Role of Eotaxin-1 Signaling in Ovarian Cancer

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Abstract

Purpose: Tumor cell growth and migration can be directly regulated by chemokines. In the present study, the association of CCL11 with ovarian cancer has been investigated.

Experimental Design and Results: Circulating levels of CCL11 in sera of patients with ovarian cancer were significantly lower than those in healthy women or women with breast, lung, liver, pancreatic, or colon cancer. Cultured ovarian carcinoma cells absorbed soluble CCL11, indicating that absorption by tumor cells could be responsible for the observed reduction of serum level of CCL11 in ovarian cancer. Postoperative CCL11 levels in women with ovarian cancer negatively correlated with relapse-free survival. Ovarian tumors overexpressed three known cognate receptors of CCL11, CC chemokine receptors (CCR) 2, 3, and 5. Strong positive correlation was observed between expression of individual receptors and tumor grade. CCL11 potently stimulated proliferation and migration/invasion of ovarian carcinoma cell lines, and these effects were inhibited by neutralizing antibodies against CCR2, CCR3, and CCR5. The growth-stimulatory effects of CCL11 were likely associated with activation of extracellular signal-regulated kinase 1/2, MEK1, and STAT3 phosphoproteins and with increased production of multiple cytokines, growth factors, and angiogenic factors. Inhibition of CCL11 signaling by the combination of neutralizing antibodies against the ligand and its receptors significantly increased sensitivity to cisplatin in ovarian carcinoma cells.

Conclusion: We conclude that CCL11 signaling plays an important role in proliferation and invasion of ovarian carcinoma cells and CCL11 pathway could be targeted for therapy in ovarian cancer. Furthermore, CCL11 could be used as a biomarker and a prognostic factor of relapse-free survival in ovarian cancer.

In western and northern Europe as well as in the United States, ovarian cancer represents the third most frequent cancer of the female genital tract. Worldwide, there are an estimated 191,000 women newly diagnosed each year (1–3). The majority of early-stage cancers are asymptomatic, and over three-quarters of the diagnoses are made at a time when the disease has already established regional or distant metastases. With presently available platinum-based chemotherapy, the 5-year survival for patients with clinically advanced ovarian cancer is only 15% to 20%, although the cure rate for stage I disease is usually >90% (1–3). Therefore, identification of factors and pathways responsible for the accelerated cancer growth is of critical importance and may lead to development of novel therapeutic targets.

It has been recently shown that tumor cell growth can be directly regulated, among others, by chemokines, a group of proteins originally discovered as chemoattractants and activators of specific subsets of lymphocytes (4–6). Chemokines could induce distribution, trafficking, and effector function of various cells. Recently, several publications reported regulation of growth and migration/invasion of several cancer types by signaling from chemokine/chemokine receptors autocrine loops (7–22). Stimulation of tumor growth and migration/invasion was reported for CXCL12 (SDF-1)/CXCR4 in ovarian (23) and breast (9) cancers, CCL21/CC chemokine receptor (CCR) 7 on thyroid tumor cell lines (13), CXCL13 (BCA-1)/CXCR5 in several mouse and human carcinoma cell lines including pancreatic and colon carcinoma cell lines (11), CCL20 (MIP-3a)/CCR6 in colorectal cancer cells (7), macrophage chemotactrant protein (MCP)-1/CCR2 (10) and CCL5 (RANTES)/CCR5 (14) in prostate cancer, GROα and GROβ/CXCR2 in esophageal and lung cancers (15), and interleukin (IL)-8/CXCR2 in epidermoid carcinoma cells (12). These results underscore a potentially critical role of chemokines in tumor growth and invasion. Several retrospective studies in lung, colorectal, and head and neck cancers and lymphoma indicate that expression of chemokine receptors in many cancers correlate with enhanced disease aggressiveness and poor prognosis (24–28). No experimental data exist on the similar effects of CCL11 (eotaxin-1) in tumor cells.

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CCL11 (eotaxin-1) was originally discovered as an eosinophil-selective chemoattractant. CCL11 is a member of the CC chemokine family most homologous to the MCP subfamily (29). Genes encoding eotaxin and MCP chemokines are located on human chromosome 17q11, a region clustered with other CC chemokines (such as MIP-1, I-309, RANTES, HCC1, and HCC2; ref. 30). CCL11 mRNA is expressed at high levels in the small intestine, colon, heart, kidney, and pancreas and at lower levels in other tissues including the lung, liver, ovary, and placenta (31–33). Expression of CCL11 and CCR3 receptor was documented in human endometrium (34). CCL11 is an early gene product induced by proinflammatory cytokines in a variety of cell types in vitro. The airway epithelial cells express CCL11 mRNA in response to tumor necrosis factor (TNF)-α, IL-1, or IFN-α (31, 32). Furthermore, CCL11 is produced by fibroblasts, and IL-4 appears to be particularly important for CCL11 induction in cutaneous tissue (35). The CCL11 promoter in mice and humans has a nuclear factor-κB-binding site, STAT-6-binding elements, IFN-α response elements, and a glucocorticoid response element. This may explain the observed up-regulation of CCL11 by TNF-α, IL-4, IFN-α, and glucocorticoids (31, 32). An important feature of chemokines is their ability to bind to the glycosaminoglycan side chains of proteoglycans, predominately heparin and heparan sulfate, an interaction that protects CCL11 from proteolysis and potentiates chemotactic activity in vivo (36).

Specific activity of CCL11 playing a central role in eosinophil trafficking is mediated by CCR3 (37, 38). Recently, CCR2b and CCR5 receptors were reported to be partial agonists of CCL11 in monocytes (39, 40). Binding of CCL11 to these receptors induces a series of biochemical changes, including activation of G proteins, transient increases in intracellular calcium concentration, cytoskeletal rearrangements, activation of mitogen-activated protein kinase pathway, and rapid and prolonged receptor internalization into an endocytic compartment (41).

These three receptors are shared among several chemokines; CCL11 shares CCR3 with MCP-1, MCP-3, and RANTES (42), CCR2 with MCP-1, and CCR5 with RANTES and MIP-1β (43).

The association of CCL11 with cancer or potential CCL11 expression by tumor has not been adequately investigated. The only study exploring this association reported the overexpression of CCL11 receptor, CCR3, in renal cell carcinomas (RCC) and potent induction of proliferation of RCC cells by CCL11 (44). The presence of CCR3 in tumor samples correlated with the grade of malignancy, indicating that CCL11 could promote progression and dissemination of CCR3-positive RCC (44). CCR2 expression by myeloma cells was reported to enhance migration of tumor cells via TNF-α-induced autocrine production of MCP-1 (13). 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 matrix metalloproteinases in breast cancer cells, thus contributing to the invasive behavior of the cells (18). The above data indicate that CCL11 could be causally involved in tumorigenesis by facilitating tumor proliferation and metastasis. Several indirect lines of evidence also suggest that CCL11 can play a role in angiogenesis and metastasis. For example, it was shown that CCL11 is able to induce migration of human microvascular endothelial cells as well as the formation of blood vessels in vivo (45). The angiogenic response to CCL11 appeared to be direct and not mediated by eosinophil products (45). Microarray analysis of human airway epithelial cell lines exposed to CCL11 showed induction of several proangiogenic molecules including fibroblast growth factor (FGF)-1, -5, and -6, IL-6, and vascular endothelial growth factor (VEGF)-A and -C (46). CCL11 was shown to induce matrix metalloproteinase-2 mRNA, protein, and activity in smooth muscle cells. This effect was CCR3 receptor-mediated and dependent on activation of the epidermal growth factor receptor (47).

In the present study, we performed a comprehensive analysis of the possible involvement of CCL11 and its receptors CCLR2, CCLR3, and CCLR5 in ovarian cancer.

Materials and Methods

Patients. Sera from 342 healthy women were provided by Gynecologic Oncology Group Blood Bank, Fox Chase Cancer Center Biorepository, and University of Pittsburgh Cancer Institute. Sera from patients with ovarian cancer stages I to II (n = 215) and III to IV (n = 118) were from the Gynecologic Oncology Group Blood Bank. Sera of age-matched women with endometrial cancer (n = 231) were provided by the Gynecologic Oncology Group and M. D. Anderson Cancer Center (Dr. Karen Lu), with lung cancer (n = 67) by the University of Pittsburgh Cancer Institute (Dr. Jill Siegfried), with breast cancer (n = 220) by the Duke University Medical Center (Dr. Jeffrey Marks), with pancreatic (n = 285) and colorectal (n = 31) cancers by the University of Pittsburgh Cancer Institute (Drs. Herbert Zeh and Randall Brand). Sera were collected and stored as described previously (48). All sera were annotated with information regarding gynecologic diagnosis, ovarian cancer staging, cancer histology, grade, and age. In addition, sera from 21 women with serous ovarian adenocarcinoma were collected postoperatively at the Duke University Medical Center and provided by Dr. Jeffrey Marks. Sera were drawn at the first postoperative visit. Samples were annotated with dates of surgery and recurrence. All sera were from postmenopausal women. All serum collection protocols were approved by local institutional review boards.
Cell lines. Human ovarian cancer cell lines, OVCAR-3 and SKOV-3, were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Millipore) with addition of 0.01 mg/mL insulin for OVCAR-3 as recommended by the American Type Culture Collection (49).

Establishing primary ovarian carcinoma cells from ascites. Ascites was obtained at University of Pittsburgh Medical Center Magee Women's Hospital from 3 patients undergoing debulking surgery. The fluid was placed on ice and centrifuged to isolate the cellular component that was resuspended in RPMI 1640 with 20% fetal bovine serum. Hypotonic lysis and sedimentation were used to remove erythrocytes. Cells were counted using a Coulter counter and were plated in 150 cm² cell culture flasks at 5 × 10⁶ per flask. Adherent cells were passaged four times before analysis.

Reagents. Hoechst 33342, doxorubicin, cisplatin, and monensin were purchased from Sigma-Aldrich. Fluorochrome-conjugated antibodies against human CCR2 and CCR5 were from R&D Systems, and antibody against CCR3 was from Abcam. Antibodies against human CCR (polyclonal goat anti-CCR2, monoclonal rabbit anti-CCR3, and polyclonal rabbit anti-CCR5) for staining of formalin-fixed tissues were from Abcam. Mouse monoclonal antibody against eotaxin-1 (CCL11) that was used for both staining of formalin-fixed, paraffin-embedded tissue and neutralization of CCL11 activity was obtained from R&D Systems. Secondary antibodies conjugated with Alexa 488 were from Molecular Probes (Invitrogen). Mouse monoclonal neutralizing antibodies against CCR2, CCR3, and CCR5 were from Genetex. Recombinant human protein, CCL11, was purchased from Invitrogen/Biosource.

Intracellular staining procedure. Cells grown in 96-well plates were pretreated with monensin (2 μmol/L) for 24 h to inhibit secretion of CCL11 (50), fixed in 2% paraformaldehyde for 20 min, washed in PBS, incubated with 0.1% Triton X-100 for 10 min, and washed with PBS containing 1% bovine serum albumin (fluorescence-activated cell sorting buffer). Cells were then incubated with primary antibody against CCL11 for 1 h followed by incubation with secondary antibody conjugated with Alexa 488 for 1 h. Cell nuclei were counterstained with 2 μg/mL Hoechst 33424 for 20 min. All incubation and fixation procedures were done at room temperature. Cell images were acquired using the Cellomics ArrayScan HCS Reader (×20 objective) and analyzed using Target Activation BioApplication Software Module (ThermoFisher).

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue microarray of ovarian carcinoma (TMA-OVC1501; 150 cores/75 cases) and normal ovarian epithelia (TMA-ON906; 60 cores/30 cases; US Bioservices, Los Angeles, CA) were deparaffinized, hydrated, and rehydrated, and H₂O₂. Antigen retrieval was done using citrate buffer (pH 6.0) with 20 min steaming followed by cooling for 20 min. After unmasking, slides were blocked with H₂O₂. Before pretreatment, EnVision+ System (DAKO) was used for staining according to the manufacturer’s instructions. In short, primary antibodies were diluted with antibody diluents (DAKO) as follows: anti-CCR2 was diluted 1:200, anti-CCR3 was diluted 1:100, and anti-CCR5 was diluted 1:25. After overnight incubation with primary antibodies, sections were first incubated with labeled horseradish peroxidase anti-rabbit antibody or with biotinylated rabbit anti-goat antibody (1:5,000) and then with horseradish peroxidase anti-rabbit antibody, respectively. Next, slides were incubated with 3,3’-diaminobenzidine. Sections were counterstained with Mayer’s hematoxylin and mounted in mounting medium. To eliminate nonspecific binding of secondary antibody, tissue sections were incubated with a serum-free protein blocker before addition of primary antibodies. Primary antibodies were omitted in negative controls. Results were evaluated by two independent investigators (M.J.S. and M.E.S.) and scored as positive or negative when the percentage of stained tumor cells or normal epithelial cells in each section was >25% or <25%, respectively. The level of staining intensity was recorded as none, weak, moderate, or strong. For digital image analysis, the software Adobe Photoshop version 7.0 was used.

Reverse transcription-PCR analysis. Total RNA isolated from normal ovarian epithelium and ovarian tumors was obtained from Applied Biosystems/Ambion. Reverse transcription-PCR was done for detection of CCL11 mRNA (40 cycles; eotaxin sense primer 5’-ACACCTT-CAGCGTCTCACAT-3’ and antisense 5’-GGGTCTTGAATACACGGTCT-3’). The size of the eotaxin amplicon corresponded to its predicted size of 182 bp.

Cellomics ArrayScan automated imaging. The Cellomics ArrayScan HCS Reader (Cellomics/ThermoFisher) was used to collect information on distribution of fluorescently labeled components in stained cells. The ArrayScan HCS system scans multiple fields in individual wells to acquire and analyze images of single cells according to defined algorithms. The scanner is equipped with emission and excitation filters (XF93; Omega Optical) for selectively imaging fluorescent signals. Data were captured, extracted, and analyzed with ArrayScan II Data Acquisition and Data Viewer version 3.0 (Cellomics), Quattro Pro version 10.0.0 (Corel), and MS Excel 2002 (Microsoft).

Proliferation assays. Cancer cells were plated onto 96-well plates at 2 × 10⁴ per well. On the next day, human recombinant CCL11 was added to final concentration of 0.5 to 100 ng/mL and cells were grown for 72 h. Cells were fixed, stained with Hoechst 333424, and counted using the Cellomics ArrayScan HCS Reader (×10 objective).

Migration and invasion assay. The chemotactic effects of CCL11 (2-10 ng/mL) on migration/invasion of tumor cells were measured in BD BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s protocol. The results were expressed as the number of cells migrating through Matrigel membrane in response to CCL11.

Apoptosis assays. Tumor cells grown in 6-well plates were preincubated with 5 ng/mL CCL11 for 2 h, and cisplatin (2 μg/mL) was added for the next 20 h. Apoptosis was analyzed by flow cytometry using FITC-conjugated Annexin V and propidium iodide as described previously (51). Cell images were acquired using the Cellomics ArrayScan HCS Reader (×20 objective) and analyzed using Target Activation BioApplication Software Module.

Multiplex analysis of cytokine production by tumor cells. Analysis of human cytokines and growth factors in cell culture medium was done using multiplexing xMAP technology (Luminex). Multiplex kits for detection of 49 human cytokines were purchased from Bio-Rad Laboratories (MCP-3, GROα, CTACK, LIF, NGF, PDGF-BB, SCF, SCGF-B, SDF-1α, TRAIL, and IFN-γ), R&D systems (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, and IL-17, GM-CSF, IFN-α, MCP-1, MCP-2, IP-10, MIP-1α, MIP-1β, RANTES, VEGF, basic GF, G-CSF, CCL11, HGF, MIF, IL-2R, M-CSF, EGF, TNEFI, TNFRII, DR5, IL-1Rα, IL-6Rβ), and Millipore (sICAM-1). Analyses of tumor supernatants were done in 96-well microplate format according to the appropriate manufacturer’s protocols. Data were plotted against standard curves of serially diluted protein standards using a four-parametric curve fit and were expressed as picogram per 1 × 10⁶ tumor cells.

Multiplex analysis of phosphoproteins. Tumor cells were stimulated with 5 ng/mL hrCCL11 for 0, 5, 15, or 30 min; cell lysates were prepared using Bio-Rad Bio-Plex Cell Lysates kit and analyzed using BioRad BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s protocol. The results were expressed as the number of cells migrating through Matrigel membrane in response to CCL11.

Multiplex analysis of transcription factors. Analysis of human cytokines and growth factors in cell culture medium was done using multiplexing xMAP technology (Luminex). Multiplex kits for detection of 49 human cytokines were purchased from Bio-Rad Laboratories (MCP-3, GROα, CTACK, LIF, NGF, PDGF-BB, SCF, SCGF-B, SDF-1α, TRAIL, and IFN-γ), R&D systems (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, and IL-17, GM-CSF, IFN-α, MCP-1, MCP-2, IP-10, MIP-1α, MIP-1β, RANTES, VEGF, basic GF, G-CSF, CCL11, HGF, MIF, IL-2R, M-CSF, EGF, TNEFI, TNFRII, DR5, IL-1Rα, IL-6Rβ), and Millipore (sICAM-1). Analyses of tumor supernatants were done in 96-well microplate format according to the appropriate manufacturer’s protocols. Data were plotted against standard curves of serially diluted protein standards using a four-parametric curve fit and were expressed as picogram per 1 × 10⁶ tumor cells.

Statistical analysis. The Mann-Whitney test was used to determine statistical significance of differences in biomarker serum concentrations between patient groups. All in vitro experiments were repeated at least three times and mean ± SD was determined. The significance of the effects of CCL11 was assessed using a two-tailed Student’s t test. For the comparison of multiple groups, a one- or two-way ANOVA test was applied. For all statistical analyses, the level of significance was set at a probability of $P < 0.05$.

**Results**

Analysis of CCL11 in sera of patients with ovarian cancer and healthy women. Bead-based sandwich immunoassay was used to analyze CCL11 in sera of patients with ovarian cancer, healthy women, women with benign pelvic disease, and women with other cancers. Concentrations of CCL11 were significantly lower in sera of patients with ovarian cancer compared with healthy women (Fig. 1A). Serum CCL11 levels did not correlate with histology of ovarian cancer (data not shown).

CCL11 was measured in sera of age-matched women with endometrial, lung, breast, pancreatic, and colorectal cancers (Fig. 1B). CCL11 concentrations were significantly lower in sera of patients with endometrial cancer compared with control. Circulating CCL11 levels were significantly elevated in postmenopausal women with breast, lung, colorectal, and pancreatic cancers. Eotaxin levels in all these cancers were significantly higher than those in ovarian and endometrial cancers. Therefore, CCL11 likely plays a distinct role in gynecologic cancers.

Prognostic significance of CCL11 in ovarian cancer. CCL11 was measured in sera of 21 ovarian carcinoma patients after surgery and correlated with relapse-free survival (RFS). Serum levels in a subgroup of women with RFS <3 years were compared with those in the group of RFS ≥3 years (Fig. 1C). CCL11 concentrations were significantly ($P < 0.001$) lower in the group with longer RFS.

Absorption of CCL11 by ovarian cancer cells. We hypothesized that decreased eotaxin serum concentrations are due to absorption of circulating ligand by cognate CCL11 receptors expressed on ovarian tumor. The absorption of human recombinant CCL11 by human carcinoma SKOV-3 cells was assessed. Concentration of CCL11 in cell culture medium after incubation with SKOV-3 cells decreased by 32%, indicating active absorption of this cytokine by cultured cells (Fig. 1D).

Decrease in CCL11 concentration in the in vitro experiment corresponded to that in patients where CCL11 is 30% lower in ovarian cancer cases than in healthy controls.

Expression of CCL11 and CCR2, CCR3, and CCR5 by primary and established tumor cell lines. OVCAR-3 and SKOV-3 ovarian carcinoma cells were stained using specific polyclonal goat anti-human CCL11 antibody. Both established (Fig. 2A) and primary (Fig. 2B) cell lines showed distinctive positive staining. Next, CCL11 mRNA expression was analyzed in ovarian tumors and normal ovarian epithelium using reverse transcription-PCR (Fig. 2C). CCL11 was equally expressed in both ovarian tumor and normal ovarian epithelium. Surface expression of CCR2, CCR3, and CCR5 receptors on established cell lines was analyzed by flow cytometry (Fig. 2D) and in primary tumor by immunohistochemistry (Fig. 2E). SKOV-3 cells expressed

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**Fig. 1.** Serum CCL11 in ovarian cancer. Serum CCL11 concentrations were analyzed in healthy controls and patients with early (I-II) and late (III-IV) stages of ovarian cancer (A) and age-matched women with ovarian (all stages), endometrial, breast, lung, pancreatic, and colon cancers (B) using bead-based immunoassay technology. Reactions were done according to the manufacturer’s protocol (Invitrogen). C, postoperative levels of CCL11 in sera of ovarian cancer patients. CCL11 was measured in postoperatively collected sera using Luminex bead-based assay. Patient group was divided into two subgroups with RFS <3 and ≥3 years. D, absorption of CCL11 by cultured ovarian carcinoma cells. Cells (10^7) were trypsinized and resuspended in 3 mL cell culture medium and CCL11 was added to a final concentration of 1 ng/mL. Cells were incubated with shaking for 1 h, supernatant was collected, and CCL11 concentration was determined by bead-based immunoassay. In this and the following figures: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, statistical significance of differences between groups.
predominantly CCR3 and to a smaller extent CCR2 receptor. CCR5 expression was very low. OVCAR-3 cells expressed all three receptors.

Expression of CCR2, CCR3, and CCR5 in tissue sections. Expression of CCL11 receptors in tissue sections was analyzed by immunohistochemistry on tissue microarray of ovarian tumors (75 cases) and normal ovarian epithelia (30 control tissues). CCR2, CCR3, and CCR5 receptors were expressed in 17%, 20%, and 30% cases of healthy epithelia, respectively. The expression was weak relative to tumor tissue. In tumor tissues, CCR2, CCR3, and CCR5 were expressed in 94%, 67%, and 72% of tumors, respectively, and the intensity of staining ranged from weak to strong (Table 1). Expression of CCR2, CCR3, and CCR5 was observed in serous, mucinous, and endometrioid histologies, with the highest expression in mucinous histology followed by serous and by endometrioid (Table 1). The analyzed tissue microarray did not contain clear cell carcinoma sections. Expression of all three receptors showed strong predominance in mucinous histology.

Table 1. Expression of CCR2, CCR3, and CCR5 in ovarian tissue

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<th>Groups</th>
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<th>CCR2</th>
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Fig. 2. Expression of CCL11 (A-C) and CCR2, CCR3, and CCR5 receptors in ovarian carcinoma. A, CCL11 protein expression in ovarian carcinoma cell lines. B, CCL11 protein expression in primary ovarian tumor cells. Primary ovarian carcinoma cells were obtained from ascites fluid. Adherent cells were cultured for 7 days in 96-well plates. These primary and established OVCAR-3 and SKOV-3 cells were preincubated with monensin for 48 h, fixed, and incubated with primary antibodies against CCL11 and secondary Alexa 488-conjugated antibodies, counterstained with antibody against cytokeratin 19 (CK) and with Hoechst 33342, and analyzed by Cellomics ArrayScan (×40 objective). C, CCL11 RNA expression in normal ovarian epithelium pooled from 5 healthy women (1), in individual ovarian tumors (2 and 3), and in cultured ovarian carcinoma cells, OVCAR-3 (4) and SKOV-3 (5), with negative control (6) and positive control (7; actin). D, CCL11 receptors expression in established cell lines. CCR2, CCR3, and CCR5 were stained in cultured cells using specific monoclonal FITC-conjugated antibody and analyzed by flow cytometry. Nonspecific antibody binding observed after staining the cells with isotype-matched FITC-conjugated IgG control was subtracted. E, analysis of CCL11 receptors in ovarian tumors. CCR2, CCR3, and CCR5 were analyzed in ovarian carcinoma tissue array using primary polyclonal antibodies optimized to work in formalin-fixed, paraffin-embedded tissues as described in Materials and Methods. Tumor sections from 75 different patients and normal ovary sections from 30 healthy women were examined for each receptor with a representative section presented (×200). 1, negative control on ovarian cancer; 2, negative control on normal ovary; 3, CCR2 in ovarian cancer; 4, CCR2 in healthy ovarian epithelium; 5, CCR3 in ovarian cancer; 6, CCR3 in normal ovary; 7, CCR5 in ovarian cancer; 8, CCR5 in normal ovary.
positive correlation with tumor grade (Table 1). No correlation with tumor stage could be observed (data not shown).

**Role of CCL11 in migration, proliferation, and apoptosis of ovarian carcinoma cells.** To ascertain potential functional role of CCL11 in ovarian cancer, its effects on cell proliferation and migration were evaluated. OVCAR-3 and SKOV-3 cells were incubated with 0 to 5 ng/mL human recombinant CCL11 for 48 h, and cell migration/invasion was assessed as described in Materials and Methods. CCL11 induced migration/invasion of both ovarian carcinoma cell lines in a dose-dependent manner (Fig. 3A). Next, we tested whether disruption of CCL11 signaling by neutralizing antibodies against CCL11 and its receptors, CCR2, CCR3, or CCR5, could affect cell migration.

Neutralizing antibodies against CCR2, CCR3, and CCR5 significantly inhibited CCL11-induced migration/invasion in OVCAR-3 and SKOV-3 cell lines (Fig. 3B). Combination of three antibodies had the most pronounced effect on migration/invasion in both cell lines (Fig. 3B).

To measure proliferative effects of CCL11, cell numbers were counted using imaging cytometry. CCL11 potently stimulated growth in OVACR-3 and SKOV-3 cell lines in a dose-dependent manner (Fig. 3C). Blocking individual CCL11 receptors significantly inhibited cell proliferation in OVCAR-3 cells (Fig. 3D). Abrogation of CCL11 autocrine loop by neutralization of CCL11 itself and all three receptors almost completely abrogated proliferation in these cells (Fig. 3D). In OVCAR-3

![Fig. 3.](image-url)
cells stimulated with CCL11, blocking CCR2, CCR3, and CCR5 receptors significantly inhibited proliferation, and simultaneous neutralization of all three receptors had the most pronounced effect (Fig. 3D). These results show the importance of CCL11 signaling for ovarian tumor cell proliferation. Next, we tested whether blocking CCL11 signaling could increase tumor cell sensitivity to cisplatin. Inhibition of CCL11 signaling by combination of anti-CCL11 and anti-CCR2, -CCR3, and -CCR5 antibodies did not induce apoptosis in untreated OVCAR-3 and SKOV-3 ovarian carcinoma cells (data not shown). Blocking each individual receptor or neutralization of the ligand did not significantly increase the apoptotic effects of cisplatin. However, in combination, these antibodies significantly increased OVCAR-3 tumor cell sensitivity to the apoptotic effects of cisplatin in these cells (Fig. 3E).

**CCL11 stimulates production of cytokines, growth and angiogenic factors, and adhesion molecules in ovarian carcinoma cell lines.** Eotaxin could elicit its proliferative effects either directly or via induction of cytokines or growth factors. We have analyzed concentrations of various cytokines and growth factors in cell culture medium of ovarian OVCAR-3 and SKOV-3 cells after 24 h incubation with CCL11 (2 ng/mL). CCL11 potently up-regulated secretion of chemokines [MIF, IL-8 (CXCL8), G-CSF, GM-CSF, and M-CSF], cytokines/receptors (IL-6R and -8), growth/angiogenic factors (VEGF and SDF-1α), and adhesion molecule (ICAM-1) in both cell lines (Fig. 4). Some chemokines were differentially up-regulated by CCL11 in these cell lines. In SKOV-3 cells, CCL11 up-regulated RANTES, MCP-1, GROα, MCP-1, IFN-α2, IP-10, and TNFRI and DR5 receptors (Fig. 4A), whereas, in OVCAR cells, MIP-1β, LIF, TNF-α, TNF-β, PDGF-BB, SCF, basic FGF, and LIF (Fig. 4B) were elevated.

**Analysis of CCL11 signal transduction pathways in ovarian carcinoma cell lines.** The mechanisms and signaling pathways regulating the biological effects of CCL11 in tumor cells remain unknown. To analyze CCL11 signaling in ovarian carcinoma cell lines, OVCAR-3 and SKOV-3 cells were incubated with 5 ng/mL hrCCL11 for 0, 5, and 15 min, and phosphorylation of 17 proteins as listed in Materials and Methods was analyzed using 8-Plex phosphoprotein kit as described in Materials and Methods. B, transcription factors expression in CCL11-treated tumor cells. OVCAR-3 and SKOV-3 cells were treated with 5 ng/mL CCL11 for 0, 5, and 30 min. Nuclear extracts were prepared and transcription factor analysis was done using 40-Plex kit. Representative transcription factors that showed the most robust response to CCL11 treatment are presented.
phosphorylation, a rapid dephosphorylation occurred by 15 min of incubation.

Next, activation of 37 transcription factors (listed in Materials and Methods) following 5 to 30 min incubation with 5 ng/mL CCL11 was analyzed using multiplex bead-based assay. Nuclear factor-κB, AP2, STAT4, ELK-1, FAST-1, p53, EST, E2F-1, RUNX, CEBP, c-myb, and IRF were activated >1.7-fold after 15 min incubation in CCL11-treated cells compared with untreated cells (Fig. 4D). Delayed activation suggests that CCL11 receptor cross-linking induced transcription factor activation not directly but rather via transactivation of some growth factor receptors.

Discussion

Until recently, eotaxin-1 or CCL11 was considered to be just an eosinophil-specific chemoattractant and consequently was studied mostly in diseases characterized by an accumulation of eosinophils in tissues, notably allergic conditions, such as asthma, rhinitis, and atopic dermatitis, and other inflammatory disorders, such as inflammatory bowel disease, eosinophilic gastroenteritis, and pneumonia (52). Because CCL11 has been poorly investigated outside the realm of lymphoid cells, little is known about its biological significance in different cell types, normal or malignant.

In this study, for the first time, we show cancer-dependent changes in serum eotaxin levels in patients with ovarian cancer, indicating possible important role of CCL11 in ovarian tumor growth and invasion. We hypothesized that this reduction could be due to sequestration of soluble CCL11 by cognate receptors expressed on ovarian cancer cells. In support of this hypothesis, we show overexpression of CCL11 receptors in cultured ovarian carcinoma cells. Our comparative analysis of CCL11 receptor expression in normal ovary and ovarian cancer revealed overexpression of all three CCL11 receptors (CCR2, CCR3, and CCR5). Furthermore, we showed that cultured ovarian carcinoma cells could absorb and deplete CCL11 exogenously added to cell culture medium. Of note, the extent of depletion corresponded to the extent of decrease of CCL11 levels in serum of ovarian cancer patients compared with healthy women. An alternative explanation of lower serum CCL11 levels in ovarian and endometrial cancers would be immunosuppression resulting in inhibition of CCL11 secretion by lymphoid cells. However, this explanation would contradict observed elevated CCL11 levels in late-stage (III-IV) ovarian cancer and in nongynecologic cancers that often associated with immunosuppression. Interestingly, eosinophils are rarely seen in ovarian tumor microenvironment (53). This could be due by the fact that most of produced CCL11 is rapidly bound to cognate receptors overexpressed by ovarian tumor and internalized making it unavailable for eosinophil recruitment.

We observed an important association of lower serum CCL11 with longer RFS. Although study population is small and presented data have to be validated in a larger set, these results indicate potential significance of CCL11 as a prognostic biomarker of ovarian cancer. It was shown that production of CCL11 is regulated by Th2- and Th1-related cytokines. IL-4 and TNF-α stimulate, whereas IFN-γ inhibits CCL11 production in fibroblasts (54). It is possible that reduction of serum CCL11 in ovarian carcinoma patients is due to decrease of IL-4 and TNF-α and/or increase IFN-γ. In fact, levels of IFN-γ were significantly (P < 0.05) higher in the group with longer RFS.7

We have reported expression of the three known CCL11 receptors, CCR2, CCR3, and CCR5, in ovarian cancer both in vitro and in vivo. CCL11 receptors in ovarian carcinoma are functionally active as CCL11 treatment manifested in phosphorylation of ERK1/2, MEK1, and STAT3 and activation of numerous transcription factors. Activation of ERK1/2 (mitogen-activated protein kinase-1) and MEK1 by CCL11 in eosinophils has been reported by Kampen et al. (55). The ability of mitogen-activated protein kinase-1 to activate STAT3 and CREB was also shown (56–58). CCR2 can rapidly activate MEK and ERK1/2 (59), and all three receptors can activate ERK1/2 and MEK via STAT3 (60). Therefore, the downstream CCL11 signaling in ovarian tumor cells and eosinophils is similar. Activation of a wide variety of transcription factors by CCL11 occurs relatively late, at least 15 min, after receptor binding and therefore most likely results from transactivation of other growth factor receptors and triggering the cascade of other cytokines and growth factors signaling.

Normal physiologic functions of chemokines include stimulation of proliferation (61), extravasation (62–64), and migration (65) of leukocytes. Limited evidence also suggests that chemokine receptor activation leads to increased resistance to cycloheximide-induced apoptosis in T cells possibly via activation of Akt and its downstream effectors (66). These processes could reflect in the role of chemokines in tumor growth, progression, and metastasis (67). Our data indicate that CCL11 stimulates proliferation and migration/invasion of ovarian carcinoma cells. These effects could be mediated by multiple cytokines that are up-regulated by CCL11. CCL11 may potentially facilitate ovarian tumor growth via up-regulation of IL-8, VEGF, basic FGF, PDGF-BB, and others and tumor dissemination at several key steps, including adherence of tumor cells to endothelium via ICAM, extravasation from blood vessels via matrix metalloproteinases, and angiogenesis via basic FGF, IL-8, and VEGF.

The biological effects of individual eotaxin receptors were not investigated in ovarian cancer cells. Strong proliferative and chemotactic effects of CCL11 in ovarian carcinoma cells were likely mediated by CCR2, CCR3, and CCR5 receptors as neutralizing antibodies to these receptors abrogate CCL11-induced proliferation and migration/invasion. All three receptors are likely important for both processes because inhibition of all three receptors most potently abrogated CCL11 effects. Mediating proliferative and metastatic activity of ovarian tumor by CCR2, CCR3, and CCR5 receptors could explain the observed strong positive correlation of expression of these receptors with ovarian tumor grade. This observation also suggests that expression of these receptors may represent a useful prognostic biomarker of aggressive phenotypic behavior of ovarian tumor.

The affinity of CCL11 binding to CCR2 and CCR5 is lower than that to CCR3 as was shown in monocytes (39, 40), although this may not reflect the real situation as G-protein-coupled receptors could function as heterodimers (68). It must be noted that CCL11, similar to other chemokines, shares its three receptors with other members of chemokine family; for example, CCR3 receptor is shared with MCP-1, -2, -3, and -4, RANTES, and HCC2; CCR2 is also receptor for MCP-1, -2, -3,
and -4, HCC4, and eotaxin-2 and -3; and CCR5 interacts with RANTES, MIP-1α, MIP-1β, HCC1, and HCC4 (69). Such redundancy indicates that lack of one chemokine can be compensated by other chemokines in cellular processes emphasizing critical importance of intact chemokine signaling for normal physiologic processes. Indeed, when individual chemokines or their receptors have been deleted in mice, defects have been relatively subtle (67).

Our findings that ovarian carcinoma cell lines produce CCL11 and overexpress all three of its receptors, CCR2, CCR3, and CCR5, indicate activation of autocrine loops of eotaxin signaling in ovarian cancer. Our experiments with neutralizing antibodies show that proliferation of ovarian carcinoma cells is seriously compromised in the absence of CCL11 signaling. Furthermore, blocking of CCL11 signaling potentiates chemotherapy-induced apoptosis in ovarian carcinoma cell lines. This important finding may open new opportunities for exploring CCL11 signaling for ovarian cancer therapy. Small-molecule inhibitors of CCL11 receptors are available. Maraviroc, an allosteric inhibitor of CCR5, is currently used for treatment of HIV-1 infection (70), and other diseases, such as rheumatoid arthritis (71) and respiratory disease (72), have been suggested as possible targets for this CCR5 antagonist. Development of UCB35625, a potent, selective small-molecule inhibitor of CCR1 and CCR3A, was recently reported (73).

It must be noted that targeting CCL11 receptors for cancer therapy is not straightforward due to their pleiotropic effects. For example, CCR5 has been reported to control antitumor responses by participating in chemotaxis of memory and activated naïve T cells, and it is required for T-cell activation (74). Similarly, transfer of MCP-3 gene that shares CCR2 and CCR3 receptors with CCL11 elicits tumor rejection by activating type I T-cell-dependent immunity (75). Furthermore, it was shown that CCR5 activated tumor suppressor p53 in breast cancer cell lines, and abrogation of CCR5 expression enhanced proliferation of tumor cells bearing wild-type p53 in a mouse breast cancer xenograft model (76). Therefore, further studies in animal models of ovarian cancer are necessary to evaluate complex systemic effects of abrogation of CCL11 signaling.

In summary, we show a strong association of CCL11 with ovarian cancer. Our experimental evidence indicates a potentially important role of CCL11 in ovarian cancer growth and metastasis. These results warrant further investigation of the role of CCL11 in the initiation and progression of ovarian cancer. Blocking the autocrine loop of CCL11 and its receptors could be targeted for ovarian cancer therapy. CCL11 and possibly CCR2, CCR3, and CCR5 could be prognostic factors of RFS in ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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