

Analysis of Chemokines and Chemokine Receptor Expression in Ovarian Cancer Ascites

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ABSTRACT

Purpose: Ascitic disease is a common occurrence in human ovarian cancer, but it is unclear how the cellular composition of ascitic fluid is determined. Because chemokines can determine host cell infiltration in solid ovarian cancer, we assessed CC chemokine protein and CC chemokine receptor expression in ovarian cancer ascites.

Experimental Design: We used reverse transcription-PCR and RNase protection assay to determine CC chemokine and chemokine receptor mRNA expression and ELISA to measure CC chemokine protein levels. Flow cytometry was used to identify cell populations and their chemokine receptor protein expression.

Results: mRNA for the CC chemokines CCL2, -3, -4, -5, -8, and -22 was expressed in cell isolates from ascites samples, and the corresponding proteins were detected in ascitic fluid. mRNA for CC chemokine receptors CCR1, -2a, -2b, -3, -4, -5, and -8 was detected in cells from ascites. Fluorescence-activated cell-sorting analysis showed variable numbers of macrophages and CD3⁺ T lymphocytes (predominantly CD4⁺) within ovarian cancer ascites. CD14⁺ macrophages within ascites consistently expressed protein for CCR1, -2, and -5. CCR1 was expressed by >60% of all T cells, but more CD4⁺ than CD8⁺ T cells expressed CCR2 and -5. A direct correlation was found between the CCL5 concentration and CD3⁺ T-cell infiltration.

Conclusions: We conclude that there is a complex chemokine/chemokine receptor network in ovarian cancer ascites. However, associations between chemokine receptor expression, chemokine levels, and cell counts were limited.

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INTRODUCTION

Chemokines are a large subfamily of chemoattractant cytokines, which are classified into four highly conserved groups, CXC, CC, C, and CX₃C, based on the position of the first two cysteines adjacent to the NH₂ terminus. Chemokines selectively regulate the recruitment and trafficking of leukocyte subsets to inflammatory sites. Chemotactic and growth signals initiated by chemokines are mediated by the activation of G-protein-coupled receptors.

Early growth of epithelial ovarian cancer is usually confined to the ovaries, whereas in advanced stages of ovarian cancer, the peritoneal wall, diaphragm, and omental structures are seeded with micro- and macrometastases of tumor cells. Growth of ovarian cancer (stages 1C–IVC) is also associated with the development of ascites (1). Ascitic fluid arises as a plasma exudate; formation results from an imbalance between the influx and efflux of fluid from the peritoneal compartment (2). This is attributable to a combination of increased microvascular permeability mediated by the angiogenic development of immature blood vessels (3); the production of vascular permeability agents, including vascular endothelial growth factor (4–6); and the blockade of lymphatic drainage by cells present in the ascites (7). Ascites consists of a proteinaceous fluid with variable numbers of suspended cells and debris. The cellular population consists of differing proportions of tumor cells, mesothelial cells, fibroblasts, macrophages, leukocytes, and RBCs (8, 9). Peritoneal dissemination of ovarian carcinoma occurs by the growth of malignant cells in the protein-rich ascitic fluid (10).

Ovarian cancer is one epithelial malignancy in which there is evidence for a complex cytokine network. Published information on individual cytokines has suggested a number of autocrine and paracrine cytokine loops in the biology of this tumor (11). Ascitic fluid is rich in cytokines and growth factors that are secreted by the tumor cells and by mesothelial cells lining the peritoneal cavity, some of which can act to directly stimulate tumor cell growth (12–15). It is possible that ascitic fluid also contains chemokines that may influence its cellular composition, but this has not been investigated in detail. Previously our group has detected mRNA for CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CCL5 (RANTES) in solid ovarian tumors by *in situ* hybridization. CCL2 was the predominant chemokine detected in both epithelial and stromal areas, and this chemokine was also found in ascites (16).

With the aim of further understanding chemokine action in human ovarian cancer, we investigated CC chemokine receptors and their ligands in ascitic fluid samples. This, to our knowledge, is the first detailed study of CC chemokines and their receptors in human ovarian cancer ascites.

MATERIALS AND METHODS

Samples. Samples of ascitic fluid were collected from patients with ovarian carcinoma at the time of surgery or by paracentesis for palliative/diagnostic purposes. Volumes of

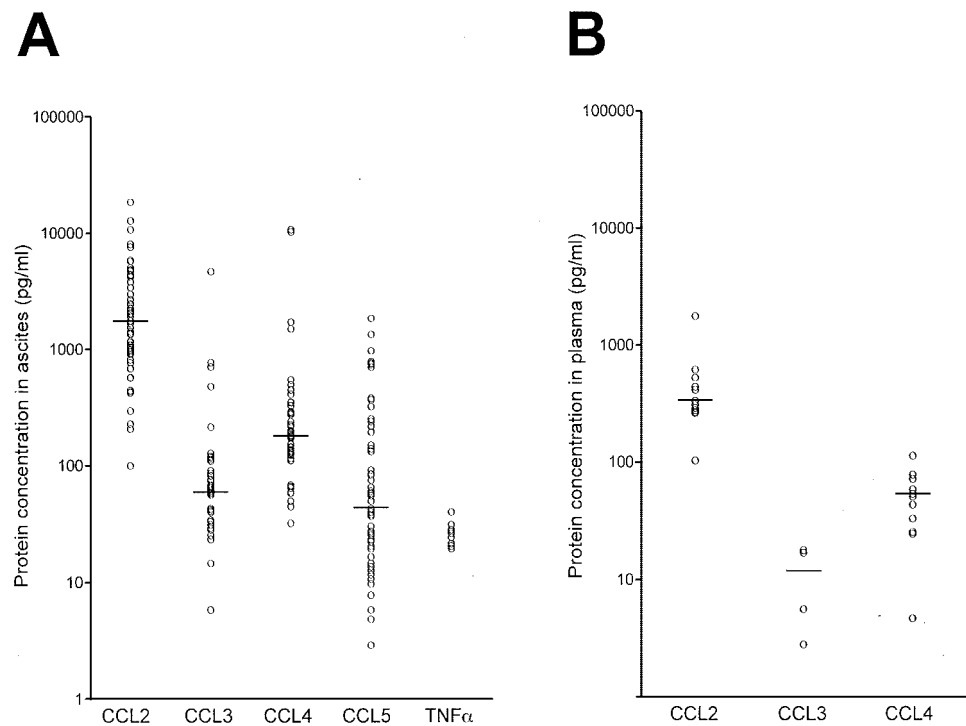


Fig. 1 ELISA for chemokine protein. **A**, ELISA data for chemokines and TNF α in 66 samples of ascitic fluid. **B**, ELISA data for chemokines in patient plasma. Horizontal bars represent median values. For correlations and statistical analysis, see Table 2.

1–1.5 liters were taken. All 66 ascites samples were from patients with epithelial ovarian cancer. Each sample was spun down, and the ascitic fluid was removed for ELISA analysis. The cell isolate was treated with ACK lysing buffer to remove RBCs before RNA extraction or flow cytometry. In some patients, a peripheral blood sample was also collected, plasma was removed and stored at -80°C , and PBMCs² were isolated by Ficoll-Hypaque density centrifugation (Lymphoprep; Nycomed, Birmingham, United Kingdom). Control PBMCs were isolated from heparinized venous blood or cytophoresis buffy coats from normal volunteer donors. In five samples, the pO₂ and pCO₂ were measured.

RNA Extraction and RT-PCR. Total RNA was prepared from all samples with Tri Reagent (Sigma Chemical Co., Poole, United Kingdom) according to the manufacturer's instructions. For RT-PCR and RNase protection analysis, total RNA was treated with DNase to remove contaminating genomic DNA, using RNase-free DNase I (Pharmacia Biotech, St. Albans, United Kingdom). cDNA was synthesized from DNase-treated total RNA using the Ready-to-Go T-primed First Strand kit (Pharmacia Biotech). The primers for CCL2 and CCL22 (MDC) were designed from sequences submitted to GenBank, using Primer 3.0. CCL3 and CCL4 sequences were from Hosaka *et al.* (17), and CCL5 and CCL8 sequences were from Mattei *et al.* (18) and Van Coillie *et al.* (19), respectively. The primer

sequences and product sizes for CCL2 and CCL22 (MCP-2) were as follows:

CCL2: forward, 5'-CAAAGTGAAGCTCGCACTCTCGCC-3'; reverse, 5'-ATTCTTGGGTTGTGGAGTGAGTGTTC-3' (product, 354 bp)

CCL22: forward, 5'-CCCTACCTCCCTGCCATTAT-3'; reverse, 5'-CAGGGAGCTAGAACCCAACA-3' (product, 338 bp)

For each sample, a 25- μl volume was used, containing 200 ng of cDNA, 1 unit of AmpliTaq DNA polymerase, GeneAmp PCR buffer, GeneAmp deoxynucleotide triphosphates (all from Perkin-Elmer, Beaconsfield, United Kingdom), and 4 μM each primer. The following protocol was used in a GeneAmp PCR System 9700 thermal cycler: 94 $^{\circ}\text{C}$ for 5 min; 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 60 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 30 s; with a final step at 72 $^{\circ}\text{C}$ for 7 min. PCR products were electrophoresed through 1.2% agarose gels and visualized by ethidium bromide. Markers (123 bp; Life Technologies, Inc., Paisley, United Kingdom) were used to estimate band sizes. PCR products were extracted from the gels and sequenced to confirm their identity.

RPA. The hCR5 template set from PharMingen (Becton Dickinson, Oxford, United Kingdom) contained DNA templates for CCR1, CCR2, CCR2a, CCR2b, CCR3, CCR4, CCR5, CCR8, GAPDH, and L32. RPA was carried out using [α -³⁵S]UTP (Amersham International plc, Aylesbury, United Kingdom) rather than [α -³²P]UTP. The RNase-protected fragments were separated on an acrylamide-urea sequencing gel (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom), which was then adsorbed to filter paper and dried under vacuum. Autoradiography was subsequently carried out using Kodak Biomax MS film with a Transcreen LE

² The abbreviations used are: PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-PCR; RPA, RNase protection assay; TNF α , tumor necrosis factor α .

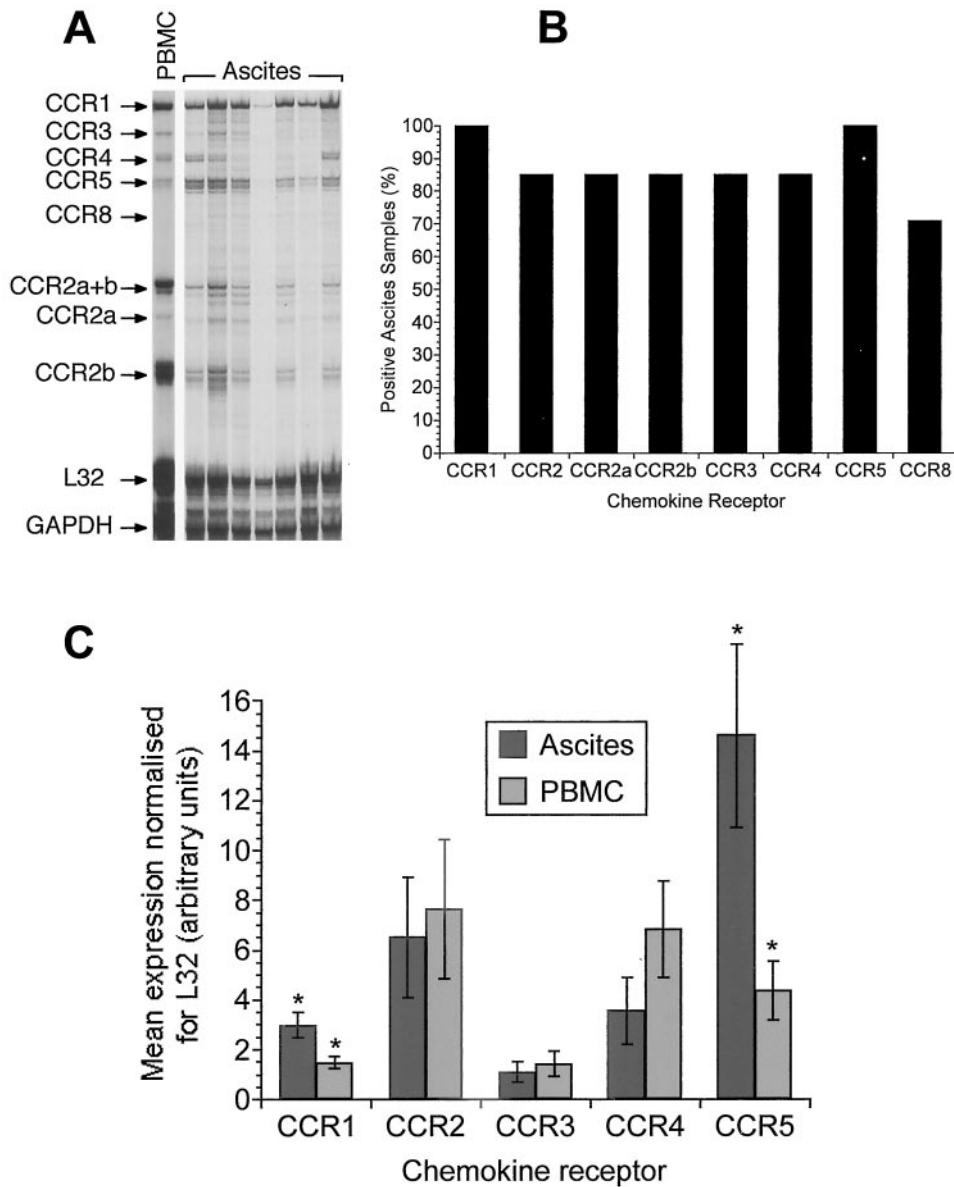


Fig. 2 Chemokine receptor expression in ovarian cancer ascites. **A**, RPA for CC chemokine receptor expression in normal PBMCs and cell isolates from ovarian cancer ascites. **B**, percentage of samples expressing CC chemokine receptor mRNA in cell isolates from ovarian cancer ascites, based on the data derived from **A**. **C**, densitometric analysis of CC chemokine receptor expression in ascites and PBMCs from the same patients. * denotes statistical significance ($P < 0.05$). Bars, SD.

intensifying screen (Sigma). Densitometry was performed using NIH Image 1.61.

ELISA of Chemokines in Ascitic Fluid and Plasma Samples. Concentrations of the chemokines CCL2, CCL3, CCL4, and CCL5 and the cytokine TNF α were measured in 66 samples of ascitic fluid and 12 patient plasma samples, using Quantikine ELISA kits (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's protocol. The sensitivities of the assays were as follows: 5 pg/ml for CCL2, 7 pg/ml for CCL3, 4 pg/ml for CCL4, 8 pg/ml for CCL5, and 4 pg/ml for TNF α .

Monoclonal Antibodies and Flow Cytometry. FITC- and phycoerythrin-labeled monoclonal antibodies against CD3 (UCHT1), CD4 (MT310), CD8 (DK25; all from DAKO, Ely, United Kingdom), CD14 (UCHM-1; Sigma), CCR1 (a kind gift

from Shixin Qin, Millennium Pharmaceuticals, Cambridge, MA), CCR2 (48607.211), and CCR5 (45531.111; R&D Systems) and isotype-matched labeled controls were obtained and used to characterize cell surface phenotypes by flow cytometry. For staining, cells were washed and resuspended in PBS supplemented with 1% heat-inactivated fetal bovine serum and 0.01% NaN₃. Antibodies were diluted in this buffer and used at a final concentration of 2–20 μ g/ml. Incubations with antibodies were carried out for 30 min on ice. After the final washing step, labeled cells were fixed with 1% formaldehyde solution (Sigma). Appropriate gates were drawn around ascites cell populations after propidium iodide staining was used to eliminate dead cells and debris; 10,000 gated cells were analyzed by flow cytometry on a FACScan flow cytometer using Cellquest software (Becton Dickinson). For determination of CD4⁺/CD8⁺

T-cell populations, only those cells within a gate drawn around the distinct CD3⁺ population were counted to minimize the inclusion of any CD4⁺ macrophages.

Statistical Analysis. To determine correlation coefficients for the ascitic fluid chemokine concentrations, the non-parametric Spearman's rank correlation was used to calculate *P* because the data were not normally distributed (20). For all other statistical analysis, we used the Welch's *t* test (a more stringent form of Student's *t* test).

RESULTS

Cell Populations within Ascitic Fluid. Fluorescence-activated cell-sorting analysis of 20 ascites samples from patients with ovarian cancer showed considerable variation within the cell populations. The numbers of CD14⁺ cells were variable (median, 27%; range, 0–66%). There were usually high numbers of CD3⁺ T lymphocytes (median, 59%; range, 1–89%) forming a discrete population of cells. The CD3⁺ population contained predominantly CD4⁺ T cells (median, 51%; range, 1–65%), rather than CD8⁺ T cells (median, 10%; range, 0–31%). The T-cell subsets in ascitic fluid differed significantly from those in solid ovarian tumors, which contain predominantly CD8⁺ T cells (with few or no CD4⁺ T cells) and macrophages (16). Because T lymphocytes and macrophages typically respond to CC chemokines, we studied a range of these and their related receptors in ascites.

Chemokine Protein Levels in Ovarian Cancer Ascites. CCL2, CCL3, CCL4, and CCL5 protein levels were measured by ELISA in 66 samples of ascitic fluid from ovarian cancer patients (Fig. 1A). The total protein concentration in ascites was reasonably constant (range, 13–75 mg/ml; median, 44 mg/ml); therefore, chemokine concentrations are expressed as pg/ml. All 66 samples contained the CC chemokine CCL2 at a range of 103–19,195 pg/ml (median, 1778 pg/ml); this was the most abundant CC chemokine. The median concentrations of CCL3, CCL4, and CCL5 were approximately one log lower, at 60, 182, and 44 pg/ml, respectively. CCL3 was found in 62 of 66 samples (range, 6–4840 pg/ml), CCL4 was found in all 66 samples (range, 33–11,040 pg/ml), and CCL5 was found in 63 of 66 samples (range, 3–1903 pg/ml). TNF α levels were also assayed in 37 of the ascites samples. Only 16 of the 37 samples had detectable levels of TNF α (range, 20–41 pg/ml).

Chemokine Protein Levels in Ovarian Cancer Patient Plasma. In addition to ascitic fluid, plasma was obtained from a few patients. Protein concentrations for CCL2, CCL3, CCL4, and CCL5 were determined by ELISA in these plasma samples. CCL2, CCL3, and CCL4 were present in low concentrations, with medians of 341, 12, and 54 pg/ml, respectively (Fig. 1B). CCL2 was present in 12 of 12 samples (range, 110–1888 pg/ml), CCL3 was present in 4 of 10 samples (range, 3–19 pg/ml), and CCL4 was present in 12 of 13 samples (range, 5–120 pg/ml). CCL5 was detected at extremely high levels, with a median of 28,950 pg/ml (data not shown); however, this was likely attributable to the release of stored CCL5 from platelets (21). These results suggest that a chemokine gradient may exist between plasma and ascites, which may partially explain the leukocyte infiltration seen in ascites.

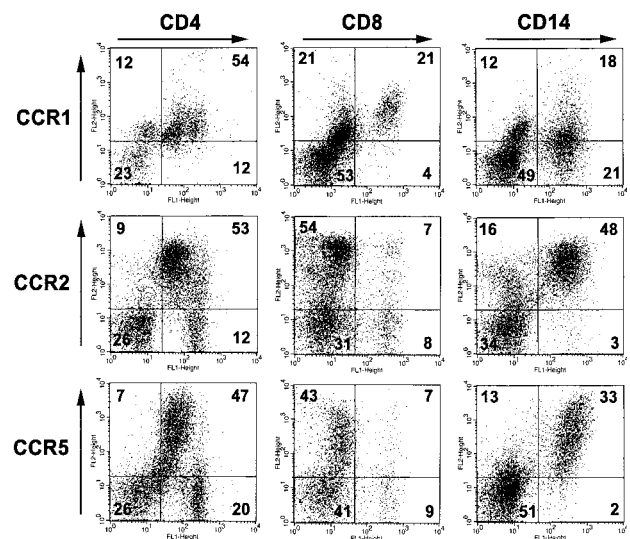


Fig. 3 Chemokine receptor expression on cells isolated from ovarian ascites as determined by flow cytometry. CCR1, CCR2, and CCR5 expression on CD4⁺ (left panels), CD8⁺ (middle panels), and CD14⁺ (right panels) cells.

CC Chemokine mRNA Expression in Cell Isolates from Ovarian Cancer Ascites. RT-PCR was used to screen for six CC chemokines (CCL2, CCL3, CCL4, CCL5, CCL8, and CCL22), which bind to the receptors studied below, in cell isolates from ascites samples. Each of these chemokines was expressed in 85–100% of the samples studied (data not shown).

Chemokine Receptor Expression in Cell Isolates from Ascitic Disease. We found that CC chemokine receptor expression in seven samples of RNA isolated from ascites was similar to that of the normal PBMC control (Fig. 2, A and B), with a majority of samples positive for each receptor. This contrasts with our findings on RNA from solid ovarian tumor biopsies (22), where CCR1 was the only CC chemokine detected in the majority of cases. In solid tumors, CCR1 localized to infiltrating leukocytes and macrophages. The level of chemokine receptor expression in five of the ascites samples was compared densitometrically with the expression in PBMCs from the same patients. When normalized for the “housekeeping” gene, *L32*, the levels of expression for CCR2, CCR3, and CCR4 were similar in PBMCs and ascites, but CCR1 and CCR5 appeared to be more strongly expressed by the ascites cells (*P* < 0.05; Fig. 2C).

CC Chemokine Receptor Protein on Ascites Cells. Two-color flow cytometric analysis of cells freshly isolated from three samples of ovarian cancer ascites detected cell surface expression of CCR1, CCR2, and CCR5 on the majority of CD14⁺ cells (Fig. 3 and Table 1). CCR1 was expressed by the majority of CD4⁺ T cells. CCR2 expression was detected on 25–80% of cells, and CCR5 was detected on 23–70% of the cells (Fig. 3 and Table 1). CCR1 was expressed by the majority of CD8⁺ T cells, and CCR2 and CCR5 were expressed by ~5–45% of cells (Fig. 3 and Table 1). Thus, more CD4⁺ than CD8⁺ T cells expressed CCR2 and CCR5, suggesting a difference in the chemokine receptor profile of these cell types.

Table 1 Percentage of each leukocyte type expressing CCR1, CCR2, and CCR5, in three samples of ascites.

Sample	Cell type	Cells positive for each receptor (%)		
		CCR1	CCR2	CCR5
A	CD4 ⁺	82	82	70
	CD8 ⁺	84	44	45
	CD14 ⁺	45	94	93
B	CD4 ⁺	88	75	42
	CD8 ⁺	87	48	27
	CD14 ⁺	80	96	68
C	CD4 ⁺	57	26	19
	CD8 ⁺	91	6	4
	CD14 ⁺	57	88	60

Table 2 Correlations between the chemokines present in ascites. Spearman correlation coefficients and (*P*) between the chemokines. *P* < 0.05 is statistically significant (**bold**).

	CCL2	CCL3	CCL4	CCL5
CCL2				
CCL3	0.04 (0.78)			
CCL4	0.11 (0.11)	0.56 (<0.0001)		
CCL5	0.32 (0.008)	0.29 (0.02)	0.28 (0.02)	

Correlations. The nonparametric Spearman's rank correlation was used to examine the relationship between the protein concentrations of the four CC chemokines studied. Significant correlations were found between the levels of CCL3 and CCL4 ($r_s = 0.56$; $P = 0.0001$; Table 2) and between the levels of CCL2 and CCL5 ($r_s = 0.32$; $P = 0.008$). Weaker associations were observed between CCL3 and CCL5 ($r_s = 0.29$; $P = 0.02$) and between CCL4 and CCL5 ($r_s = 0.28$; $P = 0.02$). No other chemokine pair gave a significant correlation. These correlations may suggest that CCL3 and CCL4 are produced by the same cell type or are induced by the same stimulus in ovarian cancer ascites. This could also be true for CCL2 and CCL5.

Despite high levels of chemokine protein, there were few correlations found between the total cell counts in the ascites and the concentration of any chemokine (Table 3). CCL5 was expressed at higher levels when CD3⁺ cells were prevalent ($r_s = 0.54$; $P = 0.01$), and there was a negative correlation between CCL5 and the presence CD14⁺ cells ($r_s = -0.47$; $P = 0.03$). There appeared to be no correlations between the stage of disease and the presence of any chemokine.

DISCUSSION

Previous studies by our group and others have addressed the relationship between leukocyte infiltration into solid tumors and chemokine expression (14, 16, 23). To our knowledge, this work is the first comprehensive study of CC chemokines and chemokine receptor expression in human ovarian cancer ascites.

We found that ascitic fluid is rich in CC chemokines and that the CD14-expressing cells and T cells present in ovarian cancer ascites express CC chemokine receptor mRNA and protein.

Is the extent and phenotype of the leukocyte infiltration in ovarian ascites related to chemokines and chemokine receptor

Table 3 Correlations between the chemokines and cell populations within ovarian ascites

Spearman correlation coefficients and (*P*) between the chemokines and cell populations within ovarian ascites. *P* < 0.05 is statistically significant (**bold**).

	CCL2	CCL3	CCL4	CCL5
CD3	-0.14 (0.57)	0.09 (0.70)	-0.12 (0.62)	0.54 (0.01)
CD14	0.08 (0.75)	-0.24 (0.31)	-0.26 (0.28)	-0.47 (0.03)

expression? Gradients of chemokines usually cause tissue recruitment of leukocytes through effects on adhesion and endothelial transmigration (24). Our data suggest that CC chemokine protein levels are significantly higher in ascitic fluid than in patient plasma samples. Therefore, chemokines present in ascites could form a gradient for leukocyte migration into the peritoneal cavity. However, we did not find many associations between chemokine concentrations and leukocyte numbers; this could be because multiple chemokines cooperate to attract a particular leukocyte. Apical production of chemokines during infection or peritoneal inflammation is well documented (25); both CC and CXC chemokines are produced by mesothelial cells, and the resulting chemokine gradient chemoattracts an infiltrate of polymorphonuclear leukocytes.

There was a correlation between CCL5 expression in ascites and the presence of CD3⁺ T cells, suggesting that this chemokine could be important for recruiting T cells. This chemokine also correlated with the presence of CD8⁺ T cells in solid ovarian tumors (16).

The fact that the CD4:CD8 ratio differs between solid tumor (16) and ascites may be related to CC chemokine receptor expression on the T cells. There were differing proportions of CD4⁺ and CD8⁺ T cells expressing CCR2 and CCR5 (the receptor for CCL5). CCR2 was expressed more by CD4⁺ T cells than by CD8⁺ T cells, and the same was true of CCR5. The lower number of CD8⁺ T cells expressing CCR5 could account for the defective recruitment of these cells to the ascites compared with the solid tumor. We cannot rule out that a small proportion of the CD4⁺ cells may be CD14⁺ macrophages rather than T cells; however, the CD3⁺ T-cell population was clearly distinct from the CD14⁺ population by flow cytometry.

Overall, the profile of CC chemokine receptor expression in ascites was similar to that of leukocytes in peripheral blood and contrasted with the restricted expression of CC chemokine receptors seen in solid ovarian tumors (22). This may be attributable to differences in the solid tumor and ascitic microenvironment. TNF α levels in ascites are low; therefore, this cytokine is unlikely to be responsible for the stimulation of chemokine production or down-regulation of CC chemokine expression (26). Another important stimulus regulating chemokine production is hypoxia (27, 28). Gas analysis of four ascites samples revealed that pO₂ (range, 59–95 mm Hg) and pCO₂ (range, 41–52 mm Hg) were similar to that of arterial blood (~95 and 40 mm Hg, respectively). Solid tumors are likely to be hypoxic and have high TNF levels, whereas ascites is normoxic with low TNF levels. These factors may contribute to the differences in CC chemokine receptor expression between the two tumor states.

There was a negative correlation between CCL5 and CD14⁺ cells. A majority of CD14⁺-expressing cells within ascites consistently expressed CCR1, CCR2, and CCR5. CCR5 and CCR1 are both receptors for CCL5, suggesting that CCL5 may have an antagonistic effect under certain circumstances. Alternatively, we found a correlation between the concentrations of CCL2 and CCL5, although CCL2 levels were consistently one log higher. Thus, at high CCL5 concentrations, CCL2 levels may become antagonistic, as has been shown for CXCL12 (SDF-1 α ; Ref. 29).

CCR1 was the predominant receptor found on leukocytes within the solid tumor (22); this receptor was also found on the majority of leukocytes within the ascites. The expression of CCR2 on CD14⁺ cells within the ascites contrasts with the results obtained by Sica *et al.* (26), who suggested CCR2 down-regulation on ovarian tumor infiltrating macrophages. However, this may reflect the fact that the cells used in this study are freshly isolated from ascitic fluid and not purified or cultured. Recently, we have demonstrated that ovarian tumor cells express functional CXCR4 (30), and high concentrations of the ligand CXCL12 have been detected in ovarian cancer ascites. Similar observations in breast and pancreatic carcinoma (31, 32) lead us to suggest that ovarian cancer cells may respond to chemokine gradients in the process of metastasis.

We conclude that there is a complex chemokine/chemokine receptor network in ovarian cancer ascites. We found few associations between chemokine receptor expression, chemokine levels, and cell composition; this is in contrast to the associations found by us and others in solid tumors. It is possible that high levels of chemokine protein in ascites cause receptor desensitization.

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REFERENCES

- Hirabayashi, K., and Graham, J. Genesis of ascites in ovarian cancer. *Am. J. Obstet. Gynecol.*, 106: 492–497, 1970.
- Nagy, J. A., Herzberg, K. T., Dvorak, J. M., and Dvorak, H. F. Pathogenesis of malignant ascites formation: initiating events that lead to fluid accumulation. *Cancer Res.*, 53: 2631–2643, 1993.
- Garrison, R. N., Galloway, R. H., and Heuser, L. S. Mechanisms of malignant ascites production. *J. Surg. Res.*, 42: 126–132, 1987.
- Nagy, J. A., Masse, E. M., Herzberg, K. T., Meyers, M. S., Yeo, K. T., Yeo, T. K., Sioussat, T. M., and Dvorak, H. F. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. *Cancer Res.*, 55: 360–368, 1995.
- Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science (Wash. DC)*, 219: 983–985, 1983.
- Nagy, J. A., Herzberg, K. T., Mase, E. M., Zientara, G. P., and Dvorak, H. F. Exchange of macromolecules between plasma and peritoneal cavity in ascites tumor-bearing, normal, and serotonin-injected mice. *Cancer Res.*, 49: 5448–5458, 1989.
- Feldman, G. B. Lymphatic obstruction in carcinomatous ascites. *Cancer Res.*, 35: 325–332, 1975.
- Haskill, S., Becker, S., Fowler, W., and Walton, L. Mononuclear-cell infiltration in ovarian cancer. I. Inflammatory-cell infiltrates from tumour and ascites material. *Br. J. Cancer*, 45: 728–736, 1982.
- Sheid, B. Angiogenic effects of macrophages isolated from ascitic fluid aspirated from women with advanced ovarian cancer. *Cancer Lett.*, 62: 153–158, 1992.
- Veatch, A. L., Carson, L. F., and Ramakrishnan, S. Differential expression of the cell-cell adhesion molecule E-cadherin in ascites and solid human ovarian tumor cells. *Int. J. Cancer*, 58: 393–399, 1994.
- Burke, F., Relf, M., Negus, R., and Balkwill, F. A cytokine profile of normal and malignant ovary. *Cytokine*, 8: 578–585, 1996.
- Li, B. Y., Mohanraj, D., Olson, M. C., Moradi, M., Twigg, L., Carson, L. F., and Ramakrishnan, S. Human ovarian epithelial cancer cells cultures *in vitro* express both interleukin 1 α and β genes. *Cancer Res.*, 52: 2248–2252, 1992.
- Mills, G. B., May, C., Hill, M., Campbell, S., Shaw, P., and Marks, A. Ascitic fluid from human ovarian cancer patients contains growth factors necessary for intraperitoneal growth of human ovarian adenocarcinoma cells. *J. Clin. Investig.*, 86: 851–855, 1990.
- Punnonen, R., Teisala, K., Kuoppala, T., Bennett, B., and Punnonen, J. Cytokine production profiles in the peritoneal fluids of patients with malignant or benign gynecologic tumors. *Cancer (Phila.)*, 83: 788–796, 1998.
- Stromberg, K., Collins, T. J., Gordon, A. W., Jackson, C. L., and Johnson, G. R. Transforming growth factor- α acts as an autocrine growth factor in ovarian carcinoma cell lines. *Cancer Res.*, 52: 341–347, 1992.
- Negus, R. P., Stamp, G. W., Hadley, J., and Balkwill, F. R. Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. *Am. J. Pathol.*, 150: 1723–1734, 1997.
- Hosaka, S., Akahoshi, T., Wada, C., and Kondo, H. Expression of the chemokine superfamily in rheumatoid arthritis. *Clin. Exp. Immunol.*, 97: 451–457, 1994.
- Mattei, S., Colombo, M. P., Melani, C., Silvani, A., Parmiani, G., and Herlyn, M. Expression of cytokine/growth factors and their receptors in human melanoma and melanocytes. *Int. J. Cancer*, 56: 853–857, 1994.
- Van Coillie, E., Froyen, G., Nomiya, H., Miura, R., Fiten, P., Van Aelst, I., Van Damme, J., and Opdenakker, G. Human monocyte chemotactic protein-2: cDNA cloning and regulated expression of mRNA in mesenchymal cells. *Biochem. Biophys. Res. Commun.*, 231: 726–730, 1997.
- Altman, D. G. *Practical Statistics for Medical Research*. London: Chapman and Hall, 1991.
- Kameyoshi, Y., Dorschner, A., Mallet, A. I., Christophers, E., and Schroder, J. M. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med.*, 176: 587–592, 1992.
- Scotton, C., Milliken, D., Wilson, J., Raju, S., and Balkwill, F. Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours. *Br. J. Cancer*, 85: 891–897, 2001.
- Yoong, K. F., Afford, S. C., Jones, R., Aujla, P., Qin, S., Price, K., Hubscher, S. G., and Adams, D. H. Expression and function of CXC and CC chemokines in human malignant liver tumors: a role for human monokine induced by γ -interferon in lymphocyte recruitment to hepatocellular carcinoma. *Hepatology*, 30: 100–111, 1999.
- Baggiolini, M. Chemokines and leukocyte traffic. *Nature (Lond.)*, 392: 565–568, 1998.
- Robson, R. L., McLoughlin, R. M., Witowski, J., Loetscher, P., Wilkinson, T. S., Jones, S. A., and Topley, N. Differential regulation of chemokine production in human peritoneal mesothelial cells: IFN- γ controls neutrophil migration across the mesothelium *in vitro* and *in vivo*. *J. Immunol.*, 167: 1028–1038, 2001.
- Sica, A., Sacconi, A., Bottazzi, B., Bernasconi, S., Allavena, P., Gaetano, B., Fei, F., LaRosa, G., Scotton, C., Balkwill, F., and Mantovani, A. Defective expression of the monocyte chemotactic protein-1

receptor CCR2 in macrophages associated with human ovarian carcinoma. *J. Immunol.*, *164*: 733–738, 2000.

27. Kunz, M., Hartmann, A., Flory, E., Toksoy, A., Koczan, D., Thiesen, H. J., Mukaida, N., Neumann, M., Rapp, U. R., Brocker, E. B., and Gillitzer, R. Anoxia-induced up-regulation of interleukin-8 in human malignant melanoma. A potential mechanism for high tumor aggressiveness. *Am. J. Pathol.*, *155*: 753–763, 1999.

28. Negus, R. P., Turner, L., Burke, F., and Balkwill, F. R. Hypoxia down-regulates MCP-1 expression: implications for macrophage distribution in tumors. *J. Leukocyte Biol.*, *63*: 758–765, 1998.

29. Poznansky, M. C., Olszak, I. T., Foxall, R., Evans, R. H., Luster, A. D., and Scadden, D. T. Active movement of T cells away from a chemokine. *Nat. Med.*, *6*: 543–548, 2000.

30. Scotton, C. J., Wilson, J. L., Milliken, D., Stamp, G., and Balkwill, F. R. Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res.*, *61*: 4961–4965, 2001.

31. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. *Nature (Lond.)*, *410*: 50–56, 2001.

32. Koshiba, T., Hosotani, R., Miyamoto, Y., Ida, J., Tsuji, S., Nakajima, S., Kawaguchi, M., Kobayashi, H., Doi, R., Hori, T., Fujii, N., and Imamura, M. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. *Clin. Cancer Res.*, *6*: 3530–3535, 2000.