Expression of CC Chemokine Receptor-7 and Regional Lymph Node Metastasis of B16 Murine Melanoma

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Background: CC chemokine receptor-7 (CCR7), which plays a critical role in the migration of activated dendritic cells to regional lymph nodes via afferent lymphatic vessels, is also expressed by human breast and melanoma cell lines. Because neoplastic cells also enter lymphatic vessels before metastasis to the lymph nodes, we investigated whether CCR7 expression enhances metastasis of B16 murine melanoma cells to regional lymph nodes. Methods: B16 cells were transduced with a retroviral vector containing CCR7 complementary DNA (CCR7-B16 cells) or with vector alone (pLNCX2-B16 control cells). The functional assay for CCR7 protein was Ca\(^{2+}\) flux stimulated by the chemokine CCL21, a CCR7-specific ligand produced by lymphatic endothelial cells. B16 tumor cells were injected into the footpad of mice. Tumor cell metastasis to draining lymph nodes was assessed by measuring messenger RNA (mRNA) for tyrosinase-related protein-1 (TRP), a melanocyte-specific enzyme, with real-time, quantitative reverse transcription-coupled polymerase chain reaction. All statistical tests were two-sided. Results: One week after injection into the footpad, 701-fold (95% confidence interval [CI] = 64- to 1336-fold) more TRP mRNA was detected in draining lymph nodes from CCR7-B16 cell-injected mice than in those from control cell-injected mice. Three weeks after footpad injection, 58% (11 of 19) of the draining lymph nodes from CCR7-B16 cell-injected mice and 5% (one of 19) of those from control mice showed gross metastases (P<.001). CCR7-B16 cells isolated from lymph node metastases retained functional CCR7 expression. Lymph node metastasis of CCR7-B16 cells was blocked by neutralizing anti-CCL21 antibodies (metastasis in none of five lymph nodes) but not by control immunoglobulin G (three of five). Enhanced metastasis of CCR7-B16 cells was specific for a lymphatic route because both CCR7-B16 and control cells co-injected intravenously metastasized to the lung at the same frequency. Conclusion: Expression of a single chemokine receptor gene, CCR7, increased B16 cell metastasis to draining lymph nodes, suggesting that cancer cells may co-opt normal mechanisms of lymph node homing during metastasis. [J Natl Cancer Inst 2001;93:1638–43]

Malignant melanoma is the ninth most common cause of cancer among Caucasians in the United States, with an incidence that appears to be increasing (1). Metastases occur in 15%–36% of patients (2) and are the primary cause of morbidity and mortality, as observed for many other forms of cancer. In malignant melanoma, about 60% of metastases occur in regional lymph nodes (3); thus, the status of regional lymph node metastases is the most powerful predictor of survival for this disease (4).

Our knowledge of the molecular determinants of metastasis is clearly limited, although a small number of gene products have been reported to suppress (5) or to increase (6) metastasis. For example, stimulation of intratumor lymphangiogenesis with growth factors such as vascular endothelial growth factor-C (7) markedly stimulates lymph node metastasis. Most of these studies (8,9) focused on dissemination of cells through blood vessels to organs, such as lungs or liver, although lymphatic vessels are clinically important routes for metastatic melanoma and breast cancer cells.

The migration of cancer cells to regional lymph nodes via lymphatic vessels resembles the normal migration of antigen-presenting cells such as dendritic cells during the course of inflammation. Antigen-bearing dendritic cells from tissues enter afferent lymphatic vessels and migrate to draining lymph nodes. Dendritic cell migration has been studied extensively. Although several proteins (i.e., E-cadherin, P-glycoprotein, and α\(_5\)β\(_1\) integrin) are potentially important to the migration of dendritic cells to draining lymph nodes [reviewed in (10)], the role of CC chemokine receptor-7 (CCR7) has been established conclusively. When dendritic cells are activated by a variety of inflammatory stimuli (such as lipopolysaccharide) or cytokines (such as tumor necrosis factor-α), the cell surface expression of CCR7 is increased and the cells become responsive to CCL21 (also termed secondary lymphoid tissue chemokine and 6Ckine/exodus-2) (11). CCL21 is a chemokine that is produced constitutively by lymphatic endothelial cells in the skin (12) and other organs (13), and CCL21 induces a CCR7-mediated Ca\(^{2+}\) flux. Targeted deletion of the CCR7 gene (14), spontaneous mutations that prevent the expression of CCL21 (15), and inhibition of CCL21 with neutralizing antibodies (12) show that interference with either CCR7 or CCL21 prevents the migration of dendritic cells from peripheral tissues to regional lymph nodes.

Of interest, CCR7 is expressed by human adult T-cell leukemia cells with lymph node involvement (16) and by some human breast and melanoma cell lines (17). Thus, CCR7 may play a role in lymph node metastasis.

Herein we investigate whether functional expression of CCR7 enhances murine melanoma cells to metastasize to regional lymph nodes at a higher rate than control melanoma cells and explore the mechanism used.

Materials and Methods

Cell Lines, Animals, and Reagents

B16/F1 melanoma cells (18), derived from C57BL/6 mice, were provided by the cell repository of the National Cancer Institute (NCI)-Frederick Cancer Research and Development Center (Frederick, MD). The cells were grown in Dulbecco’s modified Eagle medium (MEM) (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) with 10% heat-inactivated fetal calf serum, 10 mM MEM nonessential amino acids, 100 mM sodium pyruvate, and 200 mM l-glutamine. Recombinant chemokines were purchased from Peprotech (Rocky Hill, NJ). Female C57BL/6 mice (6–10 weeks old) from the NCI-Frederick Cancer Research and Development Center Animal Facility were used in all in vivo experiments, which were approved by the NCI Animal Care and Use Committee.

Retroviral Transduction of B16/F1 Melanoma Cells

Murine CCR7 complementary DNA (cDNA) (from A. Iwasaki, Yale University School of Medi-
luminal melanoma cells that had metastasized to a lymph node
(21,22). The fold increase of TRP mRNA in lymph nodes from CCR7-B16- or CXCR5-B16-injected mice relative to those from pLNCX2-B16-injected mice was calculated according to the expression $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is the difference between $\Delta C_T$ (chemokine receptor-B16) and $C_T$ (pLNCX2-B16) with the following primers.

Enhanced Migration of CCR7-B16 Melanoma Cells to Draining Lymph Nodes In Vivo

B16 melanoma cells have a low spontaneous rate of metastasis from tissue to local draining lymph nodes (see Fig. 3). To assay migration of B16 melanoma lines from the periphery to draining lymph nodes, we injected CCR7-B16, pLNCX2-B16, and CXCR5-B16 melanoma cells into the footpad of C57BL/6 mice. One week later, when tumors in the footpad were not grossly visible, we collected popliteal lymph nodes (Fig. 2, A–C) and isolated RNA for quantitative, real-time RT–PCR. Although visible lymph node metastases were occasionally observed for CCR7-B16-injected cells at that time (Fig. 2, B), they were never observed for pLNCX2-B16 (Fig. 2, A) or CXCR5-B16 cells (Fig. 2, C). We assessed expression of TRP messenger RNA (mRNA), a melanoma/melanocyte-specific marker, by quantitative, real-time RT–PCR. This assay is a sensitive, quantitative measure of melanoma metastasis that has been used clinically to stage human melanoma (21). In five experiments, lymph nodes collected from mice given an injection of CCR7-B16 cells showed 701-fold (range, 200- to 1400-fold; 95% confidence interval [CI] = 64- to 1336-fold) more TRP mRNA than lymph nodes from mice given an injection of pLNCX2-B16 cells (Fig. 2, G). In two experiments, lymph nodes from CXCR5-B16-injected mice showed only a minimal threefold increase (95% CI = -9. to 16-fold) of TRP mRNA over that of the vector control. Comparison of the groups injected with CCR7-B16 and CXCR5-B16 cells provided evidence of a trend toward increased metastasis in the CCR7-B16 group, despite the lack of statistical significance ($P = .095$ by a two-tailed exact Wilcoxon rank sum test) because of a limited number of observations with CXCR5-B16-injected animals. CXCR5 was chosen as negative control because its ligand, CXCL13, is expressed within the lymph node within B-cell zones (24). However, unlike CCR7, CXCR5 apparently has no role in the entry of dendritic cells into lymphatic vessels. In pLNCX2-B16 mice, the levels of TRP mRNA in draining lymph nodes and in contralateral popliteal lymph nodes were comparable, indicating that very few of the control B16 melanoma cells migrated to lymph nodes soon after injection (data not shown).
To determine whether CCR7-B16 melanoma cells metastasized to draining lymph nodes via blood vessels or lymphatic vessels, we determined metastasis to the lymph nodes and the lungs of mice given an intravenous injection of CCR7-B16 cells (four C57BL/6 mice) or pLNCX2-B16 cells (four mice). After 2 weeks, the animals were killed, and in the lungs and peripheral lymph nodes were examined for metastases. Peripheral lymph nodes from the four CCR7-B16-injected mice did not show visible metastases either by gross inspection (Fig. 2, D, lymph nodes from one representative mouse) or by histology (data not shown). For calcium flux assays, vector (pLNCX2-B16 cells) (B) and CCR7 (CCR7-B16 cells) (D)-transduced B16 cells were loaded with fura-2 acetoxyethyl ester dye and exposed to CCL21 in a continuously stirred fluorimeter at 37 °C as described previously (31). Arrows show points of injection of phosphate-buffered saline (PBS), CCL21 (500 ng/mL), and ionomycin (ION) (1 μg/mL), as indicated, to demonstrate appropriate dye loading.

Two weeks after tumor cell injection into footpads, B16 tumors increased rapidly in size. Three weeks after injection, when these tumors were 5–7 mm in diameter, the animals were killed, and the draining lymph nodes were removed for visual inspection. The tumor volumes in CCR7-B16- and pLNCX2-B16-injected animals were similar (data not shown). However, in four experiments, large metastases (multiple in some lymph nodes) were found in lymph nodes of CCR7-B16-injected mice (Fig. 3, A). In four experiments, 11 (58%) of 19 (range, 25%–100%) lymph nodes from 19 CCR7-B16-injected mice had obvious metastases, but only one (5%) of 19 lymph nodes from 19 pLNCX2-B16-injected mice had a metastasis that was relatively small (Fig. 3, A) (P<.001, χ² test).

Because retroviral transduction may lead to heterogeneous expression of the gene of interest, we repeatedly subcultured B16 melanoma cells from one of the metastatic lymph nodes shown in Fig. 3, A. If CCR7 expression contributed to the lymph node migration of CCR7-B16 cells, metastatic cells within the lymph node should express CCR7. Indeed, even
node was removed from each experimental animal. and two experiments were performed with CXCR5-B16-injected mice. After 1 week, lymph nodes were recovered, visually inspected (A–C), and used for real-time reverse transcription–coupled polymerase chain reaction (RT–PCR) analysis (G). A–C) Lymph nodes from groups of five mice injected on the same day (described above) were recovered 1 week later. White arrowheads = gross visible metastases, verified microscopically by staining with hematoxylin–eosin. D) CCR7-B16 cells (1 × 10⁶ cells) were injected intravenously via the tail vein of mice that were killed 2 weeks later. Proliferative, inguinal, and axillary lymph nodes from one representative mouse are shown with no gross metastases. Microscopic metastases were not observed by routine staining with hematoxylin–eosin (data not shown). Increments of 1 mm are shown at the bottom of each panel (E and F). Representative lungs (2 weeks after injection) are shown from mice given an intravenous injection of 1 × 10⁶ pLNCX2-B16 (E) or 1 × 10⁶ CCR7-B16 (F) tumor cells. G) Lymph nodes from mice given an injection in the footpad with tumor cell lines, as in panels A–C, were pooled from each group of five mice, and RT–PCR was performed. Levels of tyrosine-related protein-1 (TRP) messenger RNA (mRNA) in lymph nodes from CCR7-B16-injected mice or CXCR5-B16-injected mice relative to that in pLNCX2-B16-injected mice were calculated as described in the “Materials and Methods” section. Horizontal bar = mean fold increase in TRP mRNA in the lymph nodes as indicated; 95% confidence intervals are shown with a vertical line.

Five experiments were performed with CCR7- and pLNCX2-B16-injected mice with five mice per group, and two experiments were performed with CXCR5-B16-injected mice. A single draining popliteal lymph node was removed from each experimental animal.

after seven passages, CCL21 induced a Ca²⁺ flux in these B16 melanoma cells (Fig. 3, B), indicating the presence of active CCR7. Thus, CCR7 expression enhanced the metastatic potential of B16 melanoma cells at both early and late times and was functionally maintained during the metastatic process.

Effect of Anti-CCL21 on CCR7-B16 Metastasis to Draining Lymph Nodes

To demonstrate that the metastasis of CCR7-B16 cells to draining lymph nodes was dependent on the CCR7/CCL21 pathway used by dendritic cells to migrate to draining lymph nodes (12,32), we repeatedly injected neutralizing antiserum CCL21 antibodies (12) or a control goat immunoglobulin G (IgG) into the mouse footpads containing CCR7-B16 cell implants. At the end of 10 days, metastases were detected in three of five lymph nodes from five IgG-treated mice, but none were detected in the lymph nodes from the five mice treated with anti-CCL21 antibodies (Fig. 4). Thus, inhibition of CCL21 blocks the metastasis of CCR7-B16 cells to draining lymph nodes.

DISCUSSION

Because activated dendritic cells home to lymph nodes through lymphatic vessels by a CCR7-dependent mechanism, we asked whether expression of CCR7 could play a role in tumor metastasis to regional lymph nodes by inserting the CCR7 gene into B16 melanoma cells. Migration of CCR7-B16 cells to regional lymph nodes was enhanced at both early and late times, and control experiments showed that CCR7 expression neither enhanced proliferation in general nor increased nonspecific metastasis to the lungs after intravenous inoculation. More important, inhibition of CCL21 with neutralizing antibodies blocked CCR7-mediated metastasis. Thus, to the best of our knowledge, our data are the first to demonstrate that induced expression of a chemokine receptor is sufficient to specifically alter the metastatic fate of cancer cells.

A number of genes are likely to be involved in metastasis, including genes that suppress metastasis (5,23) or genes that enhance metastasis, such as RhoC (a small guanosine triphosphatase) (6) or vascular endothelial growth-factor-C (7). Transcription factors, such as AP-2 (26) and CREB (27), may also be involved in the regulation of metastasis.

The role of chemokine receptors, however, in metastasis has been explored only recently. Chemokine receptors have been identified in human breast carcinoma lines that respond chemotactically to various chemokines in vitro (28). Furthermore, interleukin 8 has been reported to induce haptotactic migration of A2058 human melanoma lines (29). In a study of adult T-cell leukemia/lymphoma (ATLL) patients with and without lymph node involvement, Hasegawa et al. (16) showed that ATLL cells from patients with lymph node involvement had increased expression of CCR7 and enhanced functional responses to CCL21, suggesting that the expression of CCR7 may alter the trafficking patterns of ATLL cells by recruiting these cells via CCL21-rich high endothelial venules within lymph nodes. Finally, Müller et al. (17) showed functional expression of CCR7 and CXCR4 in breast cancer cells and of these receptors and also CCR10 (30) in several melanoma cell lines. Although the functional role of CCR7 was not explored, striking inhibition of breast cancer metastasis was obtained with a function-blocking anti-CXCR4 antibody but not with a nonbinding isotype control antibody, raising the
cultured in B16 clearly visible metastasis. A single lymph node with a metastasis and with 2×10^5 CCR7-B16 cells was injected at the same time with CCR7-B16 cells or 2×10^5 pLNCX2-transduced B16 cells (five mice group). Three experiments had metastases compared with one of 19 lymph nodes from control mice; that one lymph node had only a single visible metastasis. Thus, CCR7-B16-injected mice had 58% positive lymph nodes, whereas pLNCX2-B16-injected mice had 5% (P<.001, χ² test). A single lymph node with a clearly visible metastasis from CCR7-B16-injected mice was dissected, and its cells were cultured in B16 culture medium. After seven in vitro passages, the resulting melanoma cells (free of B or T cells) were loaded with fura-2 acetoxy methyl ester dye as described previously (31). After exposure to phosphate-buffered saline (PBS), CCL21 (500 ng/mL), or ionomycin (ION) (1 μg/mL, as a positive control), Ca²⁺ flux was assayed (31).

In summary, our data demonstrate that the expression of a single chemokine receptor gene (CCR7) by murine melanoma cells increases metastases to lymph nodes, raising the possibility that cancer cells may use normal mechanisms of lymph node homing for metastatic dissemination.

REFERENCES


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NOTES

H. E. Wiley and E. B. Gonzalez contributed equally toward this work.

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