA (black bar) or ATRA (hatched bar) treated cells/mean intensity of untreated cells (open bar) × 100] ± S.E.M. $^{*}P < 0.05$ and $^{**}P < 0.01$ are assessed as critical difference between the groups. (C) Serum starved THP-1 cells were incubated in the absence (dotted line; control) or presence of 100 nM 9-CRA (thick line; 9-CRA) or ATRA (thin line; ATRA) for 12 h, and loaded with Fluo3-AM. Dye-loaded cells were stimulated with various chemokines (200 ng/ml) and subsequently monitored for 120 s. Data are expressed as representative of four independent experiments.

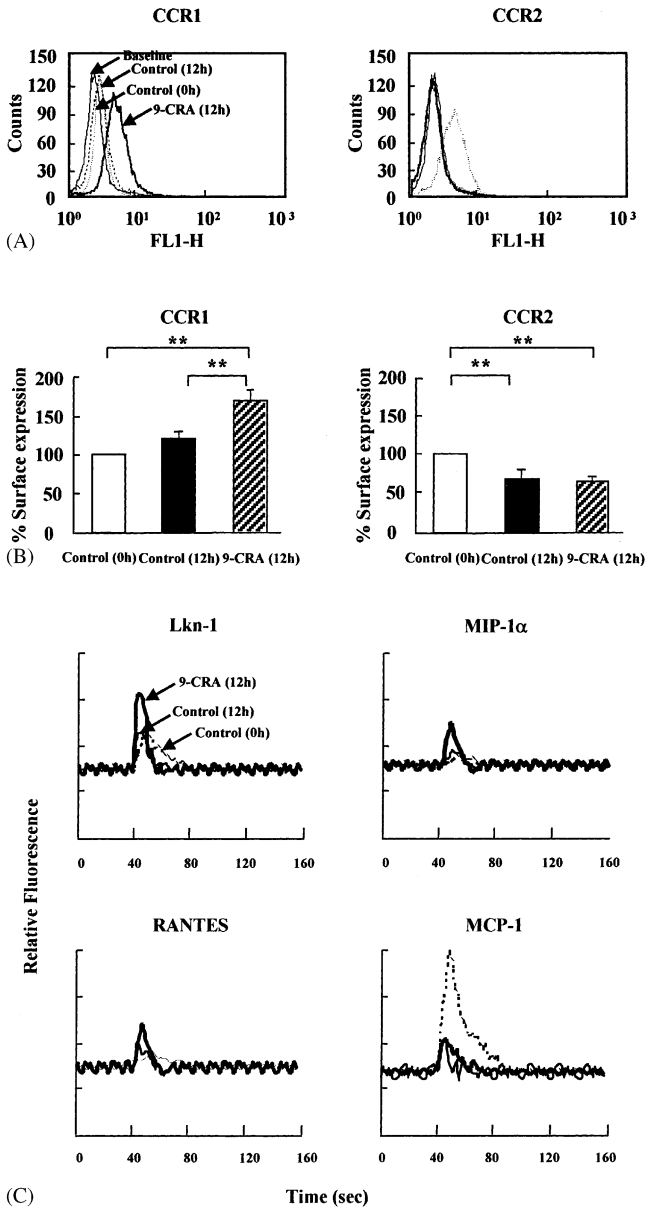

Fig. 7. 9-CRA increases intracellular Ca^{2+} concentration in response to CCR1-dependent chemokines in human peripheral blood monocytes. (A) Isolated monocytes were incubated in the absence (freshly isolated monocytes; short dotted line; control 0 h, cultured monocytes for 12 h, long dotted line; control 12 h) or presence of 9-CRA (thick line; 9-CRA 12 h) for 12 h. Baseline fluorescence was obtained by incubating with normal mouse IgG (thin line; baseline). Data are expressed as representative of five individual experiments. (B) Data are expressed as mean percentages [mean intensity of control 12 h (black bar) or 9-CRA 12 h (hatched bar)/mean intensity of control 0 h (open bar) × 100] ± S.E.M. $^{**}P < 0.01$ is assessed as critical difference among three groups. (C) Monocytes were incubated in the absence (dotted line; control 0 h, thin line; control 12 h) or presence of 100 nM 9-CRA (thick line; 9-CRA 12 h) for 12 h, then loaded with fluo3-AM. Dye-loaded cells were stimulated with various chemokines (200 ng/ml) and subsequently monitored for 120 s. Data are expressed as representative of three independent experiments.

microvascular endothelial cells [34,35]. An anti-inflammatory role for RA is further supported by a previous study showing that RA inhibits cytokine production in macrophages [36]. A recent study shows that 9-CRA increases mRNA expression, but not protein expression of CCR2, in THP-1 cells and reduces THP-1 cell migration [37]. It has been demonstrated that RXR ligands inhibit MCP-1-directed migration of monocytes [38]. These contradictory results led us to determine whether RXR ligands increase CCR1 and CCR2 expression and THP-1 cell

migration, similar to the effects of 9-CRA. We found that RXR ligands show the same effect as 9-CRA (data not shown). These conflicting results cannot be presently explained. However, the different cell culture and assay system may be affecting the results.

9-CRA is a stereoisomer of ATRA that is expressed in the liver and kidney [39]. Both ATRA and 9-CRA bind to RARs (RARα, RARβ, RARγ) but 9-CRA only binds to RXR (RXRα, RXRβ, RXRγ) [39]. The RA-mediated cellular mechanism is generally believed to be mediated by RARs. There are few comparative studies of different RAs, however recent studies have shown that 9-CRA is more effective than ATRA for induction of ICAM-3 expression in HMC-1 mast cells [24], and for inducing differentiation in these cells [25]. Our results show that 9-CRA enhances CCR1 expression and the Ca^{2+} influx in response to CCR1-dependent chemokines more effectively than does ATRA. 9-CRA has a more extensive impact on THP-1 cells than ATRA because 9-CRA mediates its effect through both RAR and RXR, whereas ATRA-induced signal is transduced only through RAR. However, there is no significant difference in CCR2 expression between 9-CRA- and ATRA-treated cells, indicating that both 9-CRA and ATRA probably induce CCR2 expression through the same signal pathway. Also, it is possible that we did not detect a significantly different effect between 9-CRA and ATRA in CCR2 induction because CCR2 expression is very low compared with CCR1. THP-1 cells and peripheral blood monocytes highly express both RARα and RXRα [40,41]. We found that RXRα expression decreased after 9-CRA treatment, indicating that 9-CRA may exert a negative feedback after stimulation (data not shown). However, we do not know whether a specific isoform of RXR is activated by 9-CRA stimulation. The exact mechanism of 9-CRA-induced RXR activation is currently under investigation.

Regulation of CCR expression following cell activation is essential for the inflammatory responses. Especially, CCR1 and CCR2 expression affects monocyte recruitment from the blood into inflamed tissues and contributes to efficient removal of invading pathogens, and is involved in the pathogenesis of the inflammatory diseases rheumatoid arthritis, atopic dermatitis, and atherosclerosis [42,43]. 9-CRA-mediated CCR1 and CCR2 expression probably elicits an increased inflammatory response, which correlates with the RA syndrome. Up-regulation of CCR expression is mediated through a variety of signal pathways depending on several stimulators. CCR1 expression induced by GM-CSF and IL-10 via activation of PKB/Akt and $p70^{S6k}$ and PMA-mediated CCR7 expression both require ERK [44,45]. RA exerts molecular actions mainly through nuclear receptors. After binding a nuclear receptor, RA can directly bind to the promoter of a target gene and subsequently evoke transcription and protein expression. RA can also increase expression of transcription factors and activate signaling molecules, which indirectly result in target protein expression. We do not understand the exact mechanism of CCR1 and CCR2 expression due to 9-CRA. A detailed signal transduction mechanism is under investigation.

To further understand the effect of 9-CRA, peripheral blood monocytes were examined under in vitro culture conditions to initiate cell differentiation. Since previous reports have indicated that RA inhibits survival and differentiation of monocytes, and expression of CCRs is altered during differentiation [28,34], we were interested to determine whether expression of CCRs can be modified by 9-CRA treatment during spontaneous differentiation. 9-CRA stimulation increased CCR1 expression, which was not altered during the differentiation process, and the intracellular Ca^{2+} concentration due to CCR1-dependent chemokines in agreement with in THP-1 cells without a differentiation signal. However, CCR2 expression and Ca^{2+} mobilization, which were both markedly reduced in the differentiation process, were not affected by 9-CRA treatment. These results indicate that CCR2 was induced by 9-CRA stimulation but CCR2 expression was inhibited by spontaneous differentiation signal at the same time. Despite a controversial report to the contrary, MCP-1 secretion probably blocks CCR2 induction in 9-CRA-treated monocytes by CCR2 internalization after ligand binding during differentiation [46]. Taken together, 9-CRA stimulation increased CCR1 expression and probably also increases CCR2 expression in peripheral blood monocytes without a differentiation signal.

We have demonstrated that exposure of THP-1 cells and human peripheral blood monocytes to 9-CRA increases CCR1 expression, resulting in an increased Ca^{2+} influx in response to CCR1-dependent chemokines and cell migration. Both CCR1 and CCR2 expression induced by 9-CRA may alter CCR1- and CCR2-dependent chemokine responses in monocytes. Our results provide further evidence of modulating immune responses due to RA.

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