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CCR4 mediates CCL17 (TARC)-induced migration of human colon cancer cells via RhoA/Rho-kinase signalling

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Abstract

Background: Accumulating data suggest a role of chemokines in tumor cell metastasis. CCR4 has been implicated in hematologic malignancies and recently also in solid tumors. Herein, we hypothesized that CCR4 might be expressed and support migration of colon cancer cells.

Methods: We used quantitative RT-PCR and flow cytometry to determine mRNA and surface expression of CCR4 on colon cancer cell lines (HT-29) and (AZ-97). Total RhoA and active RhoA protein levels in CCL17 stimulated colon cancer cells were quantified using ELISA and GLISA assays. Migration assays were performed to evaluate colon cancer cells chemotaxis. Invitro tumor growth was assessed using proliferation assay.

Results: our results show clear-cut mRNA levels and surface expression of CCR4 on a colon cancer cell line (HT-29) and on tumor cells (AZ-97). CCR4 ligand CCL17 (TARC) was a potent stimulator of colon cancer cell migration. This CCL17-induced colon cancer cell migration was inhibited by pre-incubation of the colon cancer cells with an antibody directed against CCR4 or an antagonist against CCR4. CCL17-induced signalling in colon cancer cells revealed that CCL17 increased mRNA formation of RhoA-C in colon cancer cells. Our results also found that CCL17 increased total RhoA and active RhoA protein levels in colon cancer cells. The Rho-kinase inhibitor Y-27632 abolished CCL17-induced colon cancer cell chemotaxis. In addition, inhibition of isoprenylation by GGTI-2133 markedly reduced colon cancer cell migration triggered by CCL17.

Conclusions: Our novel data indicate for the first time that the CCL17-CCR4 axis might be involved in the spread of colon cancer cells.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death in Europe [1]. Distant organ metastasis is the major cause of mortality in patients with CRC. The mechanisms behind cancer cell metastasis are not fully understood but convincing data suggest that increased expression of adhesion molecules and capacity to respond to chemotactic stimuli are necessary for the spread of tumor cells [2]. Accumulating data indicate that chemokines regulate multiple aspects of tumor cell biology, including proliferation, survival, angiogenesis and migration [3]. Chemokines belong to a superfamily of small molecules (8–14 kDa) that were initially discovered because their interaction with chemokine receptors was found to regulate trafficking of leukocytes to sites of inflammation and recirculation in secondary lymphatics [4,5]. Colon cancer cells can also express chemokine receptors, including CXCR3, CXCR4 and CCR6 and respond to specific chemokines [5]. For example, it has been demonstrated that CXCR3 is expressed on colon cancer cells and mediate lymph node and lung metastasis [6]. These findings underline the importance of studying the expression and function of chemokine receptors in colon cancer cells to better understand the molecular mechanisms controlling CRC metastasis.

CCR4 is a key receptor for regulating chemokine-dependent immune homeostasis and is selectively expressed on regulatory T cells and Th2 cells [7]. One recent study forwarded that CCR4 is involved in tumor cell evasion by stimulating accumulation of regulatory T-cells in the tumor microenvironment [8]. Several investigations have reported that CCR4 plays an important role in hematologic malignancies, such as adult T-cell leukemia, acute myeloid lymphoblastic leukemia and chronic lymphocytic leukemia [9]. Recent studies have reported that gastric and breast cancer cells express functional CCR4 receptors. Lee et al [10] showed that CCR4 is expressed in 17% of primary gastric cancers and is associated with a poor prognosis in patients with gastric cancer. Another investigation reported that only CCR4-positive cancer cells had the capacity to metastasize to the lung in the metastatic 4T1 mouse mammary carcinoma model [11]. Although these emerging evidences suggest a role of CCR4 in gastric and mammary carcinogenesis, the role of CCR4 in colon cancer biology remains elusive.

Based on the considerations above, we hypothesized that CCR4 might be expressed and mediate migration of colon cancer cells. For this purpose, we used an established colon cancer cell line (HT-29) and a primary colon cancer cell line (AZ-97) isolated from a patient with colon cancer.

Materials and Methods

Colon cancer cells

The human epithelial colon adenocarcinoma cell line HT-29 was obtained from American Type Culture Collection (HTB-38, ATCC, Manassas, VA, USA). We have established a primary human colon cancer cell line in our laboratory at Skåne University Hospital called AZ-97, which was isolated from a 76-year-old female patient undergoing surgical resection as previously described [12]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM); (Sigma-Aldrich, Stockholm, Sweden), supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37°C and 5% CO₂.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using RNeasy mini-kit from (Qiagen, Hilden, Germany) following the manufacturer's instructions. The total RNA concentration and purity of HT-29 and AZ-97 cell lines were determined using Nano drop spectrophotometer at 260 nm absorbance and the integrity of RNA samples was confirmed by 1% agarose gel electrophoresis. Reverse transcription was conducted with Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA) on 2.5 µg of total RNA in a final reaction volume of 20 µL according to the manufacturer's instructions. qRT-PCR was conducted in a final volume of 25 µL using syber green dye (Agilent Technologies) for absolute gene quantification. The RT-PCR program was as follows: initial denaturation (10 minutes at 95 °C) followed by 40cycles of denaturation (30 seconds at 95 °C), annealing (1 minute at 55 °C) and elongation (1 minute at 72 °C). After the last cycle a final extension of (1 minute at 95 °C). Thereafter, 12 µL of each product was run on 2% agarose gel electrophoresis and stained by GelRed (Biotium Inc., Hayward, CA) and analyzed under ultraviolet light. mRNA reference sequences were used to design primers using web based primer design tools of national center of biotechnology information. The Primer sequences used in this study are listed in table 1.

Flow cytometry

Surface expression of chemokine receptors was assessed using flow cytometry. HT-29 and AZ-97 cells were detached with 0.25% trypsin-EDTA in phosphate buffer saline (PBS) when reaching 80% confluence. In order to reduce non-specific bindings which can result from the binding of Fc region of the antibodies to the Fc receptors

present on the cancer cell lines, Cells were suspended to 5×10^6 cells/ml and incubated with 1 μ g of human Fc γ R inhibitor (eBioscience, San Diego, CA, USA) for 15 min. Cells were incubated with 10 μ g PE labelled anti- human CXCR4 or CCR8, FITC labelled anti-human CCR4 or CCR6, and APC labelled anti-human CXCR3 antibodies (R&D systems Europe, Abingdon, UK) in separate tubes for single tube staining and incubated for 90 min at room temperature protected from light. For intracellular staining, cells were fixed in 0.25% paraformaldehyde and incubated for 1 h at 4°C. Cells were washed and permeabilized in 1 ml 0.2% Tween20 and incubated at 37°C for 15 min. Cells were washed, Fc blocked, and stained as above and incubated on ice protected from light for 45 min. Thereafter, cells were washed twice and resuspended in 0.4 ml final volume FACS buffer and analyzed in duplicates at least three times using BD FACSCalibur (Becton Dickinson, Mountain View, CA, USA). Unstained cells were served as negative control. Histograms were made using CellQuest software with assessment of 20 000 events per sample.

Chemotaxis assay

Chemotactic responses of HT-29 and AZ-97 cells were evaluated by using 24-well cell migration chambers with 8 μ m pore size inserts (Corning Coster, Corning, NY, USA). The colon cancer cells were serum starved overnight and resuspended in serum free DMEM with 0.5% BSA. 8×10^5 cells/ml were loaded in the inserts. DMEM with 10% FBS with or without 10, 100 and 1000 ng/ml of CCL17 (Peprotech, Rocky Hill, NJ, USA) was added in the lower chambers and incubated for 12, 24, and 48 h at 37°C (5% CO₂). Non-migrated cells were removed by cotton swabs from the upper surface of the insert and cells on the lower surface of the insert membrane were fixed in ice-cold 100% methanol and stained with 0.5% crystal violet. In separate experiments, cells were treated the same way as in migration assay except that they were also pre-incubated for 30 min with different concentrations of an anti-CCR4 antibody clone 1G1 (Santa Cruz, CA, USA) or a CCR4 antagonist; 2-[1,4'-Bipiperidin-1'-yl-N-cycloheptyl-6,7-dimethoxy-4-quinazolinamine dihydrochloride], a Rho kinase inhibitor Y-27632 (10 and 50 μ M) (R&D systems) or geranylgeranyl transferase inhibitor GGTI-2133 (1 and 10 μ M) (Sigma-Aldrich). All migrated cells were counted microscopically in at least 5 different fields. Migration index was then calculated as the ratio of the number of migrated cells divided by the number of cells in the control wells.

Proliferation assay

Cell proliferation was evaluated in triplicates using Cell Counting Kit-8 assay (CCK-8; Sigma-Aldrich). Briefly, cells were seeded in 96 wells culture plate at 1×10^5 cells/well in media with or without CCL17 (100 ng/ml) for 24, 48, and 72 h at 37°C (5% CO₂). To quantify proliferation, 10 µL of CCK-8 was added per well for 4 h and absorbance at 450 nm was recorded using a microplate ELISA reader.

RhoA activation assay

RhoA-GTP activity was measured using the G-LISA RhoA activation assay Biochem kit (Cytoskeleton Inc., Denver, CO, USA) according to manufacturer's instructions. Briefly, cells were serum starved overnight and the next day stimulated by 100 ng/ml of CCL17 for 5 min. Cells were trypsinized, washed by ice cold PBS and lysed in 0.35 ml lysis buffer of the kit on ice for 10 min. Then, cells were homogenized using a 20-gauge needle for 20 strokes on ice and centrifuged at 10 000 rpm for 20 min at 4 °C. Supernatants were collected, snap frozen in liquid nitrogen and stored at -80°C until used. 50 µL of the supernatants were used for protein determination using Precision Red Advanced Protein Assay supplied with the kit. 1 mg/ml of protein was used for quantitative detection of active and total RhoA according to the manufacturer's instructions. Absorbance was read at 490 nm using a microplate ELISA reader. For each set of experiments, a titration curve was prepared using serial dilution of the RhoA/positive control of the kit.

Statistical Analysis

All statistical analyses were performed using SigmaPlot® 10 software. Statistical comparisons between more than two datasets were made with Kruskal-Wallis One Way Analysis otherwise Mann–Whitney rank-sum test was used and *P*-value < 0.05 was considered significant.

Results

Gene expression of chemokine receptors in colon cancer cells

Quantitative RT-PCR was performed to investigate the mRNA expression levels of two human colon cancer cells. As expected, it was observed that colon cancer cells expressed increased mRNA levels of CXCR4, CCR4 and CCR8; however, no significant gene expression of CCR6 was detected. Notably, we found that HT-29 and AZ-97 cells expressed high mRNA levels of the beta chemokine receptor CCR4. Of all these chemokine receptors were the mRNA levels of CXCR3, and CCR4 highest in HT-29 and AZ-97 cells. Gene expression patterns of CCR4, CCR6, CCR8, CXCR3 and CXCR4 were similar in both cell lines (**Fig. 1**).

Chemokine receptor expression in colon cancer cells

Further studies were performed using flow cytometry to analyse protein expression of chemokine receptors on the surface of colon cancer cells. The protein expression of CCR4, CCR6, CCR8, and CXCR3 corresponded very well with the mRNA levels in HT-29 and AZ-97 cells with highest surface expression of CXCR3, and CCR4 in the colon cancer cells (**Fig. 2**). Although colon cancer cells expressed CCR8 mRNA we found very low detectable CCR8 protein on the surface of the cells. Moreover, we also examined intracellular levels of chemokine receptors which revealed similar levels of CXCR3 and CCR4 inside the cells and on the surface of HT-29 and AZ-97 cells. (data not shown).

CCR4 mediates CCL17-induced colon cancer cell migration

In vitro migration assays were performed to determine the functional role of CCR4 in colon cancer cells. It was found that the CCR4 ligand CCL17 evoked a clear-cut and dose-dependent increase in colon cancer cell migration (**Fig. 3**). Next, we examined if CCL17-induced migration of HT-29 and AZ-97 cells was dependent on CCR4. We observed that pre-incubation of colon cancer cells with an antibody against CCR4 decreased colon cancer cell migration triggered by CCL17 in a dose – dependent manner (**Fig. 4a, c**). Moreover, co-incubation of HT-29 and AZ-97 cells with a CCR4-antagonist reduced CCL17-induced colon cancer cell migration (**Fig. 4b, d**).

Rho-kinase signalling regulates CCL17-induced colon cancer cell migration

Next, we examined CCL17-provoked signalling in colon cancer cells. It was found that challenge with CCL17 increased mRNA formation of RhoA-C in HT-29 cells (**Fig. 5a, b**). RhoA is particularly important for activation of Rho-kinase [13]. Thus, we examined protein levels of RhoA in colon cancer cells stimulated with CCL17. First, it was found that CCL17 increased the total levels of the RhoA protein in colon cancer cells (**Fig. 5c**). Secondly, we observed that challenge with CCL17 increased the level of active RhoA-GTP in colon cancer cells (**Fig. 5d**). Notably, co-incubation of colon cancer cells with the Rho-kinase inhibitor Y-27632 abolished CCL17-induced colon cancer cell migration (**Fig. 6a**). Thus, we next asked whether protein geranylgeranylation was involved in colon cancer cell migration in response to CCL17 stimulation. Interestingly, we observed that the geranyltransferase inhibitor GGTI-2133 markedly reduced colon cancer cell migration triggered by CCL17 (**Fig. 6b**).

CCR4 is not involved in colon cancer cell growth

Stimulation of HT-29 and AZ-97 cells with different doses of CCL17 up to three days had no effect on the proliferation of colon cancer cells (**Fig. 7**).

Discussion

An increasing body of data indicates that chemokines and their receptors are involved in the process of tumor cell metastasis, including CRC [14]. In the present study, we demonstrate that colon cancer cells express the beta chemokine receptor CCR4. Our results show that CCR4 mediates CCL17-induced migration but is not involved in the proliferation of colon cancer cells. Moreover, we show that CCL17-evoked colon cancer cell chemotaxis is dependent on RhoA/Rho-kinase signalling. Thus, these findings might help elucidating mechanisms regulating colon cancer metastasis.

Chemotactic attraction is now considered to be a key feature in the spread of cancer cells to secondary locations [15]. Indeed, colon cancer cells have been reported to express CXCR3, CXCR4 and CCR6 [16]. Our data confirm previous findings that colon cancer cells express CXCR3 and CXCR4. Importantly, this present study documents for the first time that colon cancer cells also express CCR4. We found that both primary colon cancer cells and an established colon cancer cell line expressed CCR4 at the mRNA and protein level. These findings add colon cancer cells to the growing list of tumor cells expressing CCR4, including hematologic malignancies, gastric and breast cancer [9,17]. Herein, we found that the CCR4 ligand CCL17 was a potent inducer of colon cancer cell migration. CCL17 triggered colon cancer cell chemotaxis in a dose- and time-dependent manner. These observations suggest that CCR4 exert a functional role in colon cancer biology. A functional role of CCR4 was further emphasized by our findings showing that inhibition of CCR4 by use of a specific antagonist or a blocking antibody directed against CCR4 markedly decreased CCL17-provoked migration of colon cancer cells. It should be noted that CCL17 not only binds to CCR4 but also to CCR8 [18], however, we found very low protein expression of CCR8 on colon cancer cells, suggesting that the dominant part of CCL17-induced colon cancer cell migration was mediated by CCR4. CCL17 expression is not tissue restricted since it can be expressed in numerous cell types, including dendritic cells, fibroblasts and endothelial cells [18]. Previous studies have reported that primary tumors can trigger remote induction of CCL17 in the lungs of mice and mediate CCR4-dependent pulmonary metastasis of breast cancer cells [11]. Whether such mechanisms exist in CRC remains to be studied. In this context, it should be mentioned that although CCR4 might be directly involved in the spread of colon cancer cells, CCR4 is also abundantly expressed on regulatory T-cells. A recent study reported that the spread of breast cancer cells to the lung was promoted by CCR4-dependent accumulation of regulatory T-cells into the tumor facilitating immune evasion of the breast cancer cells [19]. In contrast to migration, we observed that CCL17 had no significant effect on HT-29 or

AZ-97 cell proliferation, suggesting that the CCL17-CCR4 signalling axis is not important in the growth of colon cancer cells.

Members of the Rho family of GTPases, including Rho, Rac, and Cdc42, exert major roles in reorganizing the cell actin cytoskeleton in response to external stimuli [20]. It is generally held that the actin cytoskeleton provides the driving force for cell migration [21]. Since overexpression of RhoA has been reported to be associated worse prognosis of patients with CRC [22], we studied the expression of Rho proteins in CCL17 stimulated colon cancer cells herein. We found that CCL17 increased gene expression of RhoA-C in colon cancer cells. Furthermore, we could document increased protein levels of total RhoA as well as active RhoA in colon cancer cells after co-incubation with CCL17. Rho-kinase is the downstream target of RhoA, which phosphorylates myosin light chain and, thereby regulate actinomyosin assembly [23]. Thus, we hypothesized that Rho-kinase activity might control CCL17-induced chemotaxis of colon cancer cells. Notably, it was found that treatment with the Rho-kinase inhibitor Y-27632 markedly decreased colon cancer cell migration triggered by CCL17. This finding is in line with the observation that enhanced expression of Rho-kinase in colon carcinoma is associated with increased tumor cell dissemination [24]. Rho proteins must undergo lipid posttranslational modifications, such as isoprenylation, in order to effectively localize at cell membranes and activate Rho-kinase. Isoprenylation of Rho proteins are mediated by two enzymes, farnesyltransferase and geranylgeranyltransferase, which catalyzes attachment of pyrophosphate groups to cysteine residues in Rho proteins [25]. We investigated the role of geranylgeranylation in CCL17-provoked colon cancer cell migration by use of the geranyltransferase inhibitor GTI-2133. Interestingly, we found that GTI-2133 greatly decreased CCL17-evoked migration of colon cancer cells, suggesting that geranylgeranylation regulates the chemotactic response of colon cancer cells to CCL17. This notion is supported by a previous study showing that geranylgeranylation is involved in random migration and invasion of colon cancer cells [22].

Taken together, our findings show for the first time that colon cancer cells express CCR4 and that CCR4 mediates CCL17-induced colon cancer cell chemotaxis. However, the CCL17-CCR4 axis was not involved in the proliferation of colon cancer cells. Moreover, these results demonstrate that CCL17-evoked migration of colon cancer cells is mediated via RhoA/Rho-kinase signalling and dependent on geranylgeranylation. We conclude that CCR4 plays a functional role in colon cancer cell migration and targeting CCR4 might open new possibilities to antagonize the spread of CRC.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Gene expression of chemokine receptors in colon cancer cells. Expression of chemokine receptor mRNAs and the house keeping gene β -actin were determined using qRT-PCR in HT-29 and AZ-97 cell lines. All gene expression products were at the expected length as summarized in table 1. The expression profile indicates high mRNA expression of CCR4. Data shown represent one of three independent experiments.

Fig. 2 Chemokines receptors expression on colon cancer cells. (a) HT-29 and (b) AZ-97 cells. Single-cell suspensions were prepared from HT-29 and AZ-97 cells and stained as outlined in Materials and Methods. Unstained cells were used as negative control. Data were analysed in duplicate at least three times.

Fig. 3 CCL17 induces colon cancer cell migration. In vitro cell migration in response to 24 h stimulation with or without 1, 10 and 100 ng/ml CCL17 in (a) HT-29 and (b) AZ-97 cells. Cells were counted microscopically using 10 High Power Fields in 5 different fields. Migration index was calculated as the ratio of the number of migrated cells on wells containing CCL17 divided by the number of cells in the control wells. Data represents mean $\pm$ SEM and $n = 5$. * $P < 0.05$ versus negative control.

Fig. 4 CCR4 mediates CCL17-induced colon cancer cell migration. Migration of HT-29 (a, c) and AZ-97 (b, d) colon cancer cells was induced by 100 ng/ml of CCL17 for 24h. Cells were pre-incubated 30 min with indicated doses of an antibody against CCR4 and an antagonist against CCR4. Migration index was calculated as the ratio of the number of migrated cells on wells containing CCL17 divided by the number of cells in the control wells. Data represents mean $\pm$ SEM and $n = 4$. * $P < 0.05$ versus negative control.

Fig. 5 CCL17 triggers RhoA activation in HT-29 cells. (a) mRNAs expression of Rho proteins after CCL17 stimulation in serum free media for 24h. The gel electrophoresis shows Rho A-C mRNAs respective bands at their expected lengths. Data shown represent one of three independent experiments. (b) Fold changes in relative quantity of RhoA-C mRNA levels. (c) Total RhoA was measured by ELISA at absorbance 490 nm. (d) G-LISA activation assay was used to quantify levels of active RhoA. serum free media served as negative control. Data represent mean $\pm$ SEM and $n = 4$. * $P < 0.05$ versus Control cells.

Fig. 6 Rho-kinase and geranylgeranyl transferase regulate colon cancer cell migration. HT-29 cell migration was stimulated by 100 ng/ml of CCL17 for 24 h. **(a)** A Rho kinase inhibitor (Y-27632, 10 and 50 μ M) or **(b)** a geranylgeranyl transferase inhibitor (GGTI-2133, 1 and 10 μ M) was co-incubated with the colon cancer cells prior to CCL17 challenge. Data represents mean $\pm$ SEM and $n = 8$. [#]P < 0.05 versus negative control and *P < 0.05 versus vehicle + CCL17.

Fig. 7 CCL17 has no effect on colon cancer cell proliferation. **(a)** HT-29 and **(b)** AZ-97 colon cancer cells were incubated with 100 ng/ml of CCL17 or vehicle (Control) for 24, 48 and 72 hr. Proliferation was determined as described in Materials and Methods. Data represents mean $\pm$ SEM and $n = 5$.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7