Autocrine and Paracrine Chemokine Receptor 7 Activation in Head and Neck Cancer: Implications for Therapy

Jun Wang, Raja R. Seethala, Qing Zhang, William Gooding, Carter van Waes, Hitoshi Hasegawa, Robert L. Ferris

Background
The chemokine receptor 7 (CCR7) mediates survival and invasiveness of metastatic squamous cell carcinoma of the head and neck (SCCHN) to regional lymph nodes. Constitutive prosurvival signaling by the phosphoinositide-3 kinase/Akt pathway has been observed in SCCHN cells independent of epidermal growth factor receptor (EGFR) signaling.

Methods
Human SCCHN cell lines were treated with agents that block or activate CCR7-mediated signaling, and Akt activation, cell viability in the presence or absence of EGFR inhibition, and cisplatin-induced apoptosis (in the presence or absence of Akt inhibition) were assessed by immunoblotting, the MTT assay, and the detection of annexin V, respectively. Expression and secretion of chemokines by primary tumors, metastatic nodes, and benign nodes of patients with SCCHN were determined by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. The role of paracrine activation of CCR7 on tumor growth was analyzed by comparing the growth of orthotopic tumors derived from B7E3 murine oral carcinoma cells in wild-type BALB/c mice, in paucity of lymphoid T cell (plt, deficient in CCL19 and CCL21 expression) mice, and in plt mice in which the implanted B7E3 cells overexpressed CCR7 (n = 14 mice per group).

Results
In the absence of exogenous ligand treatment, blockade of CCR7 signaling reduced levels of phosphorylated (activated) Akt and decreased SCCHN cell viability by up to 59% (95% confidence interval [CI] = 58.2% to 59.8%), enhancing the effect of EGFR inhibition. CCR7 stimulation protected metastatic SCCHN cells from cisplatin-induced apoptosis in an Akt-dependent manner. Metastatic nodes expressed and secreted higher levels of CCL19 than benign nodes or primary tumors. CCR7-positive murine SCCHN tumors grew more slowly in plt mice than in control BALB/c mice (mean average tumor volume on day 20 = 12.2 and 26.5 mm³, respectively; difference = 14.3 mm³, 95% CI = 12.3 to 17.1 mm³).

Conclusions
Secretion of CCL19 and CCL21 by SCCHN cells and by paracrine sources combine to promote activation of CCR7 prosurvival signaling associated with tumor progression and disease relapse. CCR7 and its cognate chemokines may be useful biomarkers of SCCHN progression, and blockade of CCR7-mediated signaling may enhance the efficacy of platinum- and EGFR-based therapies.
receptor for chemokines 19 (CCL19) and 21 (CCL21), chemokine receptor 7 (CCR7), a property that may allow them to access the lymphatic system and spread to regional lymph nodes (4). Thus, the "chemoattraction" theory of metastasis may be reflected by malignant cells expressing functional chemokine receptors that can respond to organ-specific chemoattractant molecules (chemokines) and migrate directionally along chemokine gradients to set up site-specific metastases in the target organ(s). Such chemotactic migration of tumors would mirror the physiologic mechanisms of lymphocyte homing into lymphoid organs.

The effects of CCR7 activation may be mediated in part by phosphoinositide-3 kinase (PI3K), a family of lipid kinases that play crucial roles in cell growth, migration, and survival (5, 6). For example, in immune cells CCR7 activation transmits a prosurvival signal that is mediated by the PI3K/protein kinase B (PKB) cascade (7, 8), and PI3K/PKB signaling has been observed in several kinds of cancer cells (9). We have recently shown that in SCCHN cells, CCR7 stimulation strongly activates Akt/PKB to inhibit cell death pathways by directly phosphorylating and inactivating proapoptotic factors. Furthermore, activation of Akt and PKB has been shown to be associated with poor prognosis in SCCHN (10).

Thus, the consequences of CCR7 activation are not limited to effects on cell migration. However, its importance in tumor progression and its contribution to basal activation of the downstream signaling mediators PI3K/Akt and phospholipase C γ have not been determined. The identification of constitutively active CCR7 in metastatic SCCHN cells would support the biological and clinical importance of expression of CCR7 and its ligands.

The epidermal growth factor receptor (EGFR)/PI3K axis can activate Akt and the transcription factor NF-κb in SCCHN (11) to induce expression of cellular inhibitors of apoptosis (12), and promising phase III clinical data (13, 14) reflect the efficacy of EGFR-targeted agents in definitive or salvage therapy and have led to their increasing use in treatment of SCCHN. However, EGFR-independent survival pathways in SCCHN (15) and the observation of tumors that are resistant to cisplatin (traditionally attributed to altered expression of the antiapoptotic Bcl-2 family) and cetuximab (16), an inhibitory EGFR-specific monoclonal antibody (14), suggest the need for additional targeted agents to enhance EGFR-based therapies. Using murine models of tumor growth and analyzing tumors and tumor-derived cell lines derived from SCCHN patients, we have explored how chemokines activate CCR7 and the potential usefulness of this receptor as a target for combined inhibition strategies. We hypothesized that both autocrine and paracrine activation of CCR7 may promote tumor aggressiveness and that this activation may serve as a clinical biomarker for tumor relapse. We therefore sought to determine the relative contributions of autocrine and paracrine pathways of chemokine signaling to CCR7 activity.

Materials and Methods

Cell Lines and Tumor Tissues

Human SCCHN cell lines PCI-4A/B, PCI-6A/B, PCI-15A/B, PCI-37A/B (A: derived from primary tumor; B: derived from lymph node metastasis in the same patient) were generated at the University of Pittsburgh (17). Murine B7E3 cells, derived from a syngeneic BALB/c oral carcinoma, were established from alkylating carcinogen 4-nitroquinoline-1-oxide (4-NQO)–transformed oral keratinocytes from the tongues of BALB/c male mice and established as primary cultures in serum-free keratinocyte growth medium supplemented with pituitary extract and epidermal growth factor (GIBCO BRL, Grand Island, NY). Cultures were exposed to 2.6 μM 4-NQO for 3 hours. Cells were immortalized and transformed as determined by growth in soft agar and rhodamine-123 retention after 10 passages (approximately 60 days). A subset of clones were subjected to a second cycle of exposure to carcinogen 4-NQO at passage 28 and cloned by limiting dilution (18). Tumor cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen Corp., Carlsbad, CA) that contained 8% (v/v) heat-inactivated fetal bovine serum (Equitech-Bio, Ingram, TX), 100 units/mL penicillin G, and 100 μg/mL streptomycin (Invitrogen Corp.). Cells were tested for mycoplasma at monthly intervals.

Written informed consent from SCCHN patients at the Division of Head and Neck Oncologic Surgery, University of Pittsburgh, was obtained for study of all human tumors. The protocol was approved by the Institutional Review Board, and tumors were studied in de-identified fashion. Data regarding age, sex, smoking history, and clinical characteristics (tumor location and stage and history of radiation/platinum chemotherapy) of these patients were collected retrospectively by review of the medical records in a de-identified fashion (Table 1).

Antibodies

Antibodies against human CCL20 and CCL19, antibodies for immunohistochemistry, and the blocking antihuman CCR7 anti-

CONTEXT AND CAVEATS

Prior knowledge

The expression of chemokine receptor CCR7 in metastatic squamous cell carcinoma of the head and neck (SCCHN) cells and the fact that CCR7 activation stimulated prosurvival signaling in SCCHN cells in vitro suggested that chemokine-mediated activation of CCR7 may play an important role in progression of SCCHN.

Study design

The role of CCR7-mediated signaling pathways in mediating cell survival and resistance to apoptosis was analyzed in human SCCHN cell lines, and the expression of chemokines that activate CCR7 was measured in metastatic nodes and normal tissue from SCCHN patients. The contribution of paracrine and autocrine activation of CCR7 to tumor growth was analyzed using a mouse model deficient in expression of CCR7 ligands.

Contribution

Prosurvival signaling initiated by the binding of chemokines to CCR7 may contribute to the progression and treatment resistance of SCCHN.

Implications

Further study of the role of chemokine signaling in survival and proliferation of SCCHN cells may furnish insight into the mechanisms that underlie resistance to chemotherapies.

Limitations

Insights into prosurvival mechanisms obtained from cell lines and mouse models may not apply to the behavior of human cancer cells in vivo.
body (clone 150503) were purchased from R&D Systems (Minneapolis, MN). The EGFR tyrosine kinase inhibitor AG1478 was purchased from Calbiochem (San Diego, CA). The neutralization dose (ND50, ie, the dose at which 50% of kinase activity was blocked) for this anti-human CCR7 antibody was determined to be 1–5 µg/mL in the presence of 50 ng/mL of human CCL19, and the antibody was used according to the manufacturer’s instructions. The rabbit anti-Akt antibody and rabbit antibodies specific to Akt phosphorylated at serine 473 were obtained from Cell Signaling Technologies (Beverly, MA); the monoclonal antibody to β-actin was purchased from Calbiochem.

Analysis of CCR7-Mediated Signal Transduction
Briefly, 70%–80% confluent cells were serum starved for 72 hours. The metastatic CCR7+ SCCHN cell lines (PCI-4B, -6B, or -37B) were treated or not treated at 37°C with CCR7-specific blocking antibody (10 µg/mL for 2 hours) or mouse IgG (10 µg/mL for 2 hours). Cells were harvested in lysis buffer (10 mM Tris–HCl, pH 7.6, 50 mM Na$_2$PO$_4$, 50 mM NaF, 1 mM Na$_3$VO$_4$, and 1% Triton X-100), sonicated for 3 seconds, and centrifuged at 14,000 rpm at 4°C for 30 minutes. Supernatants were collected for protein quantitation using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA). Fifty µg of total protein was loaded into each lane of 10% SDS–PAGE gels (15, 19). Blots were probed for pAkt (Ser473) or Bcl-2. They were then stripped and reprobed for total Akt and β-actin (the control for protein loading and transfer). Blots were incubated with primary antibodies overnight, washed five times in phosphate-buffered saline (PBS), and then probed with secondary antibodies for 1 hour. Bound secondary antibodies were visualized using enhanced chemiluminescence detection. Densitometric quantitation of independent experiments was performed in triplicate.

MTT Assay
The MTT assay is a colorimetric assay that relies on the ability of viable cells to convert a soluble tetrazolium salt, 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), into a viable cells to convert a soluble tetrazolium salt, 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), into a yellow-to-purple color change. In tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), into a yellow-to-purple color change. In

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
Taqman Pre-Developed Assay Reagent for human CCL19 and CCL20 was from Applied Biosystems (Foster City, CA). mRNA from normal cervical lymph nodes was used as positive control for expression of CCL19 and CCL20. This RNA sample was amplified in parallel on all reverse transcription–polymerase chain reaction (RT–PCR) plates to allow comparison of samples run at different times. Reverse transcription was performed with random hexamer primers and Superscript II reverse transcriptase (Invitrogen Corp.) as described previously (18). Quantitative RT–PCR (QRT–PCR) was then carried out on the Applied Biosystems 7700 Sequence Detection Instrument using Taqman Pre-Developed Assay Reagent for human CCL19 and CCL20 (Applied Biosystems). Reverse transcription was performed at 95°C for 12 seconds; PCR was performed at 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. Expression of the target gene relative to the endogenous control gene, β-glucuronidase, was calculated using the difference in cycle time (∆CT) method, (22) in which relative expression equaled 2$^{-\Delta\text{CT}}$ (ΔCT = cycle time of the target gene minus cycle time for the β-glucuronidase gene).

Immunohistochemistry
For immunohistochemistry, 5 µm frozen sections of primary tumor or metastatic tissue were cut onto positively charged slides, air dried overnight, fixed in acetone solution for 10 minutes, and then air dried again. Sections were sequentially incubated for 10 minutes each in avidin-biotin block (Vector Labs, Burlingame, CA), Universal block (KPL Inc, Gaithersburg, MD), and Immunoprotein block (Thermo Scientific, Waltham, MA) with 5-minute rinses between steps. Sections were incubated with goat IgG anti-human CCL20 or goat IgG anti-human CCL19 (R&D Systems) at a dilution of 1:4 (25 µg/mL) for 1 hour at room temperature. Detection was performed using the avidin–biotin–peroxidase method, color reaction was developed in diaminobenzidine solution, and counterstaining was performed with Mayer’s hematoxylin solution.

Apoptosis
To determine if CCR7-mediated Akt activation contributed to tumor cell survival, annexin V and propidium iodide (PI) staining were performed using the annexin V apoptosis detection kit (BD Biosciences, San Jose, CA). Briefly, cells were serum starved for 48 hours and then pretreated with CCL19 (500 ng/mL) for 4 hours before apoptosis was induced by addition of 40 µM cisplatin for 6 hours. In some cases, cells were treated with Akt inhibitor 1-1,6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (EMD Biosciences, Inc, Darmstadt, Germany)
at 5 µM for 30 minutes before the addition of CCL19 or cisplatin. Annexin V and PI staining were performed, and quantification of early apoptosis (annexin V-positive and PI-negative cells) was by flow cytometry using EXPO32 software.

Growth of Tumors in Mice With Altered Chemokine Expression
To determine the influence of chemokine expression on the rate and extent of tumor growth, BALB/c and paucity of lymph node T cells (plt) mice (24,25) were used. Nakano et al. (25) described the plt mouse strain as one in which a naturally occurring autosomal recessive mutation in mice led to a defect in the homing of naïve T lymphocytes and thus greatly decreased numbers of immune cells in secondary lymphoid organs (26). The plt mutation is a deletion that includes the Slc gene expressed in secondary lymphoid organs and the single functional gene, leaving only an SLC gene that is expressed in lymphatic endothelium and an ELC pseudogene. The lack of CCR7 ligands in the secondary lymphoid organs of plt mice is the basis for severe abnormalities in leukocyte migration and immune response (24). The plt mice were a generous gift from Dr Hideki Nakano, Duke University Medical Center. The mice were housed under clean conditions and cared for through an Institutional Animal Care and Use Committee–approved protocol. Wild-type BALB/c mice (Jackson Labs, Bar Harbor, ME) were used as controls. A total of 14 mice per experimental or control group were used. After subcutaneous injection of 5 × 10^6 B7E3 cells into BALB/c mice and plt mice and a similar number of B7E3-CCR7 tumor cells (B7E3 cells transfected with human CCR7) into plt mice, we observed and measured tumor volume on days 0, 10, 17, and 20. Tumor growth was estimated by the number of B7E3-CCR7 tumor cells (B7E3 cells transfected with human CCR7). Tumor growth was estimated by human CCR7) into

Results
Constitutive CCR7-Dependent Phospho-Akt and Bcl-2 Expression in Metastatic Squamous Cell Carcinoma of the Head and Neck Cells
Previously, in metastatic CCR7^+ cells, we observed constitutive phospho-Akt (21) in the absence of exogenous CCL19 or CCL21. This result was observed by probing immunoblots prepared from cell lysates from four metastatic SCCHN cell lines (PCI-4B, -6B, -15B, and -37B) grown in serum-free conditions for 48 hours with an antibody specific for the phosphorylated form of the kinase(s) (21). To investigate the downstream pathways involved in ligand-induced CCR7 signal transduction, CCR7^+ cells (obtained from nonmetastatic parental primary tumor cells) or CCR7^+ cells (obtained from metastatic nodal tissue) were treated with CCL19 (10 µg/mL for 2 hours at 37°C) or mouse IgG (10 µg/mL for 2 hours at 37°C). Whole-cell lysates were probed with antibodies to phospho-Akt (Ser^473) or Bcl-2 and then probed for total Akt and β-actin. Three independent replicates of the same experiments were performed. In metastatic CCR7^+ cells, CCR7 blockade with an anti-CCR7 monoclonal antibody reduced basal phospho-Akt activation to 35% (95% CI = 2% to 68%) of that observed in control IgG-treated cells (Figure 1). We also determined the effect of CCR7 blockade on the expression of the anti-apoptotic protein Bcl-2 and found that treatment with the blocking anti-CCR7 mAb led to reduction in its expression.

Decreased Viability of Metastatic CCR7^+ Squamous Cell Carcinoma of the Head and Neck Cells After Receptor Blockade
The constitutive activation of prosurvival signaling through the Akt pathway that we observed previously in CCR7^+ cells derived from SCCHN tumors (21) suggested that CCR7 expression might play a functional role in cancer progression. To assess the importance of this constitutive CCR7 activation in SCCHN, we used the MTT...
assay to measure the viability of metastatic SCCHN cells that had been treated with a blocking CCR7 antibody or with an inhibitory truncated variant of CCL21 (SLC-4) (22) or had been left untreated. In addition, we wished to compare the effect of CCR7 blockade on cell viability with that of an EGFR inhibitor currently used to treat cancer patients. In preliminary experiments, the truncated chemokine was found in transwell assays to block migration induced by CCR7 ligand gradients (data not shown). Treatment of the metastatic CCR7+/H11001 SCCHN cell line PCI-4B for 72 hours with CCR7 monoclonal antibody (10 µg/mL), SLC-4 (10 µg/mL), or the EGFR tyrosine kinase inhibitor erlotinib (5 µM) reduced cell viability by 31% (95% CI = 28.0% to 33.9%), 20.5% (95% CI = 17.8% to 23.2%), and 19.5% (95% CI = 16.8% to 22.2%), respectively (Figure 2). However, simultaneous treatment with SLC-4 and erlotinib at these doses for 72 hours had a statistically significantly greater effect, reducing cell viability by 59.5% (95% CI = 56.8% to 66.2%) (Figure 2B). Experiments with the two other metastatic cell lines gave similar results.

Chemokine Ligand Secretion by Cells Expressing the Cognate Chemokine Receptor
Based on our observation of the constitutive activation of CCR7 in metastatic SCCHN cells cultured under serum-free conditions, we hypothesized that secretion of CCR7 ligand(s) by these tumor cells might be a mechanism for autocrine activation of CCR7. Therefore, we performed ELISA assays to determine the chemokines secreted by cells derived from primary tumors and lymph node metastases of four SCCHN patients. Supernatants were collected after 24-hour culture in serum-free medium. In each of the four patients, higher levels of CCL19 were produced by cell lines derived from metastases compared with those from primary tumors. The mean of the four metastatic cells lines was 256 pg/mL and that of primary tumor cell lines was 13 pg/mL. The mean difference for the four patients was 243 pg/mL (95% CI = 89 to 398 pg/mL; \( P \) from a one-sample \( t \) test = .0152, Figure 3A). In contrast, approximately four to five times more CCL20, the ligand for CCR6, was secreted by primary CCR6+/H11001 tumor cell lines compared with CCR6+/H11002 metastatic cell lines (mean of primary tumor cell lines = 499 pg/mL and the mean of metastatic tumor cell lines = 27 pg/mL). The mean difference was 472 pg/mL (95% CI = 219 to 821 pg/mL, one-sample test \( P \) = .0231; Figure 3B) (4). The selective secretion of CCL19 by the CCR7+/H11001 cells may represent a mechanism for constitutive activation of this chemokine receptor and downstream survival pathway signaling.

Distinct Pattern of CCL19 and CCL21 Expression in CCR7+ Metastatic Human Squamous Cell Carcinoma of the Head and Neck Tumors
To determine whether there was a distinct pattern of chemokine expression in CCR7+ metastatic SCCHN cells in vivo, we analyzed levels of chemokine ligand mRNA in 30 primary nonmetastatic...
SCCHN tumors from patients without metastatic disease, 10 benign lymph nodes from patients without metastatic disease, and 30 metastatic lymph nodes from patients with metastatic disease. The demographic and clinical characteristics of these patients are listed in Table 1. Levels of mRNAs encoding CCL19, CCL21, and the CCR6 ligand CCL20 were compared among primary, nonmetastatic SCCHN tumors, and benign and metastatic lymph nodes by real-time QRT–PCR analysis. The expression levels of the chemokine mRNAs were normalized to that of the housekeeping gene β-glucuronidase. Expression of mRNAs for CCR7 and its ligands CCL21 and CCL19 was greater in metastatic lymph nodes than in primary tumors. For example, mean log_{10} expression of CCR7 mRNA in metastatic nodes was 0.30 and in primary tumors, 1.50; the difference in the log_{10} expression of CCR7 in metastatic nodes and primary tumors was 1.80 (95% CI = 1.62 to 1.99). For CCL21, the mean log_{10} expression in metastatic nodes was 0.60 compared with 2.04 for primary tumors (difference = 1.44, 95% CI = 1.30 to 1.54). Mean log_{10} expression of CCL19 was −0.65 in metastatic nodes vs −2.07 in primary tumors (difference = 1.44, 95% CI = 1.3 to 1.53; Figure 4A). Consistent with our result from ELISA assays, average log_{10} CCL20 expression in primary tumors was −0.79, which was higher than in metastatic lymph node nodes (average = −2.04, difference = 1.25, 95% CI = 1.17 to 1.32) or benign lymph nodes (mean = −2.31, difference between tumor and benign nodes = 1.52, 95% CI = 1.41 to 1.62). Furthermore, the expression levels of mRNAs for CCL19 and CCL21 were positively correlated with each other in the tumor samples (adjusted P = .02, Spearman rank correlation test) but not with that of CCR7 (adjusted P = .282), suggesting that the autocrine activation we demonstrated in metastatic CCR7+ SCCHN cells in vitro occurs in vivo.

Because chemokine expression in the tumor cells may be regulated posttranscriptionally, chemokine protein expression in vivo was determined by immunohistochemistry. Frozen tissue samples of normal oral squamous mucosa (n = 14), primary nonmetastatic

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**Table 1. Clinicopathologic characteristics of the 60 patients with squamous cell carcinoma of the head and neck in this study at the time of initial diagnosis**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
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<tr>
<td>Mean age, y (range)</td>
<td>64 (41–92)</td>
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<td>Male, No.</td>
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<tr>
<td>Female, No.</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Oropharynx</td>
<td>9</td>
</tr>
<tr>
<td>Larynx/hypopharynx</td>
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<tr>
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<tr>
<td>T2</td>
<td>16</td>
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<td>T3</td>
<td>14</td>
</tr>
<tr>
<td>T4</td>
<td>22</td>
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<tr>
<td>Tumor differentiation, No.</td>
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<tr>
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<td>22</td>
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<tr>
<td>No</td>
<td>38</td>
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</table>

* Thirty primary tumors were characterized.
† Determined at the time of surgery.
SCCHN tumors (n = 15), and benign (n = 10) or metastatic (n = 8) lymph nodes were probed with antibodies to CCL19 and CCL20. In metastatic tumor-positive lymph nodes, expression of CCL19 was higher than that of CCL20. Consistent with expression levels as determined by QRT–PCR, immunostained tissue sections indicated that primary tumor cells expressed high levels of CCL20 and that metastatic lymph nodes expressed higher levels of CCL19 than primary SCCHN tumors or benign lymph nodes. Lymphoid follicles of a normal (benign) lymph node were weakly positive for CCL20 (Figure 4B). Because metastatic tumor-positive lymph nodes contained 70% tumor cells, it is unlikely that contamination with immune cells contributed substantially to the disparity between CCL19 vs CCL20 expression observed in metastatic lymph nodes. Although we cannot exclude a contribution of paracrine CCL19 and CCL21 sources to constitutive CCR7 activation, we observed metastatic tumor cells that were strongly positive for CCL19 relative to immune cells in these nodes (Figure 4B, black arrow) suggesting a role for autocrine CCR7 ligand secretion and CCR7 signaling by the metastatic SCCHN cells themselves.

**CCR7 Mediates Resistance to Cisplatin-Induced Tumor Cell Apoptosis Through Akt Activation**

The activation of survival pathways via autocrine CCR7 activation in SCCHN cells raised the question of whether resistance to apoptosis induced by chemotherapeutic agents might be observed in metastatic CCR7+ SCCHN cells. To investigate this possibility, apoptosis of tumor cells was induced under serum-free conditions by treatment with cisplatin (40 µM for 4 hours at 37°C), a commonly used chemotherapeutic agent for the treatment of SCCHN. Apoptosis was measured by annexin V/PI staining. Pretreatment of CCR7-positive metastatic PCI-4B or PCI-15B tumor cells with CCL19 (500 ng/mL for 4 hours) before cisplatin exposure led to a statistically significant reduction in the percentage of
annexin V^+ /PI^− binding, apoptotic cells to 5% and 4% of gated cells, respectively (Figure 5). After cisplatin treatment (40 μM for 4 hours at 37°C), these percentages increased to 64% and 54% of PCI-4B and PCI-15B cells, respectively. However, prior treatment with CCL19 (500 ng/mL for 4 hours; apoptosis was induced by addition of cisplatin (40 μM for 6 hours). In three experiments, the cells were pretreated with the Akt inhibitor 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate at 5 μM for 2 hours before CCL19 addition. Representative histograms of the fluorescence distribution of cisplatin-treated cells (dashed line), cells treated with CCL19 before cisplatin treatment (solid line), or cells treated sequentially with Akt inhibitor, CCL19, and cisplatin (dotted line) are shown. The horizontal bar represents the gated population of cells quantified.

Figure 5. Protection of CCR7+ metastatic squamous cell carcinoma of the head and neck (SCCHN) cells from cisplatin-induced apoptosis by CCR7 activation, in an Akt-dependent manner. Annexin-V and propidium iodide (PI)-stained PCI-4B and PCI-15B cells were separated by flow cytometry. Cells were serum starved for 48 hours and pretreated with CCL19 (500 ng/mL) for 4 hours; apoptosis was induced by addition of cisplatin (40 μM for 6 hours). In three experiments, the cells were pretreated with the Akt inhibitor 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate at 5 μM for 2 hours before CCL19 addition. Representative histograms of the fluorescence distribution of cisplatin-treated cells (dashed line), cells treated with CCL19 before cisplatin treatment (solid line), or cells treated sequentially with Akt inhibitor, CCL19, and cisplatin (dotted line) are shown. The horizontal bar represents the gated population of cells quantified.

Because our results suggested a role for autocrine activation of CCR7 in SCCHN survival and metastasis, we compared the roles of autocrine and paracrine CCR7 activation in tumor growth in vivo. Syngeneic murine oral carcinoma models were constructed in wild-type (BALB/c) mouse or plt mice, which lack CCR7-specific chemokines in their secondary lymphoid organs (24, 25). Tumor growth was measured 10, 17, and 20 days after tumor cell injection. Xenografts were established by orthotopic implantation of B7E3 murine SCCHN cells (18) to create plt mice with orthotopic B7E3 tumors, wild-type BALB/c mice with orthotopic B7E3 tumors, or plt mice with CCR7-overexpressing B7E3 tumors.

On days 10, 17, and 20, the volume of orthotopic SCCHN tumors in plt mice was statistically significantly less than the volume of these tumors in control BALB/c mice (Figure 6). On the final day of measurement (day 20), mean tumor volume in the B7E3-plt mice was 14.2 mm^3 (95% CI = 11.3 to 17.1) smaller than in control BALB/c mice—an average reduction of 53%. This difference was statistically significant by a Wilcoxon test (P = .0033). On day 10, this difference was 13.1 mm^3 (95% CI = 10.1 to 16.0, P = .001). On day 17, the difference was 17.1 mm^3 (95% CI = 12.9 to 21.2, P = .001).

Transfection of CCR7 in B7E3 cells before their implantation in plt mice resulted in increased tumor growth, suggesting an important role for CCR7 signaling in tumor progression in vivo. In plt mice, the mean volume on day 20 of tumors overexpressing CCR7 was 17 mm^3, compared with 12 mm^3 in tumors that did not overexpress CCR7 (difference = 5 mm^3, 95% CI = 3.3 to 6.5, P = .0019). However, tumor growth was still statistically significantly reduced relative to that observed in BALB/c mice (difference = 9.3 mm^3, 95% CI = 5.7 to 13, P = .0033).

Figure 6. Effect of CCR7 overexpression on tumor growth in CCR7 ligand-deficient mice. After injection of 5 × 10^6 B7E3 cells into BALB/c mice and paucity of lymphoid tissue (plt) mice and a similar number B7E3-CCR7 tumor cells (B7E3 cells transfected with human CCR7) into plt mice (n = 14 mice per group) tumor volume was measured on days 0, 10, 17, and 20. The inset figure shows basal level of CCR7 expression in B7E3 oral cancer cells by flow cytometry. Overexpression of CCR7 was achieved by CCR7 transfection of B7E3 cells with the human CCR7 gene, which increased surface expression (inset). On the final day of measurement (day 20), mean tumor volume in the asterisks denote statistically significant differences based on the Wilcoxon test; error bars correspond to 95% confidence intervals.

Decreased Growth of CCR7+ Tumors In Vivo in CCR7 Ligand-Deficient plt Mice
Thus, the lack of paracrine sources of CCR7 ligands in plt mice was associated with substantial reduction, but not prevention, of tumor growth. This evidence of stimulatory effects on tumor growth by paracrine chemokine signaling supports the separate roles of both paracrine and autocrine CCR7 activation in mediating tumor growth. The fact that overexpression of CCR7 in tumor cells appeared to compensate for a relative lack of expression of its ligands CCL19 and CCL21 in secondary lymphoid organs may be due to constitutive autocrine and/or residual paracrine CCR7 activation due to CCL21/SLC gene duplication in these mice, which retain residual (20%–30% of wild type) CCL21 expression (24,28).

**Discussion**

SCCHN is characterized by a high rate of invasion and early regional metastasis, the strongest predictor of patient outcome—patients who have regional metastases have an overall 5-year survival rate of only 30% (19). The use of chemotherapeutics in initial treatment (29) or postoperative adjuvant settings (30) is increasing, and these treatments may include cisplatin and EGFR-targeted agents (13). Furthermore, the efficacy of EGFR monotherapy has been disappointing, and the combination of growth factor and anti-inflammatory targeted agents is gaining increasing attention (31,32). Thus, there is need for better understanding of potential mechanisms of tumor resistance to these therapies.

We have shown previously that CCR7 activates prosurvival pathways independent of EGFR activation (21). The results of this study offer additional evidence that this chemokine receptor plays an important role in SCCHN progression and in resistance to chemotherapies. The relatively high expression of CCR7 and its ligands in metastatic lymph nodes that we observed is consistent with a role of CCR7-mediated signaling in metastatic disease. In addition, CCR7 activation may contribute to cisplatin resistance through autocrine and paracrine stimulation, as evidenced by our observation of a CCL19- and Akt-dependent reduction of cisplatin-induced apoptosis. Because nearly all metastatic SCCHN patients receive combination cisplatin and radiation therapy (30), further studies—specifically measurement of CCR7 and chemokine ligand expression in recurrent tumors treated with radiation alone and in tumors treated with cisplatin—are needed to clarify the role of CCR7 signaling in resistance to particular forms of therapy. However, our findings would appear to support the development and testing of therapies that combine CCR7 and EGFR inhibition to enhance antitumor efficacy.

In this study, we investigated the implications of our observation that the PI3K/Akt survival pathway is constitutively activated in metastatic, CCR7+ SCCHN cells (21) and the cellular mechanisms that might underlie it. The fact that these cells selectively secreted CCL19 whereas the autologous CCR7– nonmetastatic parental cells did not was confirmed by testing a series of primary and metastatic human SCCHN samples using QRT–PCR and immunohistochemical staining. The biologic importance of this finding is supported by our demonstration that blockade of the CCR7 pathway by either anti-CCR7 blocking antibody or a specific antagonist of CCL21 decreased tumor cell viability and enhanced tumor cell death under conditions in which EGFR signaling was inhibited. Conversely, CCL19-induced CCR7 activation protected these CCR7+ SCCHN cells from cisplatin-induced tumor cell apoptosis in two metastatic SCCHN cell lines.

Our results also support the possibility that CCR7 expression may prove to be a biomarker associated with the presence of metastasis, tumor relapse, or failure of chemotherapy. In the context of initial therapy or adjuvant postoperative treatment (13,29,30,33), selection of patients most likely to respond to apoptosis-inducing chemotherapy regimens (usually used in conjunction with radiotherapy) is increasingly important. Interestingly, increased expression of CCR7 appeared to be more strongly associated with tumor aggressiveness and relapse than expression of its ligands CCL19 or CCL21 because overextension of CCR7 restored tumor growth in the plt mice. These two ligands may differ in terms of their role in CCR7 activation due to quantitative differences in localized expression in different regional lymphoid compartments, such as lymphatic endothelium or T cell zones of the lymph node (34). The fact that CCR7 overexpression partially overcame the decreased tumor growth observed in the CCR7 ligand-deficient plt mouse model suggests that increased expression of this receptor may play a role in autocrine and paracrine activation of this CCR7 survival pathway in metastatic disease. Factors responsible for regulation of CCR7 expression in metastatic SCCHN cells merit detailed study (35).

Substantial evidence suggests that lymphoid chemokines used by immune cells (2–4,36) regulate recruitment of solid tumor cells to specific organ sites. Initiation of CCR7 expression and signaling may be a stochastic process that is propagated in clonal proliferations of cells with growth and survival advantages, particularly when it is in response to immunologic signals. These inflammatory, chemokine-mediated signals are expressed along the hemogenous and lymphatic channels through which metastatic SCCHN cells traverse. Promoter elements upstream of the CCR7 gene may enable inflammatory signals to activate transcription of CCR7 and allow the premetastatic SCCHN cell to respond to these signals after an inflammatory exposure in the tumor microenvironment (37,38).

Our finding that expression of CCR7 and its ligands can propagate autocrine and paracrine survival signals, including constitutive PI3K-Akt pathway activation (21), suggests that the receptor may have potential as a novel therapeutic target. However, the mechanisms responsible for initial expression of CCR7 and its ligands in carcinogenesis are not understood. Our findings also strongly suggest that selection within the tumor microenvironment of SCCHN tumor cells with upregulated CCR7 expression and prosurvival pathway activation may provide these tumors with a mechanism for resistance to apoptosis induced by cisplatin chemotherapy and lead to tumor recurrence in patients treated with platinum-containing regimens.

Our study had several limitations. First, our use of four paired primary and metastatic cells lines depended on the assumption that these lines were adequate models for SCCHN and the metastatic phenotype of this disease. Second, because advanced, recurrent SCCHN has nearly always been treated previously with both chemotherapy (such as cisplatin) or EGFR inhibitors as well as...
radiotherapy (33), we failed to identify sufficient numbers of tumors from patients who were not given radiotherapy, thus complicating the study of the causes for resistance to treatment. Third, downstream cross talk with CCR7 with other pathways is likely because PI3K/Akt is a general mediator of proliferative and anti-apoptotic signaling. All of these limitations are often present when a novel signaling pathway is studied in vitro using retrospectively collected tissues.

The potential importance of NF-κB activation of CCR7 expression has been suggested by others (39–43), and we have obtained preliminary data that support this activation in our system (35). Future studies will be needed to elucidate the role of Akt in the context of inflammatory NF-κB–mediated pathway activation. The importance of inflammatory pathway mediators such as NF-κB or STAT-3 (44,45) (in both immune and tumor cells) for which in vivo inhibitors are in clinical evaluation suggests that these signals should be studied in relation to CCR7 expression.

References

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