Expression of Chemokine Receptor CCR7 Is Associated with Lymph Node Metastasis of Gastric Carcinoma

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INTRODUCTION

The chemokines comprise a family of small basic chemotactic proteins that mediate their effects by binding to G-protein-coupled receptors (1). They were originally identified by their ability to direct extravasation of inflammatory cells. However, several chemokines have been found recently to be expressed constitutively in lymphoid tissues, suggesting that these chemokines might exhibit homeostatic functions by regulating lymphocyte-trafficking to or within lymphoid organs. Two constitutively expressed chemokines, CCL21/6Ckine and CCL19/ELC, share a common receptor, CCR7. CCL21/6Ckine is expressed in the high endothelial venules of lymph nodes and Peyer’s patches; in the T-cell zones of spleen, lymph nodes, and Peyer’s patches; and in the lymphoid endothelium of multiple organs. CCL19/ELC is expressed predominantly by stromal cells within the T-cell zones of lymph nodes, spleen, and Peyer’s patches. Both CCL21/6Ckine and CCL19/ELC had shown the most abundant expression in lymph nodes; however, quantitative analyses showed that CCL21/6Ckine was expressed at high levels, supporting its critical role in the homing of cells into lymph nodes (2, 3). The receptor for these ligands, CCR7, is expressed on all naive T cells, some memory T cells, B cells, and mature dendritic cells and plays a central role in lymphocyte trafficking and homing to lymph nodes (4, 5). On the basis of these findings, we hypothesized that cancer cells might use chemokine-mediated mechanisms during the process of lymph node metastasis of cancer cells such as those regulating lymphocyte trafficking. A recent study reported that high levels of expression of CXCR4 and CCR7 were found in breast cancer cells and the determination of the metastatic destination of tumor cells (3, 6). We reported previously the expression of CXCR4 in gastrointestinal carcinomas (7). In this study, we investigated the expression of CCR7 in gastric cancer cells and revealed its critical roles in metastasis of lymphoid organs by gastric cancer cells.

MATERIALS AND METHODS

Cell Lines. Gastric carcinoma cell lines AZ521, Kato III, MKN7, MKN28, NUGC3, and NUGC4 were supplied by the Japanese Cancer Research Bank (Tokyo, Japan). These cell lines were maintained in RPMI 1640 (Invifort Corp., Carlsbad, CA) containing 10% fetal bovine serum (Equitech-Bio, Ingram, TX), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Invifort Corp.).

Tumor Samples. Sixty-four tumor samples were immediately frozen in liquid nitrogen after surgical resection and kept at −90°C until RNA extraction. The surgical samples were obtained from the Department of Surgery, Medical Institute of Bioregulation, Kyushu University. Written informed consent for molecular analysis of surgical samples was obtained from all patients.

Expression of RNA and Semiquantitative RT-PCR Analysis. RNA extraction and cDNA synthesis was performed as described previously (8). The presence of CCR7 cDNA was detected by PCR amplification in separate reactions, using oligonucleotide primers reported previously. The primer sequences were 5′-TCTTTCTCATCAGCAAGCTGTC-3′ (forward) and 5′-GAGGCCGACCAGTTCTGGAAG-3′ (reverse) as described previously (9). To evaluate amplified products quantitatively by PCR, preliminary experiments were carried out to determine a suitable number of cycles in the linear range of PCR amplification in representative cases as the same methods as described previously (10). Then the proper number of cycles was chosen as 32, and PCR was performed at 96°C for 1 min, 58°C for 1 min, and 72°C for 1 min. PCR products were size-fractionated on 2% agarose gel and visualized with ethidium bromide staining. To confirm the specificity of the PCR products of the genes, we cloned PCR products into pCRII vector (Invitrogen) and subsequently sequenced the cDNA by the chain-termination DNA sequencing method and determined the nucleotide sequence of representative samples of PCR products and confirmed them to be identical to the expected fragments of cDNA in the CCR7 gene. The integrity of RNA was confirmed by performing PCR amplification of each cDNA with primers for the gene for glyceraldehyde-3-phosphate dehydrogenase. The primer sequences were 5′-GTCAACGGATTGGTGCTGATT-3′ and 5′-AGCTTCTCGGTTGGCAGTGAT-3′. Flow Cytometric Analysis. Flow cytometric analysis was conducted as recommended by PharMingen as follows. After incubating with 1% FCS-PBS for 1 h, 106 cells were labeled with anti-CCR7 mouse mAb (2H4; PharMingen, San Diego, CA), washed, sequentially incubated with FITC-conjugated goat antimouse IgM (PharMingen), and washed again. Ten thousand cells were collected for each sample using FACScan, and the data were analyzed with CellQuest software (Becton Dickinson, San Jose, CA).

Ca2+ Mobilization. Ca2+ mobilization in response to CCL21/6Ckine was performed as described (9). Briefly, the cells were loaded with Fluor-3-AM (Molecular Probes) for 30 min and warmed to 37°C before analysis of flow cytometry. The fluorescence intensity was followed kinetically after addition of CCL21/6Ckine on flow cytometer. To induce maximal Ca2+ release, cells were subsequently stimulated with 2.5 μg/ml ionomycin (Sigma).

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References

3 The abbreviations used are: RT-PCR, reverse transcription-PCR; mAb, monoclonal antibody; F-actin, filamentous actin.
Actin Polymerization Assay. Actin polymerization was tested as described previously (3, 11). Gastric cancer cells were incubated with CCL21/6Ckine (R&D Systems, Minneapolis, MN). At the indicated time points, cells were fixed, permeabilized, and stained in a solution containing 1-α-lyso-phosphatidylcholine, FITC-labeled phallolidin (both from Sigma Chemical Co., St. Louis, MO) and 1% formaldehyde in PBS. The cells were analyzed by flow cytometry, and all time points were plotted relative to the mean relative fluorescence of the sample before the addition of chemokine. For analysis by confocal microscopy, gastric cancer cells were preseeded and incubated with 200 nM CCL21/6Ckine or assay buffer RPMI 1640 with 0.5% BSA for 30 min. Cells were fixed, permeabilized, and stained with rhodamine-labeled phallolidin (Molecular Probes, Eugene, OR).

Chemotaxis and Invasion Assay. Migration and invasion assay was performed in 24-well cell culture chambers using inserts with 8-μm pore size (Becton Dickinson) as described previously (12). For invasion assay, used inserts were coated with Matrigel (100 μg/cm²; Becton Dickinson). Gastric carcinoma cells were suspended in the chemotaxis buffer (DMEM, 0.1% BSA, and 12 mM HEPES) at 5 × 10⁶/ml and added to inserts, which were transferred to wells containing buffer with or without CCL21/6Ckine. After incubation for 6 or 24 h for chemotaxis or chemoinvasion assays, respectively, cells on the lower surface of the membrane were stained and counted under a light microscope in five different fields (×200). Assays were performed in triplicate.

Immunohistochemistry. The primary antibody used in this study was 2H4, an anti-CCR7 murine mAb (PharMingen). Frozen tumor sections were thawed, washed in PBS, fixed in formalin, and incubated overnight at 4°C in the presence of 2H4. 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.

Clinicopathological Data. All data including sex, histology, depth of tumor invasion, lymph node metastasis, lymphatic invasion, vascular invasion, and disease stage were obtained from the clinical and pathologic records. Disease stage was classified according to the criteria proposed by the International Union Against Cancer (1997 edition). Tumors with or without expression of CCR7 genes were then compared.

Statistical Analysis. The statistical significance of differences was determined by the χ² test or Student’s t test. Next, stepwise multivariate regression analysis was performed using significant variables. Fs >4.0 were considered significant for determining a variable to be a final independent one. Survival curves were computed according to the method of Kaplan and Meier; for differences between curves, Ps were calculated using the log-rank test. P < 0.05 were considered significant.

RESULTS

Expression of CCR7: Cultured Cell Lines. To investigate mRNA expression of CCR7 in gastric cancer cell lines, RT-PCR analysis was performed. CCR7 was expressed in four (MKN7, MKN28, NUGC3, and NUGC4) of the six cell lines (66.6%; data not shown). PCR products were cloned and then sequenced to be confirmed as identical to the expected fragments of cDNA in the CCR7 gene, as described in “Materials and Methods.” Next, to examine expression of the CCR7 protein, flow cytometric analysis was performed using anti-CCR7 mAb. According to the results of RT-PCR, NUGC3 highly expressed CCR7, and both Kato III and AZ-521 did not express CCR7 on flow cytometric analysis (data not shown). NUGC4, MKN7, and MKN28 exhibited low expression of CCR7. NUGC3 was therefore used as a positive control, and Kato III was used as a negative control in each analysis.

Ca²⁺ Mobilization Induced by CCL21/6Ckine in CCR7-expressing Gastric Carcinoma Cells. Binding of chemokines to their receptors causes a characteristic increase in cytosolic calcium. This is one of the earliest biochemical events that occur in response to chemokines (3, 11). To examine intracellular calcium flux, we labeled NUGC3, a CCR7-positive gastric carcinoma, and Kato III, a CCR7-negative gastric carcinoma, with Fluo-3-AM before adding CCL21/6Ckine. Evaluation of the fluorescence of stimulated cells showed that only NUGC3 mobilized Ca²⁺ in response to CCL21/6Ckine (Fig. 1). This result indicated that CCR7 expressed in NUGC3 was the functional receptor, which responded to its ligand.
lymphocytic contamination was considered as quite low. Next, to deter-
ing lymphocytes in the gastric carcinoma tissues; therefore, the degree of
cells) were incubated with CCL21/6Ckine (200 nM) or PMA (100 ng/ml). At the indicated
ligand, CCL21/6Ckine. NUGC3 (CCR7-positive cells) and Kato III (CCR7-negative
signaling.
might play an important role in migration and invasion, mediated through
its signaling.
CCR7 Expression in Clinical Samples. We performed semiquan-
titative RT-PCR analysis of 64 tumor samples of gastric carcinoma to
examine mRNA expression of CCR7. As a result, 44 cases (68.8\%) expressed CCR7. In the most cases, there were very few tumor-infiltrat-
ing lymphocytes in the gastric carcinoma tissues; therefore, the degree of
lymphocytic contamination was considered as quite low. Next, to deter-
mine the distribution of CCR7-expressing cells in tumor tissues, we
performed immunohistochemical analysis of the 44 samples determined
to be CCR7-positive by RT-PCR analysis (Fig. 4, A and B). Forty-two samples exhibited immunoactivity for CCR7 in carcinoma cells, and 2 samples were immunostained only in the lymphocytes infiltrating tumor tissues. The staining, which was detected mostly in the membrane and also in the cytoplasm, appeared to be limited to carcinoma cells and lymphocytes. The normal gastric mucosa did not express the strong positive staining. CCR7-immunoreactive carcinoma cells were not detected in all of the 20 RT-PCR-negative samples (Fig. 4, C and D). According to the results of RT-PCR and immunohistochemical analysis, 42 of 64 (65.6\%) cases were CCR7 positive. We also performed immu-
nohistochemical analysis in metastatic lymph nodes and detected CCR7-
positive cancer cells (Fig. 4, E and F). In most of the metastasis-positive cases, we observed the heterogeneous expression pattern of CCR7 in primary lesion, whereas the homogeneous expression pattern was ob-
erved in the metastatic lesion.
Clinicopathological Features and CCR7 Expression. Several
pathological factors were compared in cases with or without CCR7 expression (Table 1). A significant correlation was observed between the two groups in the presence of lymph node metastasis ($P < 0.001$) and lymphatic invasion ($P < 0.001$). The CCR7-expressing cancers were
characterized by significant frequent lymphatic invasion and lymph node
metastasis. There were also relatively large differences in histological

Fig. 2. A, F-actin polymerization in NUGC3 (CCR7-positive cells) induced by its
ligand, CCL21/6Ckine. NUGC3 (CCR7-positive cells) and Kato III (CCR7-negative
cells) were incubated with CCL21/6Ckine (200 nM) or PMA (100 ng/ml). At the indicated
time points, cells were fixed, permeabilized, and stained with FITC-labeled phalloidin,
and the fixed cells were analyzed by flow cytometry. All time points are plotted relative
to the mean relative fluorescence of the sample before addition of CCL21/6Ckine. A
transient increase in intracellular F-actin was observed in NUGC3 cells within 15 s.
Similar results were obtained for three separate experiments. These results indicated that
the interaction between CCR7 and CCL21/6Ckine might induce an early event in the
migratory response of NUGC3 (CCR7-positive cells). B, confocal microscopy analysis
of polymerized F-actin in NUGC3 (CCR7-positive cells) and Kato III (CCR7-negative cells).
NUGC3 cells were stimulated with (a) or without (b) 200 nm CCL21/6Ckine and were
incubated for 30 min. Kato III cells were also stimulated with (c) or without (d)
CCL21/6Ckine. Cells were fixed and permeabilized, and F-actin in these cells was stained
with rhodamine-phalloidin and observed by confocal microscopy (×1000). After stimu-
lation with CCL21/6Ckine, in NUGC3 cells, intense F-actin staining was visible near the
periphery of the cells, and distinct pseudopodia formation was observed. However, these
changes were not observed in Kato III. These results suggested that the interaction
between CCR7 and its ligands might induce morphological changes needed for efficient
metastasis formation by NUGC3 (CCR7-positive cells).

Fig. 3. CCL21/6Ckine-mediated migration and invasion by CCR7-positive gastric
carcinoma cells. To assess the chemotactic responses of NUGC3 (CCR7-positive cells) or
Kato III (CCR7-negative cells) to different concentrations of CCL21/6Ckine, gastric
carcinoma cells (2.5 × 10^5) were placed onto the upper compartment with 8-μm pore size
membrane, and CCL21/6Ckine was added to the lower chamber at the indicated concen-
trations (A). Migration across the membrane was assessed after 6 h. B, gastric carcinoma
cells (2.5 × 10^5) were placed onto the inserts coated with Matrigel, and CCL21/6Ckine
was added to the lower chamber at the indicated concentrations. Invasion across the
membrane was assessed after 24 h. The cells on the lower side of the membrane were
counted by microscopy from five fields. Data are expressed as the mean number of
migrated or invading cells/well in a representative experiment performed in triplicate and
repeated three times with similar results; bars, SD.
type \((P < 0.05)\) and stage \((P < 0.05)\) between the two groups. Table 2 demonstrated the results of stepwise multivariate regression analysis including the significant risk factors of lymph node metastasis. Significant independent risk factors for lymph node metastasis were found to be expression of CCR7 and lymphatic invasion. We also examined the association between expression of CCR7 and prognosis of patients with gastric carcinoma. Survival curves plotted by the method of Kaplan-Meier are shown in Fig. 4G. Statistical analysis of the results by the log-rank (Mantel-Cox) test revealed that patients with CCR7-positive tumors had a significantly poorer prognosis than those with CCR7-negative tumors \((P < 0.05)\). These findings demonstrated that the expression of CCR7 plays a critical role in lymph node metastasis and in poor prognosis.

**DISCUSSION**

The molecular basis of tumor cell migration and metastasis to lymph nodes *in vivo* is not fully understood, although several internal molecules in tumor cells have been reported to influence the process of cell motility. Alteration in cell-cell adhesion and the secretion and activation of proteolytic enzymes are believed to be essential for optimal tumor cell invasion and migration through and across the extracellular barriers. In these aspects, several internal molecules have been reported to be associated with lymph node metastasis of gastric carcinoma, such as matrix metalloproteinase (14), p27 (15), and vascular endothelial growth factor C (16). Although external stimulants have also been believed to be essential for the process of forming metastasis, the interaction with chemotactic factors may represent a critical step in this process. In this study, we showed that human cell-derived chemokines attracted and activated cancer cells with their receptors and the presence of this rec-
tor, CCR7, and promoted cancer cell migration. We also showed that the presence of CCR7 promoted invasion of cancer cells toward lymph nodes. As a result, we found expression of the chemokine receptor CCR7 on ~60% of gastric carcinoma cells and characterized its role in migration and invasion to lymph nodes by in vitro assays. We also showed that ~60% of gastric carcinoma samples expressed CCR7 and found significant differences in clinicopathological features and prognosis among CCR7-positive and CCR7-negative samples.

To prove the functionality of CCR7 expressed in gastric carcinoma, we evaluated the intracellular calcium flow after stimulation of CCL21/6Ckine. Previous studies showed that CC chemokine induced a rapid transient increase in intracellular free calcium in dendritic cells and malignant cells (11). This is one of the earliest biochemical events in cells that occur in response to chemokines (3, 11). We found profound elevation of intracellular free calcium after stimulation by CCL21/6Ckine in CCR7-expressing gastric cancer cells, indicating that the CCR7 expressed in NUGC3 were functional receptors. Next, to determine the cell motility in response to CCL21/6Ckine, we performed staining of actin to observe actin polymerization in stimulated gastric carcinoma cells. In tumor cells, high levels of actin polymerization are required for the formation of pseudopodia, which is needed for the invasion of malignant cells into tissues and for the efficient metastasis formation (17). Chemokine ligand-receptor interactions trigger intracellular actin polymerization in leukocytes, a process that is required for cell motility and migration (13).

In our study, a transient increase in intracellular F-actin was detected in NUGC3 cells (CCR7-positive) stimulated with CCL21/6Ckine. Confocal laser scan microscopy also detected distinct pseudopodia formation after stimulation. We also showed that the migration and invasion capabilities of NUGC3 cells were increased by CCL21/6Ckine stimulation. In contrast, invasion capability was not increased for Kato III (CCR7-negative gastric carcinoma). A natural mutation in mice designated plt that results in the loss of one of the forms of murine CCL21 (2) and targeted disruption of the CCR7 gene causes impaired homing of T cells to secondary lymphoid organs (18), suggesting that the interaction between CCL21/6Ckine and CCR7 plays a critical role for lymphocytes to migrate to lymph nodes. These results of in vitro and in vivo experiments indicated that functional CCR7 were expressed in gastric carcinoma cells and might be relevant to the process by which gastric carcinoma cells preferentially migrate to lymph nodes and subsequently form lymph node metastases. In agreement with the findings obtained in these experiments, the following clinicopathological features differed significantly between CCR7-positive and -negative cases: (a) lymph node metastasis; (b) lymphatic invasion; (c) tumor histological type; and (d) overall survival. Moreover, stepwise multivariate regression analysis revealed that the most important factor affecting lymph node metastasis was the expression of CCR7, and we also detected CCR7-positive tumor cells in some metastatic lymph nodes.

Recent studies found that the chemokine receptor CXCR4 was highly expressed in breast and ovarian carcinomas, and the interaction between the receptor and its ligand, CXCL12/SDF-1, resulted in chemotaxis or directed migration of tumor cells from their primary site via the circulation to preferential sites of metastasis (3, 6, 19, 20). Wiley et al. (21) showed that expression of CCR7 enhanced metastasis of murine melanoma cells to draining lymph nodes in mouse models and that inhibition of CCL21/6Ckine blocked the metastasis to draining lymph nodes. These studies strongly supported our hypothesis. The interaction between CCR7 and CCL21/6Ckine may play crucial roles in the metastasis of cancer cells by direct effects on tumor cell migration and invasion.

In conclusion, the chemotactic interaction between CCR7 and its ligand, CCL21/6Ckine, may be a potent mechanism for induction by cancer cells of lymph node metastasis and tissue invasion. This hypothesis was supported by the findings that expression of CCR7 was observed in ~60% of gastric carcinoma tissues and was significantly correlated with the presence of lymph node metastasis and lymphatic invasion. These findings and those of previous studies suggest that CCR7 could be associated with lymphatic invasion and metastasis of gastric carcinoma. Recently, several antagonists of chemokine receptors have been identified (22). Our results suggested that an antagonist of CCR7 might be useful in controlling lymph node metastasis by gastric cancer cells.

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