Expression Pattern of Chemokine Receptor 6 (CCR6) and CCR7 in Squamous Cell Carcinoma of the Head and Neck Identifies a Novel Metastatic Phenotype

Jun Wang,1,5 Liqiang Xi,3,5 Jennifer L. Hunt,2 William Gooding,4 Theresa L. Whiteside,2,5 Zhuo Chen,5 Tony E. Godfrey,3,5 and Robert L. Ferris1,5

1Departments of Otolaryngology and Immunology, and 2Pathology, University of Pittsburgh School of Medicine, and 3Departments of Surgery and 4Biostatistics, 5University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

ABSTRACT

Squamous cell carcinoma of the head and neck (SCCHN) metastasizes predictably to cervical lymph nodes, with low rates of distant metastases. Tumor cells can express various receptors that facilitate such metastatic spread to lymph nodes and other nonlymphoid organs. Chemokine receptors (CCR), normally expressed on lymphocytes, control immune and inflammatory cell migration, providing a link between innate and adaptive immunity. Chemokine receptor expression was evaluated in SCCHN, using paired primary and metastatic tumors cell lines, and paired primary and metastatic biopsies from the same patients. Quantitative reverse transcription-PCR showed a consistent pattern of CCR6 down-regulation and up-regulation of CCR7 in metastatic cells and tissues. Chemotaxis assays, ligand-induced receptor down-regulation, and specific antibody blocking experiments supported the quantitative reverse transcription-PCR results, indicating that these surface receptors were functional on metastatic tumor cells. Cells derived from a highly metastatic mouse model of SCCHN were used to confirm CCR7 up-regulation in tumor cells with higher metastatic potential. CCR6 down-regulation is consistent with its decreased expression in cells emigrating from peripheral mucosal sites, whereas CCR7, important for homing of immune cells to secondary lymphoid organs, was significantly up-regulated. Thus, CCR6, CCR7, and their ligands, normally important in controlling immune cell trafficking in response to inflammatory stimuli, may have an important role in determining the metastasis of SCCHN cells in vivo.

INTRODUCTION

Head and neck cancer is the sixth most common tumor in the United States, with 40,000 new cases annually (1). The 5-year survival is only 30%, mainly due to the frequent presence of metastasis at diagnosis, and the pattern of regional cervical metastasis (as opposed to distant organs) in this disease is remarkably consistent (2). Because metastasis is the best predictor of prognosis, an urgent goal in head and neck oncology is to develop improved antitumor agents for clinical use. A better understanding of this unique metastatic process is necessary to enable the development of therapies designed to prevent tumor dissemination.

The predilection of metastases for specific organs may depend on a variety of factors (3–4). According to the one theory, all of the migrating tumor cells can enter any tissue but would form a metastasis only if all requirements for their growth were met (5). The second suggests that tissue-specific adhesion molecules on endothelial cells select migrating cells able to attach and form a premetastatic nucleus of cells (6). The most recent theory proposes that chemotrajectories, produced by stromal or immune cells, lead invasive cancer cells to the tissue of their potential secondary growth (7). Chemokine molecules constitute a superfamily of inducible, secreted, proinflammatory proteins (8–11) involved in a variety of immune responses, acting primarily as chemoattractants and activators of specific types of leukocytes (12–14). Recent studies have shown the involvement of chemokine receptors in cancer metastasis (15–17). Chemokine ligand (CCL19)/MIP3-β and CCL21/SLC, two chemokines constitutively expressed by lymph nodes (LN) and other immune cells, share a common chemokine receptor, CCR7 (16). This receptor is also expressed on mature dendritic cells (DC), naïve, and some memory T cells. A recent study reported high levels of expression of CCR7 and CXCR4 in breast cancer cells and linked the receptor expression to the metastatic destination of tumor cells (17).

Because of the unique site of cervical metastasis seen in squamous cell carcinoma of the head and neck (SCCHN) without frequent distant metastases, we hypothesized that SCCHN cells might also use a chemokine-mediated mechanism during the process of LN metastasis of cancer cells, similar to those regulating lymphocyte trafficking. However, we found that CCR7 up-regulation alone did not appear sufficient, rather loss by tumor cells of surface CCR6 enabled their LN metastasis. Interestingly, these results show that SCCHN cell metastasis displays a similar receptor pattern as manifested by immune cells, during the normal maturation and trafficking to LN away from peripheral sites of inflammation. Metastatic tumor cells appear to down-regulate CCR6 expression and up-regulate CCR7, enabling migration to secondary lymphoid tissues. SCCHN is thought to metastasize through afferent lymphatic channels, as opposed to the hematogenous route. Our data suggest that CCR7-mediated metastasis, using established gradient expression of the cognate ligand on vascular channels infiltrating tumors, may allow SCCHN cells to access the cervical LN.

MATERIALS AND METHODS

Human Tumor Samples and Cell Lines. Fourteen sets of paired human head and neck primary tumors and tumor-containing metastatic LNs were harvested at surgery and immediately snap frozen at −80°C until RNA extraction. Institutional Review Board-approved, written informed consent was obtained from all of the patients donating specimens for this study, through the Department of Otolaryngology, University of Pittsburgh. Clinical and demographic data for paired tumor/metastatic cell lines and fresh tumor/metastasis specimens are shown in Table 1. Cell lines PCI-1T/M, PCI-6T/M, PCI-15T/M, PCI 57-T/M (T: primary tumor, M: metastatic LN) were derived from the patient lesions (see Table 1) at the University of Pittsburgh (18–20). The panel of cell lines T686, 868LN, derived from a SCCHN patient’s primary (TU) and metastatic (LN) tumor, and 868LN-M3a2, 868LN-M3b2, and 868LN-M3b3 (3 different clones derived from a highly metastatic derivative of 868LN cells passed three times in nude mice; Refs. 20–22), were used to compare CCR7 expression by quantitative reverse transcription-PCR (qRT-PCR).

Cells were cultured in DMEM (Invitrogen Corp., Carlsbad, CA), which 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.).

Immunohistochemistry. Immunostaining was performed by the avidin-biotin-peroxidase method; color reaction was developed in diaminobenzidine.
solution, and counterstaining was performed with Mayer’s hematoxylin solution. Tissues were stained using 2H4 primary antibody (Ab), mouse IgM, antihuman CCR7 (BD Biosciences Inc., San Diego, CA), or 53103.111 mouse IgG2B, antihuman CCR6 (R&D Systems Inc., Minneapolis, MN). Ab titrations and isotype control Ab determined optimal staining conditions.

**DC Cultures.** DCs were generated from human peripheral blood mononuclear cells (24). Briefly, peripheral blood mononuclear cells were isolated from leukocyte-enriched buffy coats by density-gradient centrifugation through Ficoll-Paque Plus (Amersham Biosciences AB, Uppsala, Sweden). Peripheral blood mononuclear cells (5 × 10⁶) were resuspended in 10 ml of warm AIM-V medium. After 1 h at 37°C, nonadherent cells were removed, and adherent cells were cultured in 10 ml of DC medium supplemented with interleukin 4 (1 × 10⁶ units/ml) and granulocyte macrophage colony-stimulating factor (1 × 10⁶ units/ml) and granulocyte macrophage colony-stimulating factor (1 × 10⁶ units/ml) for 3 days. On day 3, additional interleukin 4 (1 × 10⁶ units/ml) and granulocyte macrophage colony-stimulating factor (1 × 10⁶ units/ml) were added. On day 6, immature DCs were harvested for use in some experiments. DC maturation was induced using AIM-V plus 10 ng/ml tumor necrosis factor α, 10 ng/ml interleukin 1β, and 10 ng/ml interleukin 6 for 18 h. Both immature DCs and mature DCs were phenotyped by flow cytometry, using Abs to CD80, CD83, CD86, HLA-DR, CD40, and CD83 for staining as described previously.

**Isolation of RNA and Reverse Transcription PCR (RT-PCR) Analysis.** Total RNA was isolated from tumor lines and biopsies using RNeasy Mini kit (Qiagen Inc.) according to the manufacturer’s protocol. All of the RT-PCR was done by using Thermoscript One-step System kit (Invitrogen Corp.), using primers as described (25). Reverse transcription was performed at 65°C for 35 min and 95°C for 5 min. PCR was performed at 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, total for 33 cycles. Reverse transcription was performed with random hexamer primers and Superscript II (Invitrogen Corp.) as described previously (26). qRT-PCR was then carried out on the Applied Biosystems 7700 Sequence Detection Instrument, using Taqman CT method described previously: (Ref. 27).

**Flow Cytometric Analysis of Chemokine Receptor Expression.** Tumor cells (10⁴ cells) were suspended in 25 μl of 1% (w/v) FCS in PBS (containing 0.1% NaN₃) for 15 min at room temperature and incubated with 5 μl aliquots of fluorescein-conjugated mouse antihuman CCR6 antibody, IgG2b (R&D Systems Inc.) or rat antihuman CCR7 antibody, IgG2a (BD Biosciences Inc.) for 1 h at 4°C. After washing the cells twice in 4-ml aliquots of the same PBS buffer, cells were resuspended and fixed in 200 μl 1% (w/v) paraformaldehyde for analysis. A murine IgG2b and a rat IgG2a antibody were used as negative controls. Ten thousand cells were collected using Beckman-Coulter Epics XL (Beckman Coulter, Inc., Fullerton, CA) and EXPO32 analysis software.
expression on 686LN cells, the human parental metastatic cell line used in the metastatic SCCHN mouse model (21), developed by passaging these cells through several serial metastases in nude mice. Standard RT-PCR analysis of these cells (TU686 and 686LN, which were both derived from a human primary and metastatic tumors) showed expression of both CCR6 and CCR7 in each cell line. CCR6 down-regulation was not measured quantitatively, due to its apparent lack of expression in M cells in our data obtained in the 14 human primary and metastatic tumor biopsies.

Significant Up-Regulation of CCR7 Expression in Metastatic Tumor Cells by qRT-PCR. As described above, CCR7 mRNA was detected in both primary and metastatic tumor lines and fresh specimens by RT-PCR analysis (see Fig. 1), raising the question as to whether levels of expression were similar between primary and metastatic tumor cells. Therefore, using real time qRT-PCR, we determined the relative CCR7 expression between primary tumors and metastases, in paired cell lines and fresh tumor tissues. We carefully dissected tumor from surrounding nodal architecture to remove contaminating immune cells. Because CCR6 expression was not detected in M cells in our data obtained in the 14 human primary and metastatic tumor biopsies.

Confirmation of Chemokine Receptor Expression Pattern by Flow Cytometry. To analyze the T/M cell lines by flow cytometry, surface CCR6 but not CCR7 was stained on primary tumors (T), whereas metastases (T), had low if any expression of this receptor (Fig. 4). On the other hand, metastatic tumors expressed surface CCR7, but not CCR6 (see Fig. 4A). Increased expression of CCR7 in 686LN and 686LN-M3 cells compared with TU686 cells was also observed using this technique (data not shown). Fig. 4B also shows CCR7 responses after treatment with its ligand, MIP-3β, at a concentration of 500 ng/ml for 4 h by flow cytometry. Metastatic (M) cells manifest the expected CCR7 down-regulation only after treatment with the appropriate ligand (MIP-3β). However, down-regulation of CCR6, after treatment of primary (T) tumor cells, was observed after MIP3α treatment (data not shown). Treatment of each M cell line with the CCR6 ligand, MIP-3α, as a control did not result in CCR7 down-regulation, as shown in Fig. 4B.
Immunohistochemical Staining of CCR6 and CCR7 in Fresh Tumor Biopsies Shows That a Novel Metastatic Expression Pattern Is Present in Vivo. To study chemokine receptor expression in vivo, paraffin tumor blocks were stained for CCR6 and CCR7 (Fig. 5). Using immunohistochemistry, differential CCR6 and CCR7 expression in primary versus metastatic head and neck tumors (n = 4 patients) was confirmed (P1–P4 in Table 1) from patient tumors studied using qRT-PCR. Immunostained tissue sections indicated that primary tumors expressed high levels of CCR6, but metastatic tumors expressed high levels of CCR7 (Fig. 5, A–F). These results rule out the possibility that high CCR7 expression in metastatic tumors was due to lymphoid cells in tumor-metastatic LN specimens, and is consistent with the retention of different migratory responses of primary (T) and metastatic (M) tumor cell lines, even after culture in vitro for months.

Tumor Cell Chemokine Receptors Mediate Chemotaxis in Response to Selective Chemokines. We next analyzed the capability of paired T/M cells to migrate in vitro in response to the respective ligands. Transwell migration assays followed by blocking of the receptors with receptor-specific monoclonal Abs showed that both CCR6 (on T cells) and CCR7 (on M cells) surface receptors are functionally active. Media-pulsed and isotype-matched monoclonal Ab-pulsed wells (data not shown) were used to control for CCR-specificity of the blocking effect. Increase in migration of primary tumor cells was also observed in all three of the cell lines examined, in response to MIP-3α (Fig. 6A). Enhanced responsiveness to both CCR7 ligands, MIP3β and SLC (Fig. 6B), was observed in three of three cell lines tested, consistent with CCR7 up-regulation seen by qRT-PCR.

MATRIGEL invasion assays using 686LN (poorly metastatic) cells and 686LN-M3a2 (highly metastatic derivative) as described previously (22), indicated a correlation between increased CCR7 expression detected by qRT-PCR in 686LN-M3a2 cells, with invasive potential.

DISCUSSION

Metastatic sites have been correlated with chemokine receptor expression in some metastatic tumor cells (15, 17, 31, 32, 34, 35). Our report indicates a novel role for loss of expression of the inflammatory chemokine receptor, CCR6, in addition to CCR7 up-regulation, during the metastatic process. Whether metastasis takes place via hematogenous (similar to T cells) or lymphatic channels, is not presently clear from our findings, although certainly both mechanisms may be important, depending on angiolymphatic structures invaded in proximity of CCR7-expressing tumor cells.

The consistent loss of expression of CCR6 in our system is reminiscent of the response of immune cells to inflammatory stimuli. For example, after antigen uptake or other stimuli that activate their progression to mature DCs, CCR6 expression is lost, whereas CCR7 is significantly up-regulated. Emigration from peripheral sites and trafficking to regional LNs usually leads to interaction with other immune cells. Whereas CCR7 is also used by circulating naïve and memory T cells, we would expect that metastatic SCCHN cells are likely to access cervical LNs using afferent lymphatics, similar to DCs. However, our data do not currently distinguish between vascular pathways used for this phenomenon, as our studies only used DCs as controls in qRT-PCR and migration studies. Lymphangiogenesis is
not well understood currently, including the ligand-mediated signals related to embolism and implantation into regional LN.

The abundant expression of the homeostatic chemokine SLC (a ligand of CCR7) in LN's makes it a likely candidate to attract CCR7+ tumor cells (32-35). On the other hand, CCR6 down-regulation in metastatic tumor cell derivatives indicates that the coordinate expression of CCR6 and CCR7 may be important in orchestrating SCCCHN metastasis (31).Remarkably, our observations in fresh tumor specimens were maintained in cell lines cultured in vitro for months at a time. This also helps rule out that, in fresh metastatic tumor biopsies, CCR7 up-regulation was simply the result of immune cell contamination of our tumor RNA. In addition, the pattern of this coordinate CCR expression pattern in metastases indicates that the up-regulation of CCR7 in these tumor cells is not due to the LN microenvironment in fresh tissues, because even in metastatic tumor cell lines, this effect is maintained over long periods in culture. The finding that increasing metastatic tumor derivatives in a metastatic mouse model showed quantitatively increased CCR7 expression demonstrates the in vivo importance of this phenomenon, in a completely separate experimental system.

Thus, a specific and differential expression pattern of CCR6, CCR7 is manifested by metastatic tumor cells, as their migration appears to be regulated by the loss of CCR6 expression, acting as the brake, and gain of expression of CCR7, acting as the accelerator, leading to migration of tumor cells to proximal lymphoid tissues. Although the organ predilection of metastatic cells might ultimately depend on multiple chemokine receptor-ligand interactions as we encountered here, rather than a single interaction, our data suggest that inhibition of CCR7 signaling could be a clinically useful target for therapy in the prevention of SCCCHN metastasis. Additional work is under way to determine metastatic pathways responsible for tumor cell chemokine receptor-associated metastasis, proinflammatory, nuclear factor κB-mediated signaling pathways (36–38). Preventing such a poor prognostic feature in this disease would likely enable more successful locoregional tumor control and improve survival, even with current surgical and/or chemoradiotherapeutic modalities.

ACKNOWLEDGMENTS

We thank Nicky Sieran and Dr. Andres Lopez-Albaitero for help with the RT-PCR and flow cytometry experiments, and the Daiichi Clinical Scholars Program. We also thank Drs. Olja Finn and Michael Shurin for excellent critical advice on the manuscript.

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