CCL21 Chemokine Regulates Chemokine Receptor CCR7 Bearing Malignant Melanoma Cells

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ABSTRACT

Purpose: The chemokine CC-ligand 21/secondary lymphoid tissue chemokine (CCL21/SLC) regulates the homing of naïve T cells and dendritic cells that express CC-chemokine receptor 7 (CCR7) from distant sites to lymphoid tissue such as lymph nodes. We hypothesized that CCL21/SLC regulates the migration of CCR7-bearing melanoma cells from a primary lesion to regional tumor-draining lymph nodes.

Experimental Design: Quantitative real-time reverse transcriptase-PCR (qRT) assay and immunohistochemistry (IHC) were used to assess the level of CCR7 expression in melanoma cell lines and in primary and metastatic melanoma tumors. Cell migration assay using melanoma cell lines was performed under the induction of CCL21/SLC. The CCL21/SLC expression level in tumor-draining sentinel lymph nodes (SLNs) was assessed by both qRT assay and IHC.

Results: Melanoma cell lines and tumors demonstrated heterogeneous expression of CCR7 mRNA by qRT assay. There was strong functional correlation between CCR7 mRNA expression and cell migration induced by CCL21/SLC. IHC evidence of CCR7 expression in primary melanomas significantly (P = 0.02) correlated with Breslow thickness. Assessment of SLN from 55 melanoma patients by qRT assay demonstrated that CCL21/SLC mRNA expression level was significantly (P = 0.008) higher in pathologically melanoma-negative SLNs than in melanoma-positive SLNs.

Conclusions: This report demonstrates a potential mechanism for recruitment and homing of CCR7(+) metastatic melanoma cells to tumor-draining lymph nodes, which express CCL21/SLC. The study also suggests that lymph nodes bearing metastasis may suppress CCL21/SLC production.

INTRODUCTION

The metastatic potential of primary melanoma is considerably higher than that of other primary solid tumors when comparing the size of primary lesion. Cutaneous melanoma metastasizes frequently to regional tumor-draining lymph nodes, preferentially via the lymphatics. The first evidence of metastasis is often in the regional tumor-draining lymph node characterized by lymphatic mapping as the sentinel lymph node (SLN; Refs. 1–3). Invasion of primary tumor lesions by peritumoral or intratumor lymphatic vessels can facilitate migration of tumor cells to the SLN, but the mechanism by which melanoma cells metastasize to regional draining lymph nodes remains unclear. There is evidence that antigen-presenting cells such as dendritic cells (DCs), Langerhans cells (LCs), T cells, and natural killer cells bearing chemokine receptors migrate from skin to the draining lymph node in response to specific chemotactic factors referred to as chemokines (4–10). Chemokines have been hypothesized to recruit solid tumor cells to lymph nodes (11).

Chemokines, grouped into CXC and CC subfamilies based on the arrangement of the two NH2-terminal cysteine residues, are small secreted proteins that regulate the chemotactic response for a variety of cells (4, 9). These ligands and receptors have been predominantly investigated on lymphoid cells. Of particular interest is CC-ligand 21/secondary lymphoid tissue chemokine (CCL21/SLC), also referred to as 6Ckine or exodus, which is involved in recruiting CCR7(+) naïve T cells, natural killer, memory T cells, and DCs (4–6, 8–10). CCL21/SLC is constitutively expressed in the high endothelial venules (HEVs) of lymph nodes, Peyer’s patches, thymus, spleen, and mucosal tissue (8, 12). It has a high affinity for CCR7, a member of the seven transmembrane-spanning G protein-coupled receptor family (13–16). CCR7 is prevalent in various subsets of T cells and DCs (6, 14–17). The release of CCL21/SLC by HEV cells recruits CCR7(+) cells to draining lymph nodes (6, 10, 12, 13, 17). Abnormal expression of CCL21/SLC affects lymphocyte circulation and recruitment to lymph nodes. Lymphocytes and DCs of the DDD/1-plt/plt (paucity of lymph node T cells) mouse do not migrate into peripheral lymph nodes because these nodes express no detectable SLC (17). Antigen-stimulated lymph nodes are activated and express CCL21/SLC, which in turn can induce activation of CCR7(+) immune cells such as DCs and naïve T cells (8, 10, 16).

Recently, it has been shown that breast cancer cells that express CCR7 functionally respond to CCL21/SLC (18). We hypothesized that cutaneous melanoma cells, which express CCR7 functionally, respond to CCL21/SLC in a manner that facilitates metastasis of these cells from the primary site to the SLN. This study demonstrated expression of functional CCR7 in several human melanoma cell lines and in primary and metastatic melanomas. CCL21/SLC expression levels of SLN were investigated using quantitative real-time reverse transcriptase
CCL21/SLC Regulation of CCR7(+) Melanoma Cells

Table 1  CCR7 expression in human melanoma cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CCR7/GAPDH (×10^5)</th>
</tr>
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<tbody>
<tr>
<td>MA</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>MB</td>
<td>1.8</td>
</tr>
<tr>
<td>MC</td>
<td>2.0</td>
</tr>
<tr>
<td>MD</td>
<td>2.1</td>
</tr>
<tr>
<td>ME</td>
<td>4.1</td>
</tr>
<tr>
<td>MF</td>
<td>4.5</td>
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<tr>
<td>MG</td>
<td>5.8</td>
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<tr>
<td>MH</td>
<td>6.6</td>
</tr>
<tr>
<td>MI</td>
<td>13.6</td>
</tr>
<tr>
<td>MJ</td>
<td>26.7</td>
</tr>
<tr>
<td>MK</td>
<td>28.9</td>
</tr>
<tr>
<td>ML</td>
<td>43.5</td>
</tr>
<tr>
<td>MM</td>
<td>52.4</td>
</tr>
<tr>
<td>MN</td>
<td>165.5</td>
</tr>
<tr>
<td>MO</td>
<td>208.4</td>
</tr>
<tr>
<td>Peripheral blood lymphocytes donor 1^b</td>
<td>228,453.0</td>
</tr>
<tr>
<td>Peripheral blood lymphocytes donor 2^b</td>
<td>72,018.3</td>
</tr>
<tr>
<td>Mouse muscle</td>
<td>0</td>
</tr>
</tbody>
</table>

^a CCR7 mRNA copies per 250 ng of total RNA/GAPDH mRNA copies per 250 ng total RNA × 10^7.
^b Phytohaemagglutinin-stimulated.

RNA Isolation. Total cellular RNA, from melanoma cell lines and frozen or fresh primary and metastatic melanoma specimens, was extracted using Tri-Reagent (Molecular Research Center, Inc.) as described previously (19). For paraffin-embedded tissues, 10 sections of 8–10-μm thick tissues were cut from each specimen using a microtome and disposable sterile blade. The sections were placed in a sterile container for deparaffinization with xylene. Deparaffinized tissue sections were subjected to proteinase K digestion and RNA extraction using a modified protocol of the Paraffin Block RNA Isolation kit (Ambion, Austin, TX). Briefly, tissues were digested; RNA was solubilized in a guanidinium-based buffer, separated by phenol/chloroform, and precipitated by isopropanol. Pellet Paint (Novagen, Madison, WI) was used in the precipitation procedure to enhance the recovery of RNA. RNA extraction was performed in a designated sterile laminar flow hood using RNase/DNase-free plasticware. The RNA was quantified and assessed for purity by UV spectrophotometry and by the RiboGreen detection assay (Molecular Probes, Eugene, OR). The expression of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal reference housekeeping gene, was assessed by RT-PCR on all RNA samples to verify the integrity of RNA. Specimens with undetectable GAPDH mRNA expression were not used for additional RT-PCR analysis. Tissue processing, RNA extraction, and qRT assay set-up were performed in separate designated rooms to prevent cross-contamination, as described previously (3).

Primers and Probes. Using the Oligo Primer Analysis Software, version 6.0 (National Biomedical Systems, Plymouth, MN), we selected primer and probe sequences to optimally hybridize and amplify target cDNA for RT-PCR assay and qRT assay. To avoid possible amplification of contaminating genomic DNA, primers were designed so that each PCR product covered at least one exon-exon junction. The primers and FRET probe sequences used were as follows: CCR7, 5'-AACATATTGAAAGCCTGTGCTG-3' (forward), 5'-CGAACAAAGTGTAGTCCACTCTG-3' (reverse), and 5'-FAM-ATCTGTTGCCACCTCACTTCTGAC-BHQ1-3' (FRET probe); C2L1/SLC, 5'-CAAGACACCTCCACACA-3' (forward), 5'-TGTGACGCTCAGTCTCTC-3' (reverse), and 5'-FAM-TCCCTTTGTGCAGTCTGCCAGGAC-BHQ1-3' (FRET probe); CD105, 5'-AATGAGGCTGTTGCTCAATA-3' (forward), 5'-TGGAGGAAATGTTGGGCTGA-3' (reverse), and 5'-FAM-CACTGGTCCTCACAATGGAACC-BHQ1-3' (FRET probe); CD105, 5'-GGTTGTAAGAAGAGAAGT-3' (forward), 5'-GACTGTTGCATGAGTCCCT-3' (reverse), and 5'-FAM-CAGCAAGGCTCTCGTGCACCCACA-BHQ1-3' (FRET probe).

qRT Assays. All reverse transcriptase reactions were performed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with oligo-dT (GeneLink, Hawthorne, NY) and random hexamer (Roche, Chicago, IL) priming as described previously (3). The quantitative PCR assay was performed with the iCycler iQ RealTime PCR Detection system (Bio-Rad Laboratories, Hercules, CA); cDNA from 250 ng of total RNA was used for each reaction (21). The PCR reaction mixture consisted of 1 μM of each primer, 0.3 μM FRET probe, 1 unit of AmpliTaq Gold polymerase (Applied Biosystems, Branchburg, NJ), 200 μM of each deoxynucleoside triphosphate, 4.5 mM MgCl2, and AmpliTaq buffer to a final

Materials and Methods. Fifteen established cell lines from metastatic melanoma tumors (MA to MO; Table 1) from John Wayne Cancer Institute were grown to semiconfluency in flasks containing RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with heat-inactivated 10% FBS, penicillin G, and streptomycin (100 units/ml each) as described previously (19). Frozen and paraffin-embedded tissue from primary melanomas, regional nodal metastases, and distant metastases of patients treated at John Wayne Cancer Institute were obtained from Division of Surgical Pathology, Saint John’s Health Center. SLN specimens were also obtained in consultation with the surgeon and pathologist at the John Wayne Cancer Institute and Saint John’s Health Center. Informed human subject Institutional Review Board consent was obtained from patients for the use of all specimens. All SLN specimens were from patients who underwent lymphatic mapping and SLN dissection to stage clinically localized melanoma (2). Laser Capture Microdissection. Melanoma tumor cells were microdissected from pathology-verified metastatic lesions as described previously (20). Briefly, tissues were overlaid with TissueTek (Sakura Finetek, CA) and frozen in liquid nitrogen. Ten-μm thick sections were cut from individual frozen tissue specimens using a cryostat and mounted on RNase-free slide glasses. After staining with H&E, 1000 metastatic melanoma cells were microdissected for total RNA extraction using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). No contamination of DCs or lymphocytes in the specimens was verified by two of the authors (H. Takeuchi and M. Tanaka). The PixCell II LCM System (Artcurtus Engineering, Mountain View, CA) was used for microdissection of specific cells.
volume of 25 μl. For CCR7 analysis, samples were amplified with a preamplifying hold at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min for CCR7 and CD105, annealing at 58°C for CCL21/SLC, and annealing at 55°C for GAPDH and extension at 72°C for 1 min.

The standard curve for qRT analysis was established using nine serially diluted (10^3–10^8 copies) plasmids containing CCR7, CCL21/SLC, CD105, and GAPDH cDNA. Plasmids for individual gene cDNA were constructed as described previously (21). Restriction enzyme digestion and sequencing were performed to verify the products. PCR amplification of the serially diluted cDNA standard templates of each marker cDNA showed a logarithmic signal increase. The standard curve was generated by using the threshold cycle (Ct) of templates with known numbers of copies, and the mRNA copy numbers of the samples were calculated based on the standard curve by the iCycler iQ RealTime Detection System software (Bio-Rad Laboratories).

Positive and negative controls were included in each assay set-up. For a positive control of CCR7 expression, human peripheral blood lymphocytes (PBL) from healthy donors were stimulated with 5.0 μg/ml phytohaemagglutinin (Sigma Chemical Co., St. Louis, MO) for 3 days under culture conditions and harvested for RNA extraction. Mouse muscle tissues were used as a negative control for CCR7. Inflamed tonsil tissues were used as a positive control for CCL21/SLC and CD105 expression. Reagent controls (reagent alone without template) for PCR assays were included in each assay as described previously (21). Each assay was repeated at least twice to verify the results.

Cell Migration Assay. Cell migration assays were performed using 12-mm diameter transwell double chamber with 12-μm pore size (Costar, Cambridge, MA). The membrane was coated with 35 μg of Matrigel (Becton Dickinson, Franklin Lakes, NJ) for 2 h at room temperature. The lower chamber contained fibroblast basal medium (Clonetics, Walkersville, MD) with 2% heat-inactivated FBS. Melanoma cells were removed from the culture dishes using 0.0005% EDTA in PBS and washed twice with physiological PBS. Cells were resuspended in medium with 1% heat-inactivated FBS, and 2 x 10^4 cells/well were seeded into the upper chamber. Recombinant human CCL21/SLC (1 μg/ml; R&D Systems, Minneapolis, MN) was added to the lower chamber and incubated at 37°C in 5% CO2 for 12 h. After 12 h, nonmigratory cells on the upper membrane were removed with a cotton swab; cells that migrated on the lower surface of the membrane were fixed in 100% ethanol and stained with 1% crystal violet (Sigma) in 0.1% Triton x-100 and 2% ethanol. The number of stained cells in three randomly selected fields/membrane was counted with a Nikon LABOPHOT-2 microscope (×200 objective).

Immunohistochemistry. Expression of CCR7, CCL21/SLC, and endoglin (CD105) in tissues was assessed by IHC. Specimens were fixed in 10% formalin and paraffin embedded by conventional techniques. Five-μm sections were deparaffinized in xylene and then incubated with mouse antihuman CCR7 monoclonal IgM antibody (1:200; BD Biosciences) at 4°C overnight, goat antihuman 6Ckine IgG antibody (1 μg/ml; R&D Systems) at 4°C overnight, or rabbit antihuman endoglin polyclonal IgG antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Negative control slides were treated with nonimmunized immunoglobulin fraction under equivalent conditions and with no primary antibody. Paraffin-embedded human normal spleen tissues, which contained relatively abundant DCs, were used as a positive control for CCR7 staining. Spleen and tonsil tissues were used as a positive control for CCL21/SLC and endoglin staining (8, 22). For the secondary developing reagents, biotinylated antimouse or anti-goat immunoglobulin and Vectastain ABC kit (Vector Laboratories, Burlingame, CA) were used. Slides were developed with VIP reagent (Vector Laboratories) and counterstained with methyl green. The specimens were evaluated independently by two of the authors (H. Takeuchi and A. Fujimoto) in a blinded fashion without prior knowledge of the clinicopathology. The IHC results for CCR7 were arbitrarily classified into four scores dependent on the intensity of immunoreactivity: 0, negative immunostaining; 1+, weakly positive immunostaining; 2+, moderately positive immunostaining; and 3+, strongly positive immunostaining.

Statistical Analysis. Statistical analysis of the data were performed using the unpaired Student’s t test, Mann-Whitney U test and Spearman correlation coefficient analysis. P values were two-sided at which a value of < 0.05 was considered statistically significant.

RESULTS
CCR7 mRNA Expression in Melanoma Cell Lines. CCR7 mRNA expression levels were assessed by qRT assay in 15 melanoma cell lines (Table 1). The CCR7 mRNA expression levels ranged from 4 to 3360 copies/250 ng total RNA. Peripheral blood lymphocytes from healthy donors, used as positive controls, expressed high copy levels of CCR7 (Table 1). All of the cell lines were positive for GAPDH mRNA, showing high integrity of the mRNA copy number. The CCR7 mRNA copy level was normalized with GAPDH mRNA expression level to demonstrate the relative expression. CCR7:GAPDH mRNA ratio ranged from 0.4 x 10^-2 to 208.4 x 10^-2. Four CCR7 mRNA-positive cell lines (MK, MM, MN, and MO) and one CCR7-negative cell line (MA) were selected for the subsequent studies.

Effect of CCL21 on Melanoma Cell Migration. The chemotactic response of melanoma cells to CCL21 was assessed by using recombinant human CCL21/SLC in a transwell migration assay. CCL21/SLC significantly increased migratory response of all four CCR7(+) cell lines MK, MM, MN, and MO (P < 0.01; Fig. 1). In particular, migratory responses of MN and MO cells were 1.5–2-fold higher than their respective controls. Cells with higher CCR7 mRNA expression had greater response to CCL21/SLC. However CCL21/SLC did not induce migration on MA CCR7(-) cells. These results demonstrated the correlation between a melanoma cell’s CCR7 mRNA expression and migration in response to CCL21/SLC. These studies, therefore, verified that significant CCR7 expression on melanoma cells was functional. To confirm the activation of CCR7(+) melanoma cells by CCL21/SLC, actin filaments were stained with rhodamine-conjugated phalloidin and shown to undergo cytoskeleton rearrangement by confocal microscopy (data not shown).
Expression of CCR7 mRNA in Metastatic Melanomas. CCR7 mRNA expression levels in 11 frozen specimens of metastatic melanoma from eight patients were assessed using qRT assay. The specimens were obtained from metastatic melanomas, including tumor-draining lymph node metastases. Melanoma cells were carefully microdissected from frozen-fixed tumor sections using laser capture microdissection, and RNA was isolated and analyzed. Microdissected melanoma cells were verified as melanoma cells by reference stained adjacent tissue sections. CCR7 mRNA was detected in 9 of 11 (82%) of the samples. Positive specimens had copy numbers ranged from 89 to 3270 (median, 266) ng total RNA. All melanoma specimens had verification of mRNA quality through analysis of expression of the housekeeping gene GAPDH mRNA.

Immunohistochemical Analysis of CCR7 Expression in Primary Melanomas. We examined the presence of CCR7 protein in paraffin-embedded primary melanoma tissues from 22 patients using IHC. The patients ranged in age from 19 to 87 years (mean ± SD, 63 ± 19 years); there were 13 males and 9 females. CCR7 (−) staining DCs in tumor-free spleen and lymph nodes were used as positive controls. Melanoma tissues showed a variation in CCR7 immunoreactivity (Fig. 2, A and B). IHC scores classified by the intensity of immunoreactivity were as follows: 0, 7 cases; 1−, 5 cases; 2−, 9 cases; and 3−, 1 case. When divided into two groups, CCR7 (−) (immunoreactivity, 0) and CCR7 (+) (immunoreactivity, 1−3−), the mean primary tumor thickness (Breslow) in CCR7 (−) cases was significantly higher than that in CCR7 (−) cases (4.1 mm; median, 1.8 mm; P = 0.02). Four primary tumors were ulcerated; all four ulcerated primary tumors were CCR7 (−). The presence of CCR7 was also demonstrated in metastatic melanomas found in the SLN (Fig. 2, C and D). We confirmed the IHC results using another anti-human CCR7 antibody (R&D Systems; data not shown).

CCL21/SLC mRNA Levels in Lymph Nodes. Because HEV cells in lymph nodes are the primary source of CCL21/SLC, we investigated the expression level of CCL21/SLC in SLN of melanoma patients. The SLN was assessed because it is the first draining lymph node of the primary melanoma and the most likely site of regional nodal metastasis (2, 3). Fifty-five paraffin-embedded SLNs obtained from melanoma patients were assessed by the qRT assay. Thirty-two (58%) were histopathology tumor-free (IHC verified) lymph nodes, and 23 (42%) of 55 SLNs were pathology-verified micrometastatic (tumor diameter; <2.0 mm; Ref. 23) lymph nodes. Histopathology-positive nodes with micrometastatic disease were originally selected because macroscopic metastasis replaces normal cellular contents of the lymph node. CCL21/SLC mRNA was detected in 55 of 55 (100%) SLN specimens with copy numbers ranging from 1.5 × 104 to 149 (median, 10,665 copies)/250 ng total RNA from paraffin-embedded SLN (Table 2). All specimens were positive for GAPDH mRNA, showing high integrity of the mRNA extracted from SLN specimens. The mean CCL21/SLC mRNA copy number in histopathology melanoma-free SLNs was 28,141 ± 35,183 (median, 15,035). The mean CCL21/SLC mRNA copy level in histopathology melanoma-positive SLNs was 7578 ± 7966 (median, 3850). The mean CCL21/SLC mRNA copy level was significantly (P = 0.008) higher in histopathology melanoma-free SLNs than in melanoma-positive SLNs. When mRNA copy analysis was assessed as a ratio to the housekeeping gene, the results remained highly significant. The CCL21/SLC/GAPDH mRNA copy level ratio also was significantly (P = 0.007) higher in histopathology melanoma-free SLNs (ratio, 1.16 ± 1.97) than in melanoma-positive SLNs (ratio, 0.58 ± 1.17).

CCL21/SLC mRNA copy levels in SLN also correlated with Breslow thickness of the primary tumors (Table 2). The mean Breslow thickness was 1.82 ± 1.00 mm SD for these SLN patients (n = 55). The mean CCL21/SLC mRNA copy level in SLNs of patients with small noninvasive primary tumors (Breslow thickness ≤ 1.0 mm; n = 11) was 32,039 ± 44,907 (median, 11,900). In contrast, the mean CCL21/SLC mRNA copy level in SLNs of patients with more invasive primary tumors (Breslow thickness ≥ 3.0 mm; n = 9) was 5066 ± 5511 (median, 3620). These differences showed a strong trend (P = 0.06) that metastasis to the SLN suppressed CCL21/SLC.

We validated CCL21/SLC expression in SLNs using IHC (Fig. 3, A and B). CCL21/SLC expression was detected in the endothelial cells of small vascular structures and reticular stromal cells in subcapsular and T-cell areas of the interfollicular compartment. CCL21/SLC was not expressed in germinal centers. Expression of endoglin (CD105; Refs. 24–28), a cell surface molecule in the transforming growth factor β receptor complex and a specific marker of HEVs, was used to verify the CCL21/SLC expression in HEVs of SLNs (Fig. 3C). CD105 mRNA copy levels in the 55 SLNs were assessed and correlated with CCL21/SLC mRNA expression. CD105 mRNA was detected in 55 of 55 (100%) paraffin-embedded SLN specimens in copy numbers ranging from 2.95 × 104 to 39 (mean, 4595; median, 1930)/250 ng total RNA. Spearman correlation coefficient analysis revealed a significant correlation between CD105 and CCL21/SLC mRNA copy levels (correlation coefficient, 0.390; P = 0.004). However, CD105 mRNA expression levels in the SLN did not significantly correlate with lymph node metastasis or primary tumor Breslow thickness.
DISCUSSION

There is significant evidence that tumors of specific histology metastasize to specific organ sites that promote the establishment of metastasis (29–31). This preferential metastasis cannot be explained simply by the hematogenous/lymphatic drainage pattern from the tumor. Instead, metastasis involves a series of events that are sequentially activated (29, 32, 33). Through the decades, the “seed and soil” concept of tumor metastasis has often been revisited (31); another important factor referred to as the “calling signal” has also gained attention in elucidating the events of tumor metastasis following the recent discovery and investigation of the family of chemokine ligands and their respective receptors (4, 9). Recent evidence suggests that lymphoid cells are recruited to specific organ sites through a family of soluble factors referred to as chemokines (4, 9). The recent demonstration of specific chemokine receptors on tumor cells and response to respective chemokines has provided some insight into how tumor cells may home to specific organ sites. Chemokine receptors have been suggested to play a pivotal role in regulating recruitment of solid tumor cells to specific organ sites (18, 33).

The mechanism of recruitment of lymphoid cells during immunological activation is very efficient in bringing appropriate cells to a site for a specific function. CCR7 expressing DCs, natural killer cells, memory T cells, and naïve T cells respond to CCR21/SLC and are recruited to lymph nodes (8–10). To date, CCL21/SLC ligand is known to be produced predominantly by HEV cells lining the hematogenous and lymphatic vessels of lymph nodes (12, 34, 35). CCR7-bearing immune cells are known to be recruited to draining lymph nodes during a pathological insult to the local skin region such as inflammation, infection, or tumor development (10). Activated DCs express CCR7 and are trafficked and recruited to draining lymph nodes (10). Similarly, CCR7 (+) naïve T cells are recruited to lymph

Table 2 CCL21/SLC expression in melanoma sentinel lymph node (SLN)

<table>
<thead>
<tr>
<th>SLNs</th>
<th>Patients</th>
<th>CCL21/SLC mRNA copies</th>
<th>CCL21/SLC:GAPDH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (Range)</td>
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<tr>
<td>Histopathology(−) SLNs</td>
<td>32</td>
<td>15,035 (149–153,000)</td>
<td>1.16 ± 1.97</td>
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<tr>
<td>Histopathology(+) SLNs</td>
<td>23</td>
<td>3,850 (265–25,100)</td>
<td>0.58 ± 1.17</td>
</tr>
<tr>
<td>Primary tumor Breslow thickness</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>≥1.0 mm</td>
<td>11</td>
<td>11,900 (506–153,000)</td>
<td>1.07 ± 1.60</td>
</tr>
<tr>
<td>≥3.0 mm</td>
<td>9</td>
<td>3,620 (156–15,850)</td>
<td>0.43 ± 0.80</td>
</tr>
</tbody>
</table>

a $p = 0.008$.
b $p = 0.007$.
c $p = 0.063$.
d $p = 0.21$. 

Fig. 2 Representative immunohistochemistry staining for CCR7 protein expression in paraffin-embedded primary melanomas (A and B) and sentinel lymph node with metastatic melanoma (C and D). A, negative immunostaining (0); B, strongly positive immunostaining (3+); C, weak positive immunostaining (1+); D, negative control (same specimen as C). Photograph magnification, ×200.
nodes during immune stimulation where they differentiate into antigen-specific CCR7(−) CD4+ or CD8+ T cells (4, 8, 36). This mechanism is important for antigen presentation and activation of lymph node immune responses (10, 37). CCL21/SLC is a powerful homing molecule that allows recruitment and directed migration of CCR7(+) cells from long distances.

In melanoma patients the first draining lymph node(s) (SLN) is the first site of regional nodal metastasis (2). The presence of metastasis in the SLN is important for staging and prognosis (1, 2). We hypothesized that in early stages of regional node metastasis melanoma cells are recruited to the SLN by a mechanism similar to that governing recruitment of DCs/LCs from the skin to draining lymph nodes. Functional CCR7 on melanoma cells may play an important role in facilitating melanoma cell invasion and migration to draining lymph nodes in response to CCL21/SLC produced by HEVs in lymph nodes. We demonstrated that CCR7 was heterogeneous in expression in melanoma cell lines and tumors. We then showed that CCL21/SLC promoted migration of functional CCR7(+) melanoma cells. In addition, CCR7 expression in primary melanomas significantly correlated with Breslow thickness, which is one of the strongest prognostic factors for early-stage cutaneous primary melanoma. CCR7 expression may be a potential prognostic factor for melanoma patients. These findings support previous studies that breast cancer cell lines have functional CCR7 (18). Both primary melanoma and breast cancer preferentially migrate to the SLN (38, 39). CCR7 may facilitate lymphatic metastasis to lymph nodes. However, qRT and IHC studies demonstrated that CCR7 expression levels varied in individual tumor cell lines and tumor tissue, suggesting that CCR7 expression in melanoma cells may be affected by the environment or intrinsic gene regulatory mechanisms.

Our results indicated that CCR7(+) phenotype may be important for metastasis of tumor cells to the SLN. It is apparent that the CCR7(+) phenotype plays an important role for lymphoid organ metastasis through lymphatic vessels. Coincidentally, CCL21/SLC is highly expressed in lymph nodes compared with other organs whereby the ligand source is HEV cells (8, 12, 18). CCL21/SLC is also expressed in lymphatic vessels (12). Interestingly, CCL21/SLC mRNA expression level was significantly higher in pathologically melanoma-negative SLNs than in melanoma-positive SLNs. No previous studies have assessed CCL21/SLC mRNA expression quantitatively in lymph nodes. The activation of CCL21/SLC in HEVs is likely caused by lymph node stimulation. One hypothesis is that growth of the primary tumor produces a pathological insult that activates immune and inflammatory cells, leading to activation of CCL21/SLC in the SLN. It is known that morphology and cellular activity change significantly in activated lymph nodes compared with nonactivated nodes (40). The elevated number of activated DCs/LCs was shown in the paracortical region of proximal histopathology tumor (41, 42). Metastasis to the SLN is known to suppress DCs and T-cell activity (41, 42). This is compatible with our findings of higher levels of CCL21/SLC expression in SLNs of early-stage melanoma. The migration of DCs/LCs to the draining lymph nodes via CCL21/SLC is important for activating and coordinating the T-cell immune responses (10, 15, 37). However, there is likely to be a feedback mechanism by which CCL21/SLC production is inhibited in the lymph nodes to prevent additional recruitment of T cells and DCs/LCs. Metastatic cells in the SLN may also directly downregulate CCL21/SLC expression via immune suppressive fac-

Fig. 3 Representative immunohistochemistry staining for CCL21/SLC expression (A and B) and endoglin (CD105) expression (C) in paraffin-embedded sentinel lymph nodes. A, photograph magnification, ×40; B, photograph magnification, ×200; C, photograph magnification, ×100; left upper corner, ×200.
tors. Melanoma cells have been shown to suppress DC activity (43, 44). Additionally, our results suggest that metastasis to the SLN may suppress CCL21/SLC, which in turn is potentially responsible for suppressing recruitment of DCs and naïve T cells. The entire spectrum of effects of CCL21/SLC on CCR7(+) melanoma cells remain to be studied. Future studies will investigate the potential suppression of CCL21/SLC in lymph nodes with melanoma metastasis. Endoglin (CD105), a cell-surface antigen in the transforming growth factor β receptor complex, is expressed on human vascular endothelial cells (24, 25). Expression of endoglin by HEV cells may be related to CCR7(+) T-cell trafficking (26–28). Our qRT and IHC studies demonstrated that HEV cells, which expressed endoglin, also highly expressed CCL21/SLC in the SLN.

As with lymphoid cells, tumor cells express receptors and respond to ligands (45, 46). Nonlymphoid solid tumors may develop lymphoid cell-like properties and respond to hematopoietic associated ligands for activating invasion and migration. There is a strong likelihood that tumors of different histological origin will use specific chemokine receptors for homing to specific organ sites. Metastatic melanoma cells appear to use lymphoid properties to facilitate their spread to distant sites (33). Future studies will help unravel the role of CCR7(+) phenotype of melanoma cells in the metastatic cascade.

REFERENCES


