The Chemokine (CCL2–CCR2) Signaling Axis Mediates Perineural Invasion

Shizhi He1,2, Shuangba He3, Chun-Hao Chen1, Sylvie Deborde1, Richard L. Bakst4, Natalya Chernichenko1, William F. McNamara1, Sei Young Lee1, Fernando Barajas1, Zhenkun Yu5, Hikmat A. Al-Ahmadie6, and Richard J. Wong1

Abstract

Perineural invasion is a form of cancer progression where cancer cells invade along nerves. This behavior is associated with poor clinical outcomes; therefore, it is critical to identify novel ligand–receptor interactions between nerves and cancer cells that support the process of perineural invasion. A proteomic profiler chemokine array was used to screen for nerve-derived factors secreted from tissue explants of dorsal root ganglion (DRG), and CCL2 was identified as a lead candidate. Prostate cancer cell line expression of CCR2, the receptor to CCL2, correlated closely with MAPK and Akt pathway activity and cell migration towards CCL2 and DRG. In vivo nerve and cancer coculture invasion assays of perineural invasion demonstrated that cancer cell CCR2 expression facilitates perineural invasion. Perineural invasion is significantly diminished in coculture assays when using DRG harvested from CCL2−/− knockout mice as compared with control CCL2+/+ mice, indicating that CCR2 is required for perineural invasion in this murine model of perineural invasion. Furthermore, 20 of 21 (95%) patient specimens of prostate adenocarcinoma with perineural invasion exhibited CCR2 expression by immunohistochemistry, while just 3 of 13 (23%) lacking perineural invasion expressed CCR2. In summary, nerve-released CCL2 supports prostate cancer migration and perineural invasion though CCR2-mediated signaling.

Implications: These results reveal CCL2–CCR2 signaling as a key ligand–receptor mechanism that mediates cancer cell communication with nerves during perineural invasion and highlight a potential future therapeutic target. Mol Cancer Res; 13(2); 380–90. ©2014 AACR.

Introduction

Prostate cancer currently is the most common cancer and the second leading cause of cancer-related death in American men (1). Mortality in prostate patients with cancer is generally attributable to extracapsular spread, which often results in treatment failure and is associated with poor prognosis (2). Perineural invasion is defined as the invasion of cancer cells in, around, and through nerves (3). Multiple studies have shown an association between the presence of perineural invasion in prostate cancer and both higher pathologic stage and Gleason score (4). Perineural invasion is highly prevalent in prostate cancer, observed in as many as 80% of resected prostate cancer specimens and in 20% of biopsies from patients without lymph node metastases (5–7).

Current theories suggest that perineural invasion is a key route of extracapsular spread in prostate cancer (8). The molecular mechanisms underlying perineural invasion remain poorly understood. Recent theories have suggested that nerve microenvironment may release chemotactic factors that attract cancer cells to migrate towards nerves (9–11). Our group recently demonstrated that glial-derived growth factor (GDNF), secreted by nerves, induces pancreatic cancer cell migration and perineural invasion through RET receptor phosphorylation and downstream MAPK signaling (12). These findings implicate GDNF-RET signaling as playing a central role in the process of pancreatic cancer perineural invasion. However, in these studies, the inhibition of GDNF secretion by nerves only partially blocks cancer cell perineural invasion, suggesting that other unidentified ligand–receptor interactions are also playing a role. Furthermore, when radiation is used to disrupt nerves and nerve supporting cells, perineural invasion can only be partially reconstituted by the addition of exogenous GDNF (13). These results suggest that other nerve-secreted factors likely play a contributing role in the process of perineural invasion. Other studies have also suggested that a variety of other ligand–receptor interactions play a role in perineural invasion (3, 9).

Chemokines are a family of small soluble proteins that regulate cell migration through the formation of concentration gradients (14). These proteins exhibit a high degree of conservation between mice and humans and are critical mediators of immune cell trafficking during embryonic development, wound healing, and infection (15). Interestingly, chemokines and their receptors have been implicated in tumor growth and invasion (16–18). Chemokines may elicit cancer cell mobilization and promote...
distant metastasis with organ selectivity (19–21). The chemokine receptor CXCR4 is increased in metastatic prostate cancer as compared with localized prostate cancer (22), and CXCR1 is correlated with more aggressive prostate cancer of higher Gleason score and lymph node metastases (23). CCL2–CCR2 signaling may also stimulate prostate cancer cell migration through a layer of bone marrow endothelial cells (24).

In this study, we used a chemokine profiling array to screen factors produced by dorsal root ganglion (DRG) that may potentially mediate perineural invasion. We use DRG as part of an in vitro coculture assay with cancer cells as a model of perineural invasion. The screen identified chemokine (C-C motif) ligand 2 (CCL2) as a chemokine that is expressed by DRG. Interestingly, CCL2 has been shown to be a critical modulator of inflammation, regulating monocyte recruitment during wound healing, infections, and autoimmune diseases. Chemokine (C-C motif) receptor 2 (CCR2), the receptor to CCL2, may be expressed in prostate and other cancer cell lines. These results prompted us to evaluate the role of the CCL2–CCR2 signaling in prostate cancer cells during the process of perineural invasion.

Materials and Methods

Cell lines and mice

A panel of human carcinoma cell lines was purchased from the ATCC, including prostate carcinoma (PC-3, DU145, LNCaP), pancreatic adenocarcinoma ( MiaPaCa2, Panc1), head and neck squamous cell carcinoma (SCC25), and thyroid medullary carcinoma (TIT) cell lines. A lung mucoepidermoid carcinoma cell line (H292) was a gift from Dr. Frederic Kaye (National Cancer Institute, Bethesda, MD). PC-3, DU145, MiaPaCa2, Panc1, SCC25, and H292 were grown in vitro in DMEM. LNCap was grown in RPMI1640, and TT was grown in F-12K. Cells were grown in 10% fetal calf serum (FCS) with penicillin and streptomycin and incubated in 5% CO₂ at 37°C.

All mouse studies were performed in accordance with institutional protocol guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY). Mice were maintained according to NIH Animal Care guidelines, under protocols approved by the MSKCC Institutional Animal Care Committee. Athymic nude mice were purchased from Harlan Laboratory, and Balb/c mice from Charles River Laboratory. C57BL/6 mice were purchased from The Jackson Laboratory. C57BL/6 CCL2−/− mice were obtained from Dr. Eric G. Pamer (Memorial Sloan-Kettering Cancer Center, New York, NY; ref. 25). DRG were isolated from mice as previously described (26).

Reagents

Recombinant human CCL2 was purchased from R&D Systems. A rabbit polyclonal anti-CCL2 antibody for Western blotting was purchased (ab7202, Abcam). A monoclonal anti-CCL2 antibody was obtained for neutralization of CCL2 (MAB479, R&D Systems). A rabbit polyclonal anti-CCR2 antibody for Western blotting was purchased (ab21667, Abcam). A rabbit monoclonal anti-CCR2 antibody was obtained for immunohistochemistry (E68, Novus Biologicals). Anti-GAPDH antibody was purchased from EMD Millipore. Anti-pMEK1/2, anti-MEK1/2, anti-pAkt, and anti-Akt antibodies were obtained from Cell Signaling Technology. Alexa Fluor 568 Goat Anti-Rabbit IgG (H+L) was purchased from Life Technologies. Growth factor–depleted Matrigel matrix was purchased from BD Biosciences.

Chemokine array

Chemokines secreted by DRG were screened using a Proteome Profiler Mouse Chemokine Array Kit (R&D Systems) according to the manufacturer’s instructions. The array consists of 25 different mouse chemokines spotted in duplicate onto four membranes. Conditioned media using DMEM without FCS was collected after 24 hours of exposure to 7-day-old murine DRG explants. Array membranes were incubated for 1 hour in blocking buffer and then incubated overnight with 1 ml of conditioned media or DMEM with no FCS as control, and then combined with the detection antibody cocktail. The membranes were washed and incubated with streptavidin–horseradish peroxidase and Chemi Reagent Mix.

Boyden chamber migration assays

Polycarbonate transwell inserts with 8.0 μm pores (BD Biosciences) were used in 24-well plates for migration assays. Cells were grown in 0.1% FCS media overnight and 5 × 10⁴ cells were added in 0.5 ml of media into each insert. Media (0.7 ml) with 0.1% FCS were added to each of the lower wells using CCL2 (0, 10, or 25 ng/ml) or DRG explants as attractants. In additional experiments, DRG were treated with anti-CCL2 antibody (1 or 10 μg/ml) or with 10 μg/ml isotype IgG as a control. The inserts were removed after 18 hours. Nonmigrating cells were wiped off from the superior aspect of the membranes with a cotton swab. Migrating cells on the undersurface of the membrane were fixed in 100% alcohol for 10 minutes and stained with 1% methylene blue in 1% borax for 20 minutes. Membranes were excised and mounted on glass slides. Cells were counted at five high-power fields (×200) at predetermined areas of the membrane.

Western blotting

Cells or DRG from CCL2−/− and CCL2+/+ mice were placed in serum-free media for one day. Cells were treated with CCL2 (25 ng/ml) for varying periods. Cell lysates were prepared using IP lysis buffer (Thermo Fisher Scientific). DRG were lysed using neuronal protein extraction reagent (Thermo Fisher Scientific). Conditioned media were collected from the DRG culture with or without anti-CCL2 antibody (1 or 10 μg/ml) after 15 minutes. All samples were measured for total protein content using a Bradford assay (Bio-Rad) to insure equal loading. Protein was subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in 0.1% Tween in Tris-buffered saline (Bio-Rad), and incubated with primary antibody (CCR2 antibody at 1 μg/ml) overnight followed by a secondary antibody conjugated to horseradish peroxidase. Protein–antibody complexes were exposed to light-sensitive film using an ECL Plus Detection System (GE Healthcare BioSciences). GAPDH was used as a loading control.

Lentiviral shRNA transfection

MISSION shRNA bacterial glycerol stocks containing four different constructs targeting CCR2 were purchased from Sigma-Aldrich. Plasmids were packaged with MISSION Lentiviral Packaging Mix and transfected using Lipofectamine 2000 Transfection Reagent (Life Technologies) into HEK293T cells (ATCC). After transfection, media were changed and lentivirus was collected 48 hours later and filtered through 0.45 μm filters. Cells were infected with lentivirus in the presence of polybrene (8 μg/ml). Forty-eight hours later, cells were treated with 1 mg/ml
of puromycin to select resistant clones. MISSION shRNA Control Transduction Particles (SHC001V) were used as a control. The following shRNA targeting CCR2 were tested: sh1676 5'-CCG GCC CAC GAA TCA TCT TTA CTA ACT CGA CTT AGT AAA GAT GAT TCC TGG GTT TTT-3'; sh1677 5'-CCG GCC GCC TCC TCT TAA TAA ACT GCA ACT CGA GTT GCA GTT TAT TAA GAT GAG GTT TTT-3'; sh1678 5'-CCG GCC TCC TCT TAA TAA ACT GCA ACT CGA GTT GCA GTT TAT TAA GAT GAG GTT TTT-3'; sh1679 5'-CCG GCC CAG AAA GAA GAT TCT GTT CGA GAA ACA GAA TCT TTC TCT TGG CTT TTT-3'.

**In vitro DRG coculture model of perineural invasion**

The *in vitro* DRG coculture model of perineural invasion is based on a technique originally described by Ayala and colleagues (27), and refined by our group (12, 13). Excised murine DRG are implanted in the center of a 10 μL drop of growth factor–reduced Matrigel (BD Biosciences). PC-3, shControl PC-3, and shCCR2 PC-3 cells were labeled with 25 μmol/L of the fluorescent CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes, Invitrogen) for 1 hour at 37°C. Areas of nerve invasion, defined as fluorescent cancer cells in association with DRG neurites, were outlined and quantified by MetaMorph software (MetaMorph 7.7.4; Molecular Devices).

**In vivo model of sciatic nerve invasion**

Nude athymic mice were anesthetized with isoflurane (5% for induction, 2% for maintenance; Baxter Healthcare Corp.) and their right sciatic nerves were surgically exposed. Ten mice were distributed randomly into two groups. shControl PC-3 cells (2 x 10^6) and shCCR2 PC-3 cells (6 x 10^5) in 3 μL volume of PBS were microscopically injected into the distal sciatic nerve, under the epineurium, using a 10 μL Hamilton syringe as previously described (12, 13). Additional mice underwent sciatic nerve injection with PBS as non–tumor bearing controls. The sciatic nerve innervates the hind limb paw muscles. Sciatic nerve function was measured weekly as described previously (12, 28) using the following: (i) sciatic neurological score, which grades hind limb paw response to manual extension of the body, from 4 (normal) to 1 (total paw paralysis) and (ii) sciatic nerve function index, which measures the spread width between the first and fifth toes of the hind limbs. These measures were normal in all mice immediately after surgical implantation of the cancer cells.

**MRI assessment of *in vivo* perineural invasion**

Murine sciatic nerve tumors generated from shControl PC-3 cells and shCCR2 PC-3 cells were assessed by MRI. A Bruker USR 4.7T 40 cm bore scanner (Bruker Biospin MRI GmbH) equipped with a 400 mT/m 12 cm bore gradient, using a custom-designed active decoupled radiofrequency surface coil (Stark MRI Contrast Research) was used. After the mice were anesthetized with isoflurane, sciatic nerves were localized by a scout fast spin-echo scan in three orientations, followed by a coronal T2-weighted fast spin-echo image acquired with: TR/TE 1.9 s and 40 ms, 117 x 186 μm in-plane resolution, 16 slices of 0.8 mm slice thickness and 12 averages. Images were used to visually assess the caliber of the sciatic nerve as it courses proximal to the primary tumor invasion site. Sciatic nerve invasion length based on MRI imaging was assessed. Images showing a thickened sciatic nerve were analyzed with ImageJ.

**Histology of perineural invasion by prostate cancer in vivo**

Sciatic nerve and tumor specimens were excised from mice, frozen in Tissue-Tek O.C.T. (optimum cutting temperature) compound (Sakura Finetek), and cut into 8 μm thick sections on glass slides. Slides were fixed and stained with hematoxylin and eosin. To assess CCR2 expression, immunofluorescence staining of adjacent cuts was performed at a dilution of 1:100. Slides were fixed with 2% formaldehyde and 0.2% glutaraldehyde, quenched, blocked, and incubated with primary antibodies overnight at 4°C. Slides were incubated with Alexa Fluor 568 Goat Anti-Rabbit IgG at a 1:500 dilution for 1 hour at room temperature, washed again, and treated with 5 μg/mL DAPI (4', 6-diamidino-2-phenylindole) solution for 5 minutes at room temperature. Images were captured on a Zeiss microscope (Zeiss LSM510 Inverted Confocal, AxioVert 200M) and acquired by Mirax slide scanner (20 x/0.8 NA objective) using Mirax scan software (Carl Zeiss).

Five human prostate cancer specimens exhibiting perineural invasion were formalin fixed and paraffin embedded and cut into 4 μm thick sections. A Ventana XT platform was used for immunohistochemistry, using a primary anti-CCR2 antibody (E68, Novus Biologicals) at 1:400 dilution, and a 1-hour incubation period. Detection was performed with the DAB Map Ventana detection system.

**Statistical analysis**

A Student t test was used for statistical analysis as appropriate. All P values were calculated using two-sided tests. Statistical significance was determined as *P* < 0.05. Error bars in the graphs represent SEM.

**Results**

**Dorsal root ganglia express CCL2**

To identify chemokines produced by DRG, which support cancer perineural invasion in our *in vitro* coculture assays, we used a chemokine array kit to screen conditioned media from DRG cultures. Of 25 chemokines included in the array, we identified the overexpression of six: CCL12, CCL6, chemerin, IL16, CXCL1, and CCL2 (Fig. 1A). Densitometry analysis of the chemokine array signal was performed using ImageJ (rsweb.nih.gov/ij) and normalized to the average intensity of the 3 controls (Supplementary Fig. S1). CCL12 and CCL6 were excluded, as they have been identified only in rodents. Of the remaining candidates, chemerin is an adipokine that regulates adipogenesis and adipocyte metabolism, while IL16 is a T-lymphocyte attractant. CXCL1 is a chemokine that recruits neutrophils and has been implicated in melanoma pathogenesis. CCL2 is a chemokine involved in macrophage recruitment, inflammation, and may also induce prostate cancer migration. CCL2 was selected for further evaluation. CCL2 expression by DRG was validated by Western blot analysis (Fig. 4A).

**Expression of CCR2 by cancer cell lines correlates with CCL2-induced cell migration**

CCL2 exhibits strong binding affinity to the chemokine receptor CCR2, and signals primarily through CCR2 in monocytes and macrophages to regulate migration. We assessed CCR2 expression in the cancer cell lines PC-3, DU1145, LNCap, MiaPaCa2, Panc1,
Western blot analysis con-

migrated successful depletion of CCL2 protein by shRNA1678, as compared with three other hairpins targeting CCR2 and shControl (Fig. 2A). Therefore, shRNA1678 was selected to silence CCR2 in PC-3. There were no significant differences in cell proliferation between shControl PC-3 and shCCR2 PC-3 over 4 days, although by later days, the shControl cells exhibited a more rapid growth (Fig. 2B). As migration assays were concluded by 18 hours, these findings eliminate a proliferation deficit of shCCR2 cells as a confounder in these studies. In migration assays, shControl PC-3 cells remain responsive to CCL2 in a dose-dependent manner (Fig. 2C). In contrast, shCCR2 PC-3 cells demonstrated a lack of migration in response to CCL2 stimulation (Fig. 2D).

When live explants of DRG in Matrigel were used to attract PC-3 (Fig. 2E), far more robust migration was than with CCL2 alone (Fig. 2C). We tested whether antibody inhibition of CCL2 secreted from the DRG could inhibit migration of prostate cancer cells. Adding anti-CCL2–neutralizing antibody at 10 μg/mL to the DRG in the lower chamber reduced PC-3 migration by 33% (Fig. 2E), suggesting a significant role of CCL2 signaling in mediating prostate cancer cell migration towards nerves. The addition nonspecific rat IgG at 10 μg/mL showed no inhibitory effects, in contrast to the anti-CCL2 antibody (Supplementary Fig. S2). The partial inhibition with the anti-CCL2 antibody suggests either incomplete CCL2 inhibition by this antibody, or the presence of factors other than CCL2 secreted by the DRG that may support PC-3 migration. We next used DRG as an attractant for shControl and shCCR2 PC-3 cells. The loss of CCL2 expression reduced PC-3 cell migration toward nerves by 43% (Fig. 2F), which is slightly more pronounced than the migration reduction noted by the blocking anti-CCL2 antibodies (Fig. 2E). These findings suggest that CCL2 plays a significant, though partial, role in inducing PC-3 cell migration towards DRG.

CCL2 activates the MAPK and Akt pathways in prostate cancer

We sought to identify potential signaling pathways involved in CCL2-induced cell migration. CCL2 phosphorylates Akt in PC-3 cells in protecting cells against autophagy (29). We demonstrate that p-MEK1/2 and p-Akt expression are both increased in PC-3 cells by 15 minutes after exposure to CCL2 at 25 ng/mL (Fig. 2G). To validate this effect using DRG, PC-3 cells were exposed to conditioned media collected from DRG, with or without anti-CCL2 antibody, and then underwent protein isolation. Both conditioned media showed no inhibitory effects, in contrast to the anti-CCL2 antibody (Supplementary Fig. S2). The partial inhibition with the anti-CCL2 antibody suggests either incomplete CCL2 inhibition by this antibody, or the presence of factors other than CCL2 secreted by the DRG that may support PC-3 migration. We next used DRG as an attractant for shControl and shCCR2 PC-3 cells. The loss of CCL2 expression reduced PC-3 cell migration toward nerves by 43% (Fig. 2F), which is slightly more pronounced than the migration reduction noted by the blocking anti-CCL2 antibodies (Fig. 2E). These findings suggest that CCL2 plays a significant, though partial, role in inducing PC-3 cell migration towards DRG.

C2021 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from mcr.aacrjournals.org on September 14, 2021. © 2015 American Association for Cancer Research.
CCL2 is required for CCL2-induced cancer migration, and CCL2 released by DRG induces cancer migration. A, expression of CCR2 was measured in PC-3 cells after undergoing transfection with a panel of shRNAs targeting CCR2, or control shRNA. The 1678 sh hairpin was used to create stably silenced shCCR2 PC-3 cells. B, cell proliferation of shControl and shCCR2 PC-3 cells is similar through 4 days, followed by a mildly increased rate by the shControl cells. C. Boyden chamber migration assays over 18 hours were performed using CCL2 as an attractant for shControl PC-3 cells, which show dose-response migration. (t, P < 0.05; t test). To exclude the possibility of cancer cells randomly adhering to or invading the Matrigel, we seeded shControl and shCCR2 PC-3 cells on Matrigel without any DRG, as a negative control (Fig. 3A and B).

**CCL2 release by DRG facilitates prostate cancer perineural invasion in vitro**

To confirm that CCL2 released from DRG is inducing migration and perineural invasion, we obtained CCL2+/− homzygous deceased mice. Western blot analysis confirmed lower CCL2 expression by the DRG of CCL2−/− mice as compared with the DRG from wild-type CCL2+/− mice (Fig. 4A). Cultured DRG from both wild-type and CCL2−/− mice generate equitable neurite areas (Supplementary Fig. S4B), although the CCL2−/− DRG occasionally fail to grow in culture. Boyden chamber assays were performed with PC-3 cells using CCL2−/− DRG or CCL2+/− DRG as a chemoattractant. Quantification demonstrated a 35% reduction quantifying cancer–nerve interactions. In this model, surgically excised murine DRG are grown in a drop of growth factor-depleted Matrigel, and fluorescent cancer cells are subsequently added to the media. Cancer cells may then invade into the Matrigel, attach to the DRG neurites (axonal-like projections), and migrate towards the center of the DRG along these neurites (12, 13) in a model of perineural invasion (Supplementary Fig. S3A). The fluorescent cancer cells which extend along neurites may be outlined, and the area measured with Meta Morph software (Fig. 3C–E).

We found a significant reduction of the area of perineural invasion by the shCCR2 PC-3 cells as compared with shControl PC-3 cells (Fig. 3A, B, and F; P < 0.05, t test). To exclude the possibility of cancer cells randomly adhering to or invading the Matrigel, we seeded shControl and shCCR2 PC-3 cells on Matrigel without any DRG, as a negative control (Fig. 3A and B).
of migrating PC-3 towards CCL2−/− DRG as compared with CCL2+/+ DRG (Fig. 4B; \( P < 0.05 \), t test). We then performed DRG coculture assays to assess for perineural invasion in vitro. DRG harvested from CCL2+/+ and CCL2−/− mice exhibited similar morphology and neurite growth patterns. However, the area of nerve invasion by the PC-3 cells was reduced by 58% in the CCL2−/− DRG group, as compared with the CCL2+/+ DRG group, by day 4 of the coculture assay (Fig. 4C–E; \( P < 0.05 \), t test). These
results demonstrate that CCL2 expression by nerves facilitates both prostate cancer cell migration and perineural invasion.

**CCR2 expression facilitates prostate cancer perineural in vivo**

We next assessed the contribution of prostate cancer CCR2 expression in perineural invasion using a murine in vivo model of sciatic nerve invasion. Sciatic nerve tumors generated from shControl PC-3 cells grew more rapidly than those generated from shCCR2 PC-3 cells, and shControl tumors at week 5 were of comparable volume to shCCR2 tumors at week 7 (Fig. 6A). To control for this growth differential, we compared perineural invasion between these groups at these two different time points, when overall tumor volume was equitable.

Functional assessment demonstrated that shControl tumors developed progressive, complete, ipsilateral hind limb paralysis over 5 weeks (Figs. 5B, 6B, and C). In contrast, mice injected with shCCR2 PC-3 cells maintained intact sciatic nerve function over 5 weeks, with minimal additional functional deficits noted by 7 weeks (Figs. 5C, 6B, and C). MRI of shControl tumors showed anatomically thickened sciatic nerves at week 5 extending proximal to the primary tumors toward the spinal cord, consistent with perineural invasion (Fig. 5E). In contrast, MRI imaging of shCCR2 tumors showed preservation of thin proximal sciatic nerves at week 7 (Fig. 5F), similar to those of normal mice lacking any tumor (Fig. 5A and D). Sciatic nerve invasion length was significantly greater for shControl tumors as compared with shCCR2 tumors ($P < 0.05$; Supplementary Fig. S5).

Clinical evaluation at the time of surgical exploration confirmed the MRI findings, demonstrating thickened and infiltrated proximal sciatic nerves for the shControl tumors at week 5 (Fig. 5H), as compared with normal appearing proximal sciatic nerves for the shCCR2 tumors at week 7 (Fig. 5I) and normal sciatic nerves in non-tumor bearing animals (Fig. 5G). Histologic evaluation of excised sciatic nerves demonstrates extensive cancer...
CCR2 is expressed in human prostate cancer specimens with perineural invasion

We assessed 24 human prostatectomy specimens, in which there were 34 distinct prostate adenocarcinomas that were spatially separated by normal prostatic tissue. These specimens were assessed with hematoxylin and eosin (H&E) staining and CCR2 immunohistochemistry (Fig. 8). Of the 34 distinct prostate cancers, there were 21 with histologic evidence of perineural invasion and 13 without evidence of perineural invasion. For the 21 cancers with perineural invasion, 20 showed positive cytoplasmic expression of CCR2 (95%) by immunohistochemistry. For the 13 cases without perineural invasion, just 3 stained positive for CCR2 (23%). The distribution of CCR2 expression within the positive specimens was fairly uniform. Expression intensity was assessed to be 1+ for all of the positive specimens. Macrophage expression of CCR2 served as a positive control with 3+ expression intensity.

Discussion

Perineural invasion is the process through which cancer cells invade and extend along nerves. Perineural invasion is an under-recognized and poorly understood route of metastatic spread (3). Perineural invasion is a marker of poor prognosis for many malignancies, including pancreatic, prostate, head and neck, skin, salivary, colon, and other cancers (3, 30–32). Signaling between cancer cells and nerves through ligand–receptor interactions appears to be a key mechanism through which cancer cell migration towards and along nerves may be induced (3, 9, 12). Chemokines are a family of small secreted molecules that play important roles in regulating immune cell recruitment during inflammatory responses and defense against foreign pathogens (33). Chemokines also may play roles in enabling cancer cell migration and metastases (15–17). More than 50 chemokines have been discovered and arranged into four different chemokine subfamilies, including the CC, CXC, XC, and CX3C subfamilies, depending on the number and spacing of the first two cysteine residues in the amino-terminal part of the protein (34). In addition to their roles in the immune system and in cancer progression, chemokines are also involved in nerve development, maintenance of nerve homeostasis, nerve injury repair, neuropathic pain, and nerve inflammation (35). Because these varied roles may potentially intersect in perineural invasion, we hypothesized that chemokine signaling may play a role in this process.

To identify novel chemokine ligands involved in perineural invasion, we performed a chemokine screen of explants of murine DRG, which contain primarily neurons and glial cells. DRG explants in Matrigel sprout axonal-like projects which are analogous to tiny nerves, which in cocultures with cancer cells serve as a model that recapitulates perineural invasion (12, 27). Our screen identified CCL6 and CCL12, but these proteins have been identified only in rodents, and were therefore excluded from further consideration (36, 37). Chemerin, an adipokine regulating adipogenesis and adipocyte metabolism (38), was identified, as well as IL16, which is expressed in bone marrow and spleen and acts as a T-lymphocyte chemoattractant (39). However, the most plausible candidate identified on our chemokine screen to play a role in perineural invasion was CCL2.

CCL2, also known as monocyte chemoattractant protein 1 (MCP-1), has demonstrated potent chemoattractant activity for monocytes, memory T cells, natural killer (NK) cells, and dendritic cells, resulting in the recruitment of these cells to sites of infiltration in shControl tumors at week 5 (Fig. 7B), as compared with dramatically less cancer infiltration in shCCR2 tumors at week 7 (Fig. 7C) and normal nerves (Fig. 7A). Immunofluorescence microscopy demonstrated the presence of robust CCR2 expression in the shControl PC-3 tumors (Fig. 7D and E), but the absence of CCR2 expression in the shCCR2 PC-3 tumors (Fig. 7F and G), demonstrating that CCR2 silencing was sustained in shCCR2 PC-3 prostate tumors over the course of the animal experiments.
tissue injury and inflammation. CCL2 may activate both CCR2 and CCR4 receptors, although CCR2 is the predominant functional receptor for CCL2 (40, 41). CCL2–CCR2 signaling may activate p42/44 MAPK and PKC through G proteins to regulate cellular adhesion and motility in macrophages (42). In the nervous system, CCL2 is predominantly produced by microglial cells, astrocytes, and, to a lesser extent, by endothelial cells and neurons (43). In response to injury, CCL2 expression may be upregulated by various cell types of the nervous system, including Schwann cells, macrophages, endoneurial fibroblasts, and neurons. CCL2 may also play an important role in enhancing pain sensitivity and inducing chronic pain (44, 45).

Interestingly, CCR2 is expressed in a variety of cancer types and appears to play a role in cancer progression. Using immunohistochemical staining, 84% of prostate cancer samples express CCR2 (46). Higher CCR2 expression has also been associated with higher Gleason score and higher clinical pathologic stages (46). CCL2 may support prostate cancer survival and motility through binding to its receptor CCR2 (47). Similar effects of CCL2 have also been found in breast cancer and hepatoma cells (48, 49).

We reasoned that the varied roles of CCL2 function as a (i) chemoattractant, (ii) factor released with neural injury, and (iii) factor promoting cancer progression, might all plausibly intersect in the process of perineural invasion. Cancer infiltration into nerves might induce a form of nerve injury that releases CCL2, which may then induce a nerve repair inflammatory response, induce the migration of CCR2-expressing cancer cells towards these nerves, and ultimately promote perineural invasion.

We demonstrate here that CCR2-expressing prostate cancer cells are able to migrate towards CCL2 and DRG. In contrast, cells lacking CCR2 fail to migrate towards CCL2, and migration towards DRG is diminished, but not abrogated, suggesting that other nerve-secreted factors additionally support cancer cell migration. Using an in vitro coculture assay of perineural invasion with DRG harvested from CCL2−/− mice, we demonstrate that both (i) CCL2 release by the DRG and (ii) CCR2 expression by the prostate cancer cells significantly enable PNI. Using an in vivo murine sciatic nerve model of perineural invasion, prostate cancer cells lacking CCR2 show a dramatically reduced ability to invade nerves and impair nerve function. Finally, human prostate cancer specimens demonstrated a very high rate of CCR2 expression (95%) in tumors with perineural invasion, but a considerably lower rate of CCR2 expression in tumors lacking perineural invasion (23%). This correlation between the rate of CCR2 expression and the presence of perineural invasion in human clinical specimens further supports the concept that CCR2 is likely playing a mechanistic role in the process of perineural invasion.

Clearly, other neurotrophic factors may be additionally contributing to the mechanisms underlying perineural invasion. When live explants of DRG in Matrigel are used to attract PC-3, more robust migration is demonstrated than with CCL2 alone (Fig. 2E), suggesting the presence of other nerve secreted factors.
CCR2 is expressed in human prostate cancer specimens with perineural invasion. Tumors 17 and 20 are human prostate adenocarcinomas with perineural invasion, which demonstrate positive staining of CCR2 by immunohistochemistry (N, nerve). In contrast, tumor 18 is a human prostate adenocarcinoma without perineural invasion that fails to express CCR2. Of 21 prostate adenocarcinomas with histological evidence of perineural invasion, 20 showed positive cytoplasmic expression of CCR2 (95%) by immunohistochemistry (IHC). In contrast, out of 15 cases without perineural invasion, just 3 stained positive for CCR2 (20%).

facilitating migration. Our group previously demonstrated the role that GDNF-RET signaling plays in pancreatic cancer perineural invasion, and others have explored the contributions of other nerve-released ligands including NGF, artemin, CX3CL1, BDNF, and others (9). It is possible that a spectrum of mechanisms of cell surface receptors dictating a chemotactic response to each nerve-secreted ligand. Other nerve-released factors may also potentiate perineural invasion by having effects on cancer cell adhesion, motility, and proliferation. The recent development of CCR2 antagonists raises the possibility that CCL2–CCR2 signaling may be pharmacologically targeted (50). Therapeutically blocking the molecular mechanism underlying a cancer’s particular adverse phenotype, rather than simply targeting cell viability, represents an innovative approach towards cancer management that merits further study.

In conclusion, we identify CCL2–CCR2 signaling as playing a significant role in perineural invasion by prostate cancer. Perineural invasion holds clinical significance as an adverse prognostic factor for a variety of malignancies; however, its underlying mechanisms remain complex and poorly understood. Communication between cancer cells and the nerve microenvironment may occur through a variety of mechanisms, and CCL2–CCR2 signaling appears to play a fundamental role. These results reveal a novel molecular target in perineural invasion for potential future therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. He, R.L. Bakst, N. Chernichenko, Z. Yu, R.J. Wong
Development of methodology: S. He, S. He, C.-H. Chen, N. Chernichenko, R.J. Wong
Acquisition of data (providing animals, acquired and managed patients, provided facilities, etc.): Shizhi He, C.-H. Chen, R.L. Bakst, S.Y. Lee, F. Barajas, H.A. Al-Ahmadie, R.J. Wong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. He, C.-H. Chen, R.L. Bakst, W.F. McNamara, S.Y. Lee, H.A. Al-Ahmadie, R.J. Wong
Writing, review, and/or revision of the manuscript: S. He, S. He, S. Deborde, R.L. Bakst, N. Chernichenko, H.A. Al-Ahmadie, R.J. Wong
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Shizhi He, C.-H. Chen, S.Y. Lee, R.J. Wong
Study supervision: R.J. Wong

Acknowledgments

The authors thank Ke Xu and Tatiana A. Omelchenko for assistance with microscopic imaging. Shizhi He is grateful to Dr. Demin Han and Dr. Jatin P. Shah for supporting his fellowship.

Grant Support

R.J. Wong is supported by R01CA157686. Shizhi He and Z. Yu were supported by the Beijing Municipal Health Bureau Grant 2009208. Shuangba He was supported by Anhui Provincial Natural Science Foundation 1308085MH131.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

Received May 27, 2014; revised September 21, 2014; accepted September 29, 2014; published OnlineFirst October 13, 2014.

References

The Chemokine (CCL2–CCR2) Signaling Axis Mediates Perineural Invasion

Shizhi He, Shuangba He, Chun-Hao Chen, et al.