Up-Regulation of Functional Chemokine Receptor CCR3 in Human Renal Cell Carcinoma

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Abstract

There is increasing evidence that chemokines and chemokine receptors are causally involved in tumorigenesis by facilitating tumor proliferation and metastasis. Little is known about the possible function of chemokine receptors in the development and progression of renal cell carcinoma (RCC). We, therefore, analyzed the expression of chemokine receptors in tumor specimens and adjacent healthy kidney tissues [normal kidney cell (NKC)] from 10 RCC patients. We also characterized the permanent RCC cell line A-498. CCR6, CXCR2, and CXCR3 were consistently expressed by both malignant cells and NKC. A-498 displayed additional expression of CXCR4. Importantly, the expression of CCR3 was almost absent on NKCs but clearly enhanced in a substantial proportion of RCC specimens. The primary CCR3 ligand, eotaxin-1/CCL11, induced intracellular Ca2+ mobilization, receptor internalization, and proliferation in A-498 cells confirming signaling competence of RCC-associated CCR3. In addition, we screened tumor tissue sections of 219 patients and found that 28% (62 of 219) expressed the CCR3 receptor. The presence of CCR3 in tumor samples seemed to correlate with the grade of malignancy. Previous work has established that eotaxin-1 expression is induced by tumor necrosis factor-α, a cytokine known to be present in RCC tissue. Our data, therefore, supports a scenario in which eotaxin-1 as part of tumor-associated inflammation promotes progression and dissemination of CCR3-positive RCC.

Cancers of the kidney account for 2% to 3% of all malignancies and have a peak incidence in the fifth and sixth decades of life. Renal cell carcinoma (RCC) is the most common malignant renal tumor with an incidence of 4 to 5 in 100 people. At diagnosis, 20% to 30% of the patients have metastatic disease and even a higher percentage of patients develop metachronous metastases after nephrectomy. When in metastatic progression, systemic therapies of RCC are largely ineffective in impacting disease response or patient survival. Therefore, defining the factors involved in disease progression and metastasis constitutes a necessary approach in the development of effective therapies.

Multiple factors are known to contribute to the development and progression of neoplasias in general. Among them, chemokines are increasingly being recognized as key players (for reviews, see refs. 1, 2). Chemokines constitute a family of small (8-10 kDa) secreted proteins that act in paracrine or autocrine fashion. Most chemokines are expressed in response to stimuli [e.g., tumor necrosis factor-α (TNF-α) or IFN-γ], but constitutive expression might also occur in a tissue-specific manner. Chemokines exert their activity via interaction with a large family of seven-transmembrane-spanning G protein–coupled receptors. There are ~20 chemokine receptors described thus far and many of the receptors exhibit widespread binding properties. Thus, several chemokines can signal through the same receptor.

Originally, chemokines were discovered as chemoattractants and activators of specific subsets of lymphocytes. It has now been shown that chemokine function is neither confined to chemoattraction nor to lymphocytes. In fact, like leukocytes, tumor cells might also express distinct chemokine receptors that enable chemotactic responses to chemokine gradients. Furthermore, chemokine/chemokine receptor loops might directly induce tumor cell growth, or specific molecules that support tumor migration, such as metalloproteinases.

Several investigations stress the impact of chemokine receptors in cancer development. Müller et al. (4) showed the involvement of CXCR4 and CCR7 in the metastatic behavior of breast cancer cells and the neutralizing potential of antibodies against CXCR4 was confirmed in a mouse model. Involvement of CXCR4 was also described in the migration and progression of human melanoma (5) and prostate cancer (6). Besides the correlation of CXCR4 expression on tumor cells with the formation of bone marrow metastasis, metastasis to lymph nodes has been shown to depend on the regulation of CCR6 and CCR7 expression in squamous cell carcinoma of the head and neck (7), on the expression of CCR7 in esophageal squamous carcinoma (8) and
in non–small cell lung cancer (9), and on CXCR3 expression in a mouse model of melanoma (10). CXCR2 has been implicated in the metastatic behavior of murine squamous cell carcinoma cells (11) and in an in vitro prostate carcinoma migration model (12). In our previous work, we were able to show that CCR2 expression on myeloma cells facilitated enhanced migration of tumor cells via TNF-α–induced autodestructive production of MCP-1, a ligand for CCR2 (13). Autocrine activation of the CCR6/MIP-3α loop also contributed to growth and migration of pancreatic cancer cells (14), and the existence of a CCR3/ eotaxin-1 loop in T-cell lymphomas (15) has been suggested to be involved in the induction of malignant cell growth. The IL-8/CXCR1 and IL-8/ CXCR2 pathways has been implicated in the extent of aggressive growth of malignant melanoma cells (16). Activation of CCR5 was shown to influence progression of breast cancer via regulation of p53 transcriptional activity (17) and CCR5 expression was also considered a prerequisite for the induction of metalloproteinases in breast cancer cells by the chemokine RANTES, thus contributing to the invasive behavior of the cells (18).

The pleiotropic functions of chemokines in mind, we investigated a possible role of chemokine receptors in RCC. Up to now, only CXCR4 expression has been described for RCC (19). In the present work, we attempted to establish a more comprehensive chemokine receptor profile of RCC and of the corresponding NKC. We decided to investigate distinct chemokine receptors that have previously been correlated with tumor migration and growth.

Materials and Methods

Patients. Patients were enrolled in the study after having signed informed consent. RCC and the adjacent normal kidney tissue samples were collected from 10 patients immediately after tumor nephrectomy. In an effort to identify a possible preponderance of certain chemokine receptors in tumor cells with defined metastasis patterns, we used primary tumor cells from patients who had developed metastases in different organs, such as lung, bones, or lymph nodes. For patient characteristics, see Table 1.

Renal cell carcinoma cell line and preparation of tumor cell suspensions. The A-498 cell line (20), obtained from the DSMZ (Braunschweig, Germany), is HLA-A2+/+ and expresses tumor antigens, such as Her-2/neu and MUC-1 (21).

Tumor and normal kidney tissue samples were processed immediately after surgery as previously described (22). Tissues were minced, digested with 1 mg/mL type I collagenase (EC 3.4.24.3; Sigma, St. Louis, MO) and 40 units/mL type I desoxy-RNase (DNase, EC 3.1.21.1; Sigma), and washed extensively. Cells were either used immediately or frozen in 40 units/mL type 1 desoxy-RNase (DNase, EC 3.1.21.1; Sigma), and after surgery as previously described (22). Tissues were minced, digested such as Her-2/neu and MUC-1 (21).

Total RNA was isolated using TRIzol reagent and analyzed with a FACSCalibur and CellQuest software (BD Biosciences). Total RNA was isolated using TRIzol reagent and Flow cytometry. Cells were nonenzymatically detached (Dulbecco’s PBS; PAA, Pasching, Austria, containing 2 mmol/L EDTA) and resuspended at 107 cells/mL in ice-cold washing buffer (PBS containing 1% FCS). To determine surface antigen expression, cells were labeled with phycoerythrin-labeled mouse anti-human CCR2 (BD Biosciences, Franklin Lakes, NJ), CCR3 (IgG2b; 61828.111; Leukosite, Cambridge, MA), CCR5 (IgG2b; 2D7/CCR5; BD Biosciences), CCR6 (IgG1; 11A9; Pharmingen Europe, Hamburg, Germany), CCRX3 (IgG1; 49801.111; Leukosite), CXCR4 (IgG2a; 12G5; Pharmingen), and anti-human CXCR2 (IgG2b; 6C6; Pharmingen), and anti-human CCR7 (IgG2a; 3D12; kindly provided by Dr. R. Förster, Institute of Immunology, Hamburg, Germany) followed by a FITC anti-rat antibody staining, respectively, or the appropriate isotype controls for 30 minutes at 4°C. Cells were analyzed with a FACSCalibur and CellQuest software (BD Biosciences).

Ca2+ mobilization. Recombinant chemokines eotaxin-1/CCL11 and sdf-1/CXCL12 were obtained from R&D Systems (Minneapolis, MN). A 2 mg/mL stock solution of Fluo-3 acetoxy methane (Molecular Probes, Leiden, the Netherlands) in anhydrous DMSO was used. A 20% w/v stock solution of the detergent pluronic acid F-127 (Molecular Probes) was also prepared in DMSO. An equal volume of pluronic acid stock was added to the dye stock just before use to increase Fluo-3 acetoxy methane solubility and improve dye loading into the cells as previously described (23). Adherent cells were harvested nonenzymatically and resuspended in cell loading buffer (PBS, 1% FCS, 1 mmol/L MgCl2, and 1 mmol/L CaCl2) at a concentration of 0.5 × 107/mL. Four microliters of Fluo-3/Pluronic mix were added to each sample, followed by incubation for 1 hour at room temperature on a head-over-head shaker. Thereafter, cells were washed twice with cell loading buffer and incubated at 37°C for 10 minutes. First, untreated cells were examined by flow cytometric analysis for 1 minute in fluorescence versus time to establish a baseline. Then, chemokines were added as indicated and chemokine-induced fluorescence was measured. Results are expressed as time–dependence changes in the mean fluorescence intensity.

Receptor internalization. Internalization experiments were done as previously described (15). In brief, harvested cells were resuspended at

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Staging and grading</th>
<th>Metastases location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>T3a/G3</td>
<td>Lung</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>T2a/G3</td>
<td>Lung</td>
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<tr>
<td>3</td>
<td>M</td>
<td>T3a/G2</td>
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<tr>
<td>4</td>
<td>F</td>
<td>T3a/G3</td>
<td>Lung</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>T2a/G3</td>
<td>Bones</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>T3a/G4</td>
<td>Bones</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>T2a/G4</td>
<td>Bones</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>T2a/G3</td>
<td>Lung, LN</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>T2a/G2</td>
<td>LN, soft tissue</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>T3a/G4</td>
<td>Bones, liver, LN</td>
</tr>
</tbody>
</table>

NOTE: All 10 patients had histologically confirmed bidimensionally measurable metastatic RCC of the clear cell type. Tumor-node-metastasis classification was according to the International Union Against Cancer. Abbreviations: F, female; M, male; LN, lymph node.
Our laboratory recently identified the chemokine receptor CCR3 as a functional receptor on renal cell carcinoma cells (20). As a continuation of this work, we investigated whether other chemokine receptors are expressed on the cell surface of functional renal cell carcinoma cells. To this end, we tested expression of chemokine receptors (CCR2, CCR3, CCR5, CCR6, CCR7, CXCR2, CXCR3, and CXCR4) on a well-characterized cell line, A-498. We then investigated the functional competence of CCR3. In these experiments, A-498 cells were incubated with eotaxin-1, sdf-1, or medium for 60 hours in culture media with or without 100 ng/mL CCR3 ligand chemokine bound to noninternalized receptor for 1 minute at 37°C. Cells were immediately chilled on ice and stained for surface expression of CCR3 and CXCR4 cells with appropriate monoclonal antibodies. 106 cells/mL in RPMI 1640 supplemented with 10% FCS. Short-term cultures were incubated with 100 ng/mL eotaxin-1, sdf-1, or medium only for 30 minutes at 37°C in a 5% CO2 atmosphere. After chemokine incubation, cells were extensively washed in PBS. The final washing step was done in acidic glycine buffer (pH 3) to remove chemokine incubation, cells were extensively washed in PBS. The final washing step was done in acidic glycine buffer (pH 3) to remove chemokine bound to noninternalized receptor for 1 minute at 37°C. Cells were immediately chilled on ice and stained for surface expression of CCR3 and CXCR4 cells with appropriate monoclonal antibodies.

**Expression of chemokine receptors by the permanent renal cell carcinoma cell line A-498.** In a first set of experiments, we analyzed the well-characterized cell line A-498 (20). We investigated the expression of the chemokine receptors CCR2, CCR3, CCR5, CCR6, CCR7, CXCR2, CXCR3, and CXCR4. Real-time PCR showed that CCR3, CCR6, CXCR2, CXCR3, and CXCR4 were expressed on mRNA levels (Fig. 1A). Fluorescence-activated cell sorting analysis confirmed these data on protein levels (Fig. 1B). CCR2, CCR5, and CCR7 were expressed neither on mRNA nor on protein levels. mRNA levels correlated well with the amount of surface protein expression on A-498.

**Expression of chemokine receptors by primary renal cell carcinoma cells and adjacent normal kidney cells.** To determine the relevance of the above-mentioned chemokine receptors for primary RCC, we tested short-term primary cultures of RCC and the adjacent NKC from 10 different patients by fluorescence-activated cell sorting analysis (Fig. 2). Consistent with the analysis of A-498, CCR2, CCR5, and CCR7 were also undetectable in primary RCC and NKC cultures. NKC were found to express predominantly CCR6, CXCR2, and CXCR3, whereas expression of CXCR4 was not detectable in these cells on protein level (Fig. 2, NKC) although CXCR4 mRNA levels were quite high (data not shown). Expression of CCR6, CXCR2, and CXCR3 seemed to be modestly increased in tumor samples compared with the corresponding normal tissues. CXCR4 was expressed in only 1 of 10 tumor samples (Fig. 2, RCC). The most conspicuous difference between normal and tumor cells was the expression of CCR3 in tumor cell samples (9 of 10), whereas only 2 of 10 NKC samples showed CXCR3 expression and that at very low levels.

**Measurement of chemokine-induced intracellular Ca2+ mobilization and receptor internalization.** We then investigated the functional competence of CCR3. In these experiments, A-498 cells were used because of the limited availability of primary tumor cells. First, we tested intracellular Ca2+ levels after stimulation with CCR3 ligand eotaxin-1. CXCR4 ligand sdf-1 was used as a positive control because expression of functional CXCR4 in A-498 cells has been described before (19). Both chemokines induced an up-regulation of intracellular Ca2+ signaling in A-498 cells (Fig. 3A). Second, binding of ligand to its cognate receptor led to receptor down-regulation via internalization, which was assessed by flow cytometry. Thus, A-498 cells were incubated with eotaxin-1, sdf-1, or medium (control). As shown in Fig. 3B, both chemokines induced a profound decrease of the respective cell surface receptors in A-498 cells further supporting that CCR3 exerts signaling competence.

**Chemokine-induced proliferation.** Finally, we tested the role of CCR3 in the regulation of A-498 proliferation (Fig. 3C). Cells
were grown with or without the addition of eotaxin-1 and, as a negative control, with MIP-3β, the ligand of CCR7, a receptor that is not expressed by A-498 cells (see Fig. 1). A-498 cells showed enhanced proliferation when cultured in the presence of eotaxin-1 (183% proliferation of control ± 17 SD), whereas addition of MIP-3β did not affect cell growth (102% ± 4).

**CCR3 expression on paraffin-embedded tumor tissue.** To test the in vivo relevance of our in vitro findings, we investigated

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**Fig. 1.** Expression of chemokine receptors in A-498 cell line. Different chemokine receptors are expressed on RCC cell line A-498. A. mRNA levels of chemokine receptors are shown as the ratio of chemokine receptor mRNA to 18S RNA. B. Surface protein expression is shown as relative fluorescence intensities (filled histogram) compared with isotype controls (open histogram).

**Fig. 2.** Expression of chemokine receptors in RCC and NKC. Fluorescence-activated cell sorting analysis of different chemokine receptors in RCC compared with the adjacent NKC was done on primary tumor samples from 10 patients. The patient order is identical for NKC and RCC data. Results are expressed as the mean fluorescence intensity (MFI).
paraffin-embedded tumor tissue sections. The analysis of tissue arrays comprising tumor samples of 219 patients suffering from RCC revealed that 62 (i.e., 28%) scored CCR3 positive whereas 157 tumor sections (i.e., 72%) were negative. Figure 4A depicts a sample representative of the CCR3-positive tumors and Fig. 4B a sample representative of a CCR3-negative tumor. The number of CCR3-positive samples has been found to increase with the grade of malignancy: At grade 1, 19% of the samples scored CCR3 positive, grade 2 showed 29%, grade 3 showed 26%, and grade 4 showed 42% CCR3+ samples (Fig. 5).

Discussion

Chemokines and their receptors are important actors in the development and progression of human tumors by facilitating migration and proliferation of the malignant cells. In our analysis, RCC cells as well as NKCs lacked expression of CCR2, CCR5, and CCR7 on both mRNA and protein levels (Fig. 2), suggesting that the respective ligands, such as MCP-1, MCP-2 (CCR2 ligands), MIP-1α, MIP-1β (CCR5), and MIP-3β, SLC (CCR7), may not have a receptor-mediated impact on RCC tumor cell growth and migration. In contrast, CCR6, CXCR2, and CXCR3 were expressed in RCC as well as NKC (Fig. 2) pointing to a more general role of these chemokine receptors and their respective ligands in kidney physiology. However, the modest enhancement of these three chemokine receptors in RCC may be indicative of a general chemokine receptor up-regulation in RCC, possibly as a consequence of tumor-associated inflammation. We could only partially confirm the findings of Schrader et al. (19) who described CXCR4 surface expression on two of four tumor cell lines and

![Fig. 3](image-url). Signaling competence of chemokine receptor CCR3 in A-498 cells. A, chemokines induced intracellular Ca²⁺ mobilization in A-498: Transient increase in intracellular Ca²⁺ concentration after addition of eotaxin-1 (ligand for CCR3) or sfd-1 (ligand for CXCR4; control) was recorded by monitoring the change in green fluorescence intensity of the cells as a function of time. B, effect of chemokine stimulation on receptor internalization: A-498 cells were treated with eotaxin-1, sfd-1, or medium alone. Chemokines bound to surface receptors were removed by acidic wash. The decrease of surface receptor expression was detected by specific antibodies. Surface receptor expression before chemokine addition (filled histograms) and after chemokine incubation (open histograms, dark black line) is shown with the isotype control (dashed line). C, proliferation of A-498 cells in response to CCR3 ligand eotaxin-1: Proliferation was measured via [³H]thymidine incorporation within 12 hours of a 60-hour incubation period of A-498 cells with 100 ng/mL chemokines eotaxin-1 and MIP-3β (as a negative control), respectively. Columns, percentage of proliferation of control. Experiments were done in quadruplicates and repeated at least thrice.
in immunohistochemical staining of a primary renal clear cell carcinoma. Except for the cells of one patient, the primary tumor samples, although positive for CXCR4 mRNA (data not shown), did not show CXCR4 surface expression in fluorescence-activated cell sorting analysis (Fig. 2). In contrast, we found substantial surface expression of CXCR4 on the A-498 cell line (Fig. 1), suggesting that CXCR4 surface expression might be a feature of permanent RCC cell lines without broad in vivo relevance. The most prominent difference between RCC samples and adjacent NKCs of these patients was the almost exclusive expression of CCR3 on tumor samples (Fig. 2). We could confirm CCR3 expression in 62 of 219 RCC patients tested (Fig. 4). Additionally, CCR3 ligand eotaxin-1 induced proliferation in the RCC cell line A-498 (Fig. 3C). Thus far, CCR3 has been shown to be expressed by eosinophil leukocytes but was also detected on basophils and a subset of Th2 cells (24–26). The main ligand, eotaxin-1, is a chemoattractant for these receptor-bearing cells. Eotaxin is predominantly produced by epithelial and endothelial cells and is induced by TNF-α and other inflammatory cytokines, such as IL-1 (27, 28).

In RCC, patients with high serum levels of inflammatory cytokines are known to have poor prognosis and TNF-α was reported to be an independent prognostic indicator, with a normal TNF-α plasma level being highly predictive for a good prognosis in untreated patients (29). Supporting this, Yoshida et al. (30) found that elevated levels of TNF-α correlated with advanced stages of disease and poor prognosis. The TNF-α levels of the 10 patients from whom we analyzed primary tumor samples were all above normal (data not shown) and the patient showed advanced stages of RCC including metastasis at different sites. These data stress the possible in vivo relevance of functional CCR3 chemokine receptor expression in RCC. We also found that CCR3 expression in immunohistochemical analysis of a greater number of patients correlated with the degree of malignancy (Fig. 5). Expression of the chemokine receptor increased gradually according to the malignancy of the tumor doubling the percentage of CCR3-positive samples from grade 1 (19% CCR3+ tumors) to grade 4 (42% CCR3+). It seems that renal carcinoma cells gain CCR3 expression with advanced malignancy, which, in turn, enables them to utilize the respective ligand(s). These ligands might also become up-regulated in the course of tumor-associated inflammation leading to a vicious circle of tumor growth and progression.

In the context of our findings, it is also interesting to note that ligands of CXCR3 (i.e., IFN-inducible chemokines), such as IP-10, Mig, and I-TAC, have been shown to be natural antagonists for CCR3 (31). IL-12 treatment, which induced IFN-γ and IP-10 in RCC cells, led to tumor regression (32, 33) and high levels of IP-10 and Mig in tumor tissue has recently been correlated with tumor-free survival after curative surgery (34). These effects have been attributed to the activation of cells of the immune system. However, our findings suggest that IP-10 might also contribute to the inhibition of tumor growth via competitive binding of IP-10 to CCR3, thus counteracting the tumor growth–promoting effect of eotaxin. If this holds true in vivo, newly developed antagonists of CCR3, for instance described by Erin et al.

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**Fig. 4.** Immunohistochemical analysis of CCR3 expression. Tissue arrays containing tumor samples from 219 patients suffering from RCC were screened for CCR3 expression. Expression was scored as negative or positive, respectively. From 219 patients, 62 (i.e., 28%) scored positive and 157 (i.e., 72%) scored negative. A, a sample representative of the CCR3-positive tumors; B, a sample representative of a CCR3-negative tumor. Brown membranous staining, CCR3 expression.

**Fig. 5.** Correlation of CCR3 expression and grade of malignancy. Tumor samples (n = 219) were divided according to the grade of malignancy (grade 1-4, grading according to the International Union Against Cancer) and the frequency of CCR3-positive samples was analyzed. In tumor samples with higher grade of malignancy, CCR3 expression was found more frequently (i.e., from grade 1 to grade 4); the number of CCR3-positive samples doubled.

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(35), might substantially aid in the effort to control this lethal disease.

In summary, we show here that CCR3 is expressed in RCC and found that CCR3 expression correlates with a higher grade of malignancy of the tumor cells. We propose that CCR3 expression might facilitate proliferative responses of the tumor cells and that ligands of CCR3, such as eotaxin-1, possibly up-regulated as a result of tumor-associated inflammation, might thus be involved in the development and progression of the disease.

Acknowledgments

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21. Uguccioni M, Hayashi S, Miller EJ. Asynthetic peptide ligands of CCR3, such as eotaxin-1, possibly up-regulated as a result of tumor-associated inflammation, might substantially aid in the effort to control this lethal disease.
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