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Defective Expression of the Monocyte Chemotactic Protein-1 Receptor CCR2 in Macrophages Associated with Human Ovarian Carcinoma

Antonio Sica,1* Alessandra Saccani,* Barbara Bottazzi,* Sergio Bernasconi,* Paola Allavena,* Brancatelli Gaetano,† Francesca Fei,‡ Graig LaRosa,§ Chris Scotton,§ Frances Balkwill,§ and Alberto Mantovani††

Monocyte chemotactic protein-1 (MCP-1, CCL2) is an important determinant of macrophage infiltration in tumors, ovarian carcinoma in particular. MCP-1 binds the chemokine receptor CCR2. Recent results indicate that proinflammatory and anti-inflammatory signals regulate chemokine receptor expression in monocytes. The present study was designed to investigate the expression of CCR2 in tumor-associated macrophages (TAM) from ovarian cancer patients. TAM isolated from ascitic or solid ovarian carcinoma displayed defective CCR2 mRNA (Northern blot and PCR) and surface expression and did not migrate in response to MCP-1. The defect was selective for CCR2 in that CCR1 and CCR5 were expressed normally in TAM. CCR2 gene expression and chemotactic response to MCP-1 were decreased to a lesser extent in blood monocytes from cancer patients. CCR2 mRNA levels and the chemotactic response to MCP-1 were drastically reduced in fresh monocytes cultured in the presence of tumor ascites from cancer patients. Ab against TNF-α restored the CCR2 mRNA level in monocytes cultured in the presence of ascitic fluid. The finding of defective CCR2 expression in TAM, largely dependent on local TNF production, is consistent with previous in vitro data on down-regulation of chemokine receptors by proinflammatory molecules. Receptor inhibition may serve as a mechanism to arrest and retain recruited macrophages and to prevent chemokine scavenging by mononuclear phagocytes at sites of inflammation and tumor growth. In the presence of advanced tumors or chronic inflammation, systemic down-regulation of receptor expression by proinflammatory molecules leaking in the systemic circulation may account for defective chemotaxis and a defective capacity to mount inflammatory responses associated with advanced neoplasia. The Journal of Immunology, 2000, 164: 733–738.

Selective accumulation of leukocyte subpopulations is the hallmark of pathological conditions, including allergic and inflammatory reactions and tumors (1). In particular, the presence of macrophages in tumor tissues and/or their periphery has generated broad interest, and analysis of tumor-associated macrophages (TAM)† suggests that these cells have the capacity to affect diverse aspects of the immunobiology of neoplastic tissues, including vascularization, growth rate and metastasis, stroma formation, and dissolution (1, 2). On the other hand, cells of the monocyte-macrophage lineage have the potential to express tumoricidal capacity and to elicit tumor-destructive reactions (3, 4). Based on these observations, the “macrophages balance” hypothesis (1) was coined to emphasize the dual potential of TAM to influence tumor growth in opposite directions.

Macrophages represent a major component of the lymphoreticular infiltrate of tumors, and the percentage of TAM for each tumor is usually maintained as a relatively stable “individual” property during tumor growth and upon transplantation in syngeneic hosts (1, 5). The search for tumor-derived chemotactic factors, which may account for recruitment of mononuclear phagocytes in neoplastic tissues, lead to the identification of the monocyte chemotactic protein-1 (MCP-1, CCL2) as well as of other chemokines (1, 2, 6–12). MCP-1 is a member of a superfamily of cytokines called chemokines. The hallmark of this family is a conserved cysteine residue motif (13–15). According to the relative position of the first two cysteines, it is possible to distinguish two main families: the CXC (or α) chemokines, which are active on neutrophils, T and B lymphocytes (13–15), and the CC (or β) chemokines that exert their action on multiple leukocyte populations, including monocytes, basophils, eosinophils, T lymphocytes, NK, and dendritic cells (13–18). A third type of protein (the C or γ chemokines) was described, which is active on T lymphocytes and NK cells. This protein is characterized by the absence of the first and third cysteines, but shows overall sequence identity with CC chemokines (19, 20). More recently, fractalkine, CX3C (or δ motif), was described as chemotactic for monocytes, T cells, and NK cells (21, 22).

Chemokines, as well as classical chemotactic agonists, such as formylated peptides (of which fMLP is the prototype) and C5a, bind to and activate a family of rhodopsin-like, GTP-binding protein-coupled seven-transmembrane domain receptors (23–25). Nine receptors for CC chemokines, now named CCR1 through 9, have been identified and cloned (13, 14, 23–26).
MCP-1 interacts with CCR2, of which two isoforms have been cloned and termed A and B (26). In monocytes and NK cells, CCR2 is expressed predominantly as B isoform, with vanishingly low levels of A transcripts (27). In addition to MCP-1, CCR2 recognizes MCP-2 and MCP-3 (28–30). Several lines of evidence, including studies in gene-targeted mice, indicate that MCP-1 and CCR2 are important for monocyte recruitment at the site of inflammation (31, 32). There is also evidence that in a variety of murine and human tumors, including ovarian cancer, MCP-1 is a major determinant of the degree of macrophage infiltration in neoplastic tissue (1, 2, 33–40).

Recent results indicate that proinflammatory and anti-inflammatory signals regulate chemokine receptor expression in human mononuclear phagocytes (41–44). In particular, certain primary proinflammatory signals (e.g., LPS) rapidly inhibit chemokine receptor expression (41). It was speculated that inhibition of chemokine receptor expression may serve as a stop signal to arrest and retain mononuclear phagocytes at sites of infection or inflammation (41). As discussed elsewhere (2), tumors have served as a paradigm of the in vivo function of chemokines in monocyte recruitment with minimal activation. It was therefore important to investigate chemokine receptor expression in TAM, since regulation of receptor expression has emerged as a crucial set point for the action of these molecules. In this study, we report that macrophages isolated from the ascitic fluid or solid tumor from ovarian cancer patients exhibit a drastic and selective defect of expression of the MCP-1 receptor (CCR2), which correlates with the lack of chemotaxis in response to MCP-1. In addition, we identified TNF-α as a prorot factor which may contribute to the negative regulation of CCR2.

**Materials and Methods**

**Monocytes**

Human monocytes were separated from peripheral blood of human healthy donors by Percoll gradient centrifugation (45). Monocytes (>98% pure as assessed by morphology) were resuspended at 10^6/ml in RPMI 1640 supplemented with 10% of FBS, 2 mM glutamine, and antibiotics. All reagents contained <0.125 endotoxin units/ml of endotoxin as checked by Limulus amebocyte lysate assay (Microbiological Associates, Walkersville, MD). Monocyte-derived macrophages (MDM) were derived from freshly isolated monocytes (3–5 x 10^6 cells/ml) after incubation for 5 days in RPMI 1640 medium supplemented with 10% of FBS, with 2 mM glutamine, antibiotics, and 40% autologous serum on hydrophobic plates (Petriperm Hydrophobic, Heraeus Instruments GmbH, Germany) as described previously (46). PBMC of healthy donors were obtained from buffy coats, whereas PBMC from patients with ovarian carcinoma were obtained from heparinized venous blood. Blood was diluted 1/5 with saline, and 40 ml was then placed on 10 ml of Ficoll (Sermol, Berlin, Germany) in 50-ml conical tubes (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) for centrifugation at 400 g for 20 min at room temperature. PBMC were collected at the interface, washed with saline, and suspended in complete medium at 2–5 x 10^6 cells/ml in 50-ml conical tubes.

**TAM**

Blood samples (5) and ascitic fluids (10) were collected from untreated patients with histologically confirmed epithelial ovarian carcinoma admitted to the Department of Obstetrics and Gynecology, San Gerardo Hospital (Monza, Italy). All patients had cancer classified as stage II, III, or IV. Ascitic fluid was collected and centrifuged. Cell pellets were resuspended in RPMI 1640 medium without serum and layered on top of a Ficoll-Hypaque cushion to prepare mononuclear cells. Purification of peritoneal macrophages was further conducted by two subsequent adherence steps for 45 min, each in RPMI 1640 medium without serum. After adherence procedures, cells were repeatedly washed with saline to remove all nonadherent cells. The adherent cells were cultured with complete medium over night at 37°C to rest and then stimulated as indicated above. To purify TAM by flow cytometry and sorting, ascitic fluids (four patients) were incubated with anti-CD68 mAb. A total of 5 x 10^6 cells was washed in saline with 1% human serum and then incubated in 0.5 ml of anti-CD68 diluted 1/5 for 30 min at 4°C. The cells were then washed three times in saline/1% human serum. The second incubation was conducted at 4°C for 30 min with FITC-conjugated goat F(ab')2, anti-mouse Ig. The sorting process was performed with a FACStar™ apparatus (Becton Dickinson, Mountain View, CA). Purification of TAM from solid tumor was performed as described previously (34).

**FACS analysis**

Cell staining was performed using human mAb anti-CCR5 (clone 2D7; PharMingen, San Diego, CA) and its irrelevant control mouse, IgG2a, κ (UPC10-Sigma) followed by FITC-conjugated affinity-purified, isotype-matched goat anti-mouse Ig (Southern Biotechnology Association, Birmingham, AL). For phenotype analysis, indirect immunofluorescence was performed with the human anti-CCR2 mAb Ab (clone LS132.1D9) and PE-labeled goat anti-mouse Ig (Jackson ImmunoResearch, West Grove, PA) using a FACStar (Becton Dickinson).

**Cytokines and Abs**

Human recombinant IL-β was obtained through the courtesy of Dr. J. E. Simonsen (Seattle, WA) and was used at 20 ng/ml. TNF-α (BASF/Knoll, Ludwigshafen, Germany) was used at 500 U/ml. Human recombinant MCP-1, MCP-2, and RANTES (regulated on activation normal T cell expressed and secreted) were from PeproTech (Rocky Hill, NJ). Human recombinant MCP-3 and macrophage inflammatory protein-1α (MIP-1α ) were a kind gift from Dr. A. Minty (Sanofi, Labègue, France) and Dr. Czapelewski, respectively. The mAb against TNF-α (B154.2) was a kind gift from Dr. G. Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia, PA). The mAb against IFN-γ (IFGCP) was purchased from American Type Culture Collection (Manassas, VA). IL-1ra mutant DoB 0039 was kindly donated by Dr. Diana Boraschi (Dome`, L’Aquila, Italy).

**Migration assay**

Cell migration was evaluated using a chemotaxis microchamber technique (47) as described previously (48). Twenty-seven microliters of chemotactic solution (20% RPMI 1640 medium of FCS) was added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA). A polycarbonate filter (5-μm pore size; Neuroprobe) was placed onto the wells and covered with a silicon gasket and the top plate. A total of 50 μl of cell suspension (1.5 x 10^6/ml monocytes in PBMC) was seeded in the upper chamber. The chamber was incubated at 37°C in air with 5% CO2 for 90 min. At the end of the incubation, filters were removed, stained with Diff-Quick (Baxter S.P.A., Rome, Italy), and five high-power oil-immersion fields were counted.

**Northern blot analysis**

Cells were cultured in medium alone or supplemented with the indicated agents, and total RNA was purified as described (49). Ten micrograms of total RNA from each sample was electrophoresed under denaturing conditions, blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH), and cross-linked by UV irradiation. Membranes were prehybridized with the random prime-labeled PCR products (lane 1). Histone RNA was probed to rule out differences in loading (lane 2). Blots were then probed for murine CCR1, CCR2, and CCR5 mRNA (lane 3, M-DM or lane 4, TAM purified from ascites and lane 5, lane 6–8, Lack of CCR2 mRNA expression in TAM isolated from solid tissue of ovarian carcinoma. Lane 6. Fresh monocytes from healthy donors; lanes 7 and 8, TAM from ovarium and omentum of cancer patients. Total RNA was purified from cells cultured for 4 h in RPMI 1640 supplemented with 10% of FBS. Ten micrograms of total RNA was next analyzed by Northern blot. The results presented in A show one representative experiment for each population. A total of 10, 4, and 5 specimens were analyzed for either TAM (purified from tumor ascites), TAM (purified from solid ovarian carcinoma), or PBMC, respectively. R. D., relative densitometry. B, Densitometric analysis was performed on all Northern blot results, and values were normalized for the levels of mRNA expression observed in fresh monocytes (mono) or in PBMC from healthy donors, to which a value of 1.0 was assigned. C, Surface expression of CCR2 and CCR5 in TAM and monocytes. Surface expression was determined by flow cytometry using the human anti-CCR2 and anti-CCR5 Abs. Dots, Irrelevant mAbs; continuous line, cells stained with anti-CCR2 or anti-CCR5 as indicated.

**FIGURE 1.** Defective expression of CCR2 in TAM. A, Expression of CCR1, CCR2, and CCR5. Lanes 1 and 2, Expression of the CCR2, CCR1, and CCR5 mRNAs in PBMC. PBMC were obtained from peripheral blood of either healthy donors (lane 1) or tumor patients (lane 2). Lanes 3–5, Expression of CCR1, CCR2, and CCR5 mRNAs in monocytes (lane 3), M-DM (lane 4), and TAM purified from ascites (lane 5). Lanes 6–8, Lack of CCR2 mRNA expression in TAM isolated from solid tissue of ovarian carcinoma. Lane 6, Fresh monocytes from healthy donors; lanes 7 and 8, TAM from ovarium and omentum of cancer patients. Total RNA was purified from cells cultured for 4 h in RPMI 1640 supplemented with 10% of FBS. Ten micrograms of total RNA was next analyzed by Northern blot. The results presented in A show one representative experiment for each population. A total of 10, 4, and 5 specimens were analyzed for either TAM (purified from tumor ascites), TAM (purified from solid ovarian carcinoma), or PBMC, respectively. R. D., relative densitometry. B, Densitometric analysis was performed on all Northern blot results, and values were normalized for the levels of mRNA expression observed in fresh monocytes (mono) or in PBMC from healthy donors, to which a value of 1.0 was assigned. C, Surface expression of CCR2 and CCR5 in TAM and monocytes. Surface expression was determined by flow cytometry using the human anti-CCR2 and anti-CCR5 Abs. Dots, Irrelevant mAbs; continuous line, cells stained with anti-CCR2 or anti-CCR5 as indicated.
A

PBMC

Healthy donor tumor patient

- CCR2

- CCR1

- CCR5

R.D.

1 2

3 4 5

6 7 8

R.D.

R.D.

- CCR2

- CCR1

- CCR5

R.D.

R.D.

- actin

- actin

- actin

B

CCR2 mRNA expression

Relative density

Mono M-DM Ascos Solid

Healthy Donors Tumor Patients

PBMC

C

MONOCYTES

CCR2

CCR5

Relative density

Mono M-DM Ascos Solid

Healthy Donors Tumor Patients

PBMC

TAM

CCR2

CCR5
at 42°C in Hybrisol (Oncor, Gaithersburg, MD) and hybridized overnight with 1 × 10^6 cpm/ml of [32P]-labeled probe. Membranes were then washed three times at room temperature for 10 min in 0.2× SSC (1× SSC: 0.15 M NaCl; and 0.015 M sodium citrate (pH 7.0)], 0.1% SDS, and twice at 60°C for 20 min in 0.2× SSC and 0.1% SDS before being autoradiographed using Kodak XAR-5 films (Eastman, Rochester, NY) and intensifier screens at −80°C. cDNAs were labeled by random priming using a commercial kit (Boehringer Mannheim, Indianapolis, IN) and [α-32P]-deoxy-cytidine 5'-triphosphate (3000 Ci/mmol; Amersham, Arlington Heights, IL). CCR2B cDNA was obtained by PCR amplification of the reported sequence (27, 50). CCR1 and CCR5 cDNAs were obtained as described previously (51). Densitometric analysis was performed with a scanning densitometer GS300 (Hoefer Scientific Instruments, San Francisco, CA).

Results

**Lack of expression of the MCP-1 receptor (CCR2) in macrophages associated with human ovarian carcinoma**

We investigated expression of chemokine R mRNA in TAM by Northern blot analysis. Fig. 1A shows one representative experiment, and Fig. 1B summarizes a densitometric analysis of the results obtained in the examined populations. TAM from ascites (10 samples) or solid tumor (four samples) showed little or no detectable CCR2 mRNA. This defect was selective because CCR1 was normally expressed and, as shown by densitometric analysis (Fig. 1B), only in a few samples was the CCR5 mRNA level slightly decreased. Because the defective expression of CCR2 in TAM may have been the result of the separation procedure, resulting for instance in exposure of mononuclear phagocytes to cellular debris, PCR analysis of the CCR2 mRNA level was performed on 25 ovarian cancer biopsies, including 19 serous adenocarcinomas, 2 clear cell carcinomas, 1 mucinous adenocarcinoma, 1 anaplastic carcinoma, 1 signet ring carcinoma, and 1 endometrioid carcinoma. All of the examined specimens were negative for CCR2A and CCR2B mRNAs expression, whereas 80% of the biopsies were positive for MCP-1 (data not shown). In addition, the THP-1 and MonoMac6 cell lines, which were included as part of the screen, displayed detectable CCR2 mRNA. In two patients, mRNA data were confirmed by FACS analysis (Fig. 1C). Although monocytes displayed high positivity for CCR2, TAM showed almost undetectable levels of this receptor. In contrast, CCR5 surface expression in TAM was only modestly decreased.

PBMC were used as a source of monocytes to assess expression of CCR2 in circulating mononuclear phagocytes. Because CCR2 is virtually not expressed in resting T, B, and NK cells (13, 14), monocytes are the main source of these transcripts in unseparated PBMC. As shown in Fig. 1A, circulating monocytes from five ovarian cancer patients (two, stage II; two, stage IV; and one, stage III) consistently showed defective CCR2 mRNA expression, but the defect was not as drastic as in TAM.

**TAM do not migrate in response to MCP-1**

Having established that TAM and peripheral mononuclear cells from patients with ovarian carcinoma display absent or decreased expression of the CCR2, we wanted to investigate whether this defect correlated with impaired cell migration. MCP-1 (CCL2) is a high-affinity ligand for the CCR2 receptor (26), thus migration of both TAM and PBMC was assayed in chemotaxis microchamber in response to MCP-1. As shown in Fig. 2A, MCP-1 did not enhance the migration of TAM, whereas a classical chemotactic stimulus such as C5a increased it. Despite the presence of CCR5 mRNA expression, no significant increase over the spontaneous migration of TAM was obtained in response to MIP-1β, suggesting that other determinants, such as receptor desensitization and/or internalization, may control CCR5 activity in TAM (41, 42). As expected, fresh monocytes displayed high responsiveness to the chemoattractants used in the assay. As shown in Fig. 2B, in response to MCP-1, migration of PBMC from tumor patients was defective (60% of inhibition), but not as profound as that of TAM. These cells also displayed reduced migration also toward MIP-1β (40% of inhibition). In contrast, their migration in response to C5a was less affected (21% of inhibition).

**Role of TNF-α of tumor ascites on the CCR2 mRNA expression by monocytes**

Based on these observations, it was important to understand whether factors present in ascites from the ovarian cancer could suppress CCR2 expression. Tumor ascites were reported to contain IL-6 and MCP-1 do not affect CCR2 mRNA expression in monocytes and M-DM (data not shown). In Fig. 3A, we investigated whether ascites from the ovarian carcinoma may cause inhibition of the CCR2 mRNA expression. Freshly isolated monocytes (Fig. 3A, lanes 1 and 2) and M-DM (Fig. 3A, lanes 3 and 4) from healthy donors were incubated for 4 h in the presence or absence of 20% of ascitic fluid. Ascitic fluid elicited a strong inhibitory effect on the expression of CCR2 mRNA. This inhibition was specific as no alterations were observed for the levels of expression of the CCR3 transcript. Based on this observation, the identification of putative factors responsible for the suppression of CCR2 mRNA expression was conducted by using blocking Abs directed against proinflammatory cytokines. Anti-IFN-γ, anti-TNF-α Abs, and the IL-1ra were preincubated for 2 h at room temperature with ascitic fluid in an attempt to block the...
inhibitory activity for CCR2. As shown in Fig. 3B, in the presence of ascitic fluid, anti-TNF-α (lane 5) was able to efficiently preserve the levels of expression of CCR2 mRNA, whereas the anti-IFN-γ Ab (lane 3) and IL-1ra (lane 4) did not. Coincubation with anti-IFN-γ and anti-TNF-α Abs did not synergize (Fig. 3B, lane 6). These results strongly indicate TNF-α present in the ascites of ovarian carcinoma as a tumor-derived factor responsible for the inhibition of CCR2 in TAM.

Discussion

The results presented here show that macrophages associated with human ovarian carcinoma display defective expression of CCR2, a receptor for MCPs. Lack of CCR2 expression was specific and functionally relevant since TAM were unable to respond to MCP-1 chemotactically, but still migrated in response to C5a. This defect was selective in that TAM expressed basically normal levels of CCR1 and CCR5. Moreover, lower CCR2 expression and function was observed also in the periphery, as PBMC from five tumor patients expressed lower levels of CCR2 mRNA and poorly migrated in response to MCP-1. Loss of CCR2 mRNA expression was reproduced in monocytes cultured in the presence of ascitic fluid of ovarian cancer and confirmed in 25 ovarian cancer biopsies.

The selective loss of CCR2 expression in TAM resembles the specific and selective inhibition operated by LPS and certain proinflammatory cytokines (e.g., IL-1, TNF-α, and IFN-γ) on the CCR2 expression in monocytes (41, 42). We also reported that the anti-inflammatory cytokine IL-10 induces CCR2, CCR5, and CCR1 expression (43), suggesting that proinflammatory and anti-inflammatory signals have opposite and divergent actions on CC chemokine receptor expression in monocytes. In this regard, ovarian cancer is one epithelial malignancy in which there is evidence of a complex network between the tumor microenvironment and the immune system (37, 54), and there is strong evidence that TNF-α is a cytokine which strongly influences the biology of this tumor (54, 55). Despite the expression of chemokine receptors, lack of migration in response to a specific ligand has been reported in cells activated with proinflammatory signals (41–43), a condition likely shared by TAM. This may potentially be the result of a number of mechanisms such as homologous or heterologous receptor desensitization, internalization, and/or inhibition of receptor signaling. In addition, the observed difference in the number of migrated TAM vs migrated monocytes in response to C5a may be at least partially due to cell differentiation.

Previous reports have indicated that ovarian cancer cells significantly express TNF-α (55) and we pointed to this cytokine as a possible tumor-derived inhibitor of the CCR2 expression. The validity of this hypothesis is substantiated by the observation that inhibition of CCR2 mRNA expression by tumor ascites is efficiently prevented by an anti-TNF-α Ab. Despite this observation, the levels of TNF-α that we detected in tumor ascites were quite low (data not shown) and may not fully explain the drastic down-regulation of CCR2 mRNA levels, but rather suggest possible synergism with other proinflammatory signals. Indeed, since ovarian cancer cells are a rich source of cytokines (37), it is likely that other proinflammatory cytokines within the tumor microenvironment may strengthen the inhibitory action of TNF-α. The observed decrease of CCR2 mRNA levels in PBMC from cancer patients may also imply the possibility that factors released from growing tumors may, beyond a certain size, leak into the systemic circulation to control excessive recruitment and reverse transmigration. Thus, the drastic down-regulation of CCR2 in comparison to the others receptors studied here may assume particular relevance in view of evidence suggesting MCP-1 as a main determinant of macrophage infiltration in tumors (1, 2) and normal tissues (31, 32).

We previously proposed (41) that reciprocal influences exerted by proinflammatory molecules on chemokine agonist production and receptor expression may represent a crucial set point in the regulation of the chemokine system. Our data provide the first evidence that this emerging paradigm has in vivo relevance in the pathophysiology of tumors and point to TNF-α as a tumor-derived cytokine controlling the chemokine system of macrophages associated with tumors.