Cancer Microenvironment (2013) 6:241–245
DOI 10.1007/s12307-013-0130-6

ORIGINAL PAPER

Paracrine Activation of Chemokine Receptor CCR9 Enhances The Invasiveness of Pancreatic Cancer Cells

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Received: 2 November 2012 / Accepted: 21 January 2013 / Published online: 1 February 2013

Abstract Chemokine receptors mediate cancer progression and metastasis. We have previously examined chemokine receptor CCR9 expression in pancreatic cancer. Here, our objective was to evaluate pancreatic stellate cells (PSCs) as a source of CCL25, the CCR9 ligand, and as an activator of CCL25-CCR9 signaling in pancreatic cancer cells. CCL25 and CCR9 expression levels in human pancreatic cancer tissues and normal human pancreas were assessed by immunohistochemsitry. In vitro secretion of CCL25 in PSCs and PANC-1 cells was verified by enzyme-linked immunosorbent assay. Pancreatic cancer cell invasion was measured using a modified Boyden chamber assay with CCL25, PSC secreted proteins, and PANC-1 secreted proteins as the chemoattractant. There was immunostaining for CCR9 expression in human pancreatic tumor tissues, but not in normal pancreatic tissue. CCL25 expression was absent in the normal pancreatic tissue sample, but was observed in cancer cells and in the stromal cells surrounding the tumor. In vitro, both PANC-1 cells and PSCs secreted CCL25. In an invasion assay, exposure to CCL25, PSC- and PANC-1-conditioned media significantly increased the invasiveness of PANC-1 cells. Inclusion of a CCR9-neutralizing antibody in the invasion assay blocked the increase in invading cells elicited by the chemoattractants. Our studies show that pancreatic cancer invasiveness is enhanced by autocrine and paracrine stimulation of CCR9. PSCs in the tumor microenvironment appear to contribute to paracrine activation of CCR9. Investigations into CCR9 as a potential therapeutic target in pancreatic cancer must consider cancer cell autocrine signaling and also paracrine signaling from interactions in the tumor microenvironment.

Keywords Pancreatic stellate cells · Pancreatic cancer · CCR9 · CCL25

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Introduction

Despite therapeutic advances for other cancers, the prognosis for patients with pancreatic cancer remains grim and has changed very little in the last few decades [1]. Recent clinical trials using novel drug combinations have demonstrated some benefit, [2–4] but nevertheless, the overall 5-year survival rate for pancreatic cancer patients has remained low [2, 3]. These poor outcomes have been attributed to many factors, including extensive fibrosis typically surrounding the pancreatic tumor. This desmoplastic response is an inflammatory hallmark of pancreatic cancer and creates a mechanical barrier limiting the effective delivery of chemotherapy and other therapeutic agents to pancreatic cancer cells [5, 6].

Pancreatic stellate cells (PSCs) are primarily responsible for the desmoplastic response observed in pancreatic cancer [7, 8]. PSCs are myofibroblast-like cells residing in the periacinar spaces that are typically quiescent under normal physiological conditions, but can become activated in response to pancreatic injury. In addition to stress-induced activation, cancer cells can also stimulate the activation of PSCs through the release of cytokines and growth factors [8]. In return, activated PSCs stimulate the production of extracellular matrix proteins and inflammatory molecules that further drive the development of desmoplasia [5, 7, 9, 10]. PSCs have also been implicated in tumor proliferation, tumor cell migration, and resistance to chemotherapy and radiation [5, 7, 9, 11–13].

The chemokine receptor CCR9 was initially identified for its role in the immune system, where it is present on leukocytes and is critical in T-cell development and responsible for recruiting immune cells to the small intestine [14–16]. We now know that CCR9 expression is also associated with poor prognosis and increased cancer cell invasiveness in malignant conditions, including melanoma, ovarian, breast, and prostate cancers [16–18]. CCR9 shows aberrant expression on pancreatic cancer cells [19] and may be a factor in promoting pancreatic cancer progression. While the CCL25-CCR9 axis has been examined in some cancers [17, 18, 20–22], its role is not well understood in pancreatic or other gastrointestinal cancers. In an earlier investigation, we demonstrated that activation of CCR9 by CCL25 led to increased pancreatic cancer proliferation in vitro [19]. Here, we investigated interactions between pancreatic cancer cells and PSCs and whether CCL25 released by PSCs enhances pancreatic cancer cell invasiveness.

Materials and Methods

Cell Lines and Reagents

We utilized the established human pancreatic cancer cell line, PANC-1, purchased from the American Tissue Culture Collection (Manassas, VA). PANC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech; Manassas, VA) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin. Stable, non-immortalized PSCs were a gift from the laboratory of Drs. David Rowley and Dave Berger at Baylor College of Medicine (Houston, TX). The isolation of PSCs has been described previously [23]. PSCs were grown in Iscove's modified Dulbecco's medium (IMDM) (Gibco; Grand Island, NY) supplemented with 10 % FBS and 1 % penicillin/streptomycin. All cell lines were grown at 37 °C with 5 % CO_2. The chemokine CCL25 and CCR9-neutralizing antibody were purchased from R&D Systems (Minneapolis, MN).

Immunohistochemistry

With Institutional Review Board approval, we obtained formalin-fixed paraffin-embedded pancreatic adenocarcinoma tumor specimens and normal adjacent tissue ($n=50$) from patients who underwent surgical resection at City of Hope. Immunohistochemistry (IHC) to detect the expression of CCL25 and CCR9 in these specimens was performed as previously described [19] using an anti-CCL25 antibody at a concentration of 1 μg/ml with a 30 min incubation (R&D Systems), an anti-CCR9 antibody at a concentration of 2 μg/ml with an overnight incubation (AbCam; Cambridge, MA), or isotype controls (AbCam).

Enzyme-Linked Immunosorbent Assay

To determine levels of CCL25 secreted by PSCs and pancreatic cancer cells in vitro, we utilized an ELISA (R&D Systems) according to the manufacturer's protocol. In brief, PSCs and PANC-1 cells were seeded at 3,000 cells per well in a 96-well plate. 24 h later, cell culture supernatants were collected, added to the ELISA plate containing the CCL25 antibody and incubated overnight at 4 °C. After incubation with the detection antibody and streptavidin-HRP substrate solution, light absorbance was measured using a plate reader. CCL25 protein concentration was determined using a CCL25 protein standard curve. Two independent experiments were performed with samples in duplicate. Error bars represent standard deviation.

Invasion Assay

Invasion was evaluated using a modified Boyden invasion chamber (BD Biosciences; Bedford, MA) following standard methods. Briefly, PANC-1 cells (1×10^5) were seeded onto matrigel-coated membranes (pore size 8 microns). IMDM supplemented with 5 % FBS was placed in the lower chamber, +/− CCL25 (400 ng/ml). We used 5 % FBS in the lower chamber because PSCs did not grow in media with less than 5 % FBS. Alternatively, a combination of PSC and PANC-1 cells (5×10^4 each) were seeded into the bottom chambers such that the proteins secreted by the cells served as the chemoattractant. The CCR9-neutralizing antibody (25 μg/ml) was also added to PANC-1 cells in the upper chamber at the time of seeding.

Cells were allowed to invade through the membrane for 24 h at 37 °C. Non-invading cells were then removed from the top of the membrane using a cotton swab dipped in PBS. The invading cells were fixed and stained using the Diff-Quik staining kit (Siemens Healthcare Diagnostics; Deerfield, IL) according to the manufacturer's protocol. The number of invading cells was quantified under light microscopy by counting cells in five adjacent fields at 200x magnification. For each experimental condition, the total number of cells was obtained and normalized to that of the control condition. Invasion was plotted as the average number of invading cells per field over two independent experiments +/− standard deviation.

Results

CCR9 And CCL25 are Expressed in Human Pancreatic Cancer Tissues

We have previously shown that established pancreatic cancer cell lines express CCR9 protein [19]. We sought to

determine whether CCR9 and CCL25 are expressed in human pancreatic cancer and normal tissues. As shown by IHC, CCR9 was not detected in the normal pancreatic tissue (Fig. 1a). There was however, cytoplasmic immunostaining for CCR9 in the ductal cells of the pancreatic cancer tissues (Fig. 1b). CCL25 staining was also absent in the normal tissue sample (Fig. 1c). In the pancreatic tumor tissue sample staining was positive in the cancer cells as well as in the surrounding normal stromal cells (Fig. 1d).

CCL25 Is Secreted by Pancreatic Cancer Cells and PSC

To determine if pancreatic cells and PSCs secrete CCL25 in vitro we utilized an ELISA kit. Cells were plated at 3,000 cells per cell type and incubated for 24 h. The growth media, containing proteins secreted over the 24-hour incubation, was collected and analyzed. We found that PANC-1 cells and PSC secreted CCL25 at 67.4 and 77.6 pg/ml, respectively (Fig. 2). This result suggests that there may be paracrine and autocrine activation of CCR9 signaling within the tumor microenvironment.

Activation of CCR9 Signaling Increases Pancreatic Cancer Cell Invasion

Due to discovering that both pancreatic cancer cells and PSCs secrete CCL25, we investigated the effect of CCL25-CCR9 signaling on the invasiveness of pancreatic cancer cells. By matrigel invasion assay, CCL25 elicited a 121 to 185 increase in the number of PANC-1 cells invading through the matrigel membrane (Fig. 3). The addition of a CCR9-neutralizing antibody abrogated the increase, decreasing the average to 115 invading cells. Then, we wanted to confirm that CCL25 secreted by cancer cells and PSCs enhanced invasion in similar fashion. Therefore, we plated a combination of PANC-1 cells and PSCs in the lower portion of the invasion chamber so that proteins secreted by the cells would serve as the chemoattractant. Again, PANC-1 cells were placed into the Boyden chamber insert. We observed an increase in the average number of invading cells to 339. Use of the CCR9-neutralizing antibody abrogated the increase, decreasing the average number of invading cells to 196 (Fig. 3 and Table 1). This data suggests that the CCL25 released by PSCs and pancreatic cancer cells increases CCR9 mediated chemoinvasion of pancreatic cancer cells.

Discussion

Chemokines and their corresponding receptors have established roles in directing cell migration in many different types of cancer; and expression levels of these proteins have also correlated with clinical outcomes [24–29]. The role of CCL25-CCR9 signaling in modulating metastasis was initially reported by Letsch et al. who demonstrated an association between CCR9 and organ-specific metastasis from

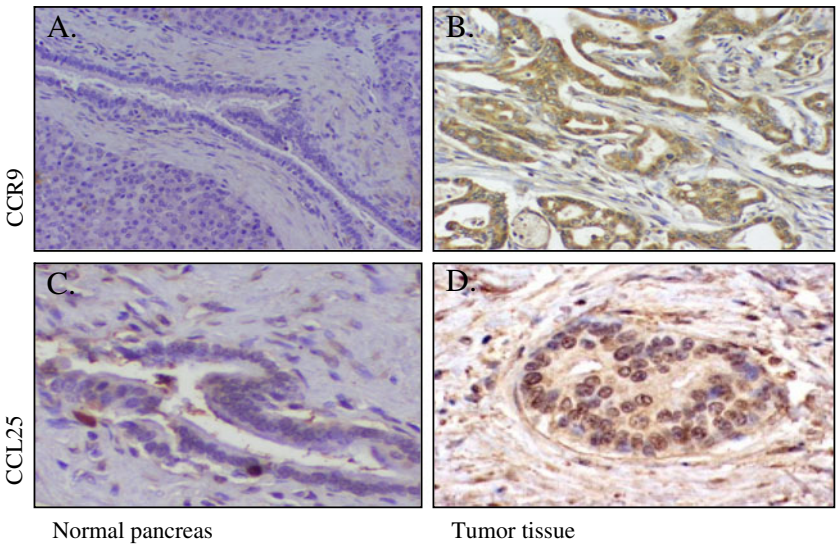

Fig. 1 CCR9 and CCL25 are expressed in pancreatic cancer tissue samples. Formalin-fixed paraffin-embedded human normal pancreatic tissue and pancreatic tumor tissue were stained with anti-CCR9 or anti-CCL25 antibodies. The normal pancreatic tissue sample shows tall columnar cells with basally located nuclei. There is an absence of CCR9 staining throughout the tissue **a**. Malignant ductal adenocarcinoma lesion demonstrates nuclear crowding and strong immunostaining for cytoplasmic CCR9 protein **b**. There is an absence of CCL25 staining within the normal architecture of the pancreatic duct and stroma **c**. In the malignant pancreatic lesion, with characteristic nuclear crowding, enlarged nuclei, and rare mitoses, there is moderate heterogeneous immunostaining for CCL25 protein in the nuclei. Moderate CCL25 staining throughout the stroma is also seen **d**. (Magnification 100x and 200x)

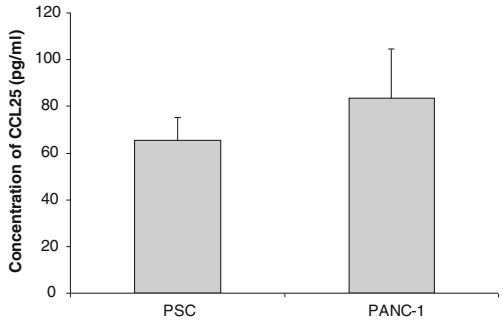

Fig. 2 PSC and PANC-1 cells secrete CCL25. PSC and PANC-1 cells were incubated for 24 h after which time cell culture media was collected. An ELISA was utilized to measure the amount of secreted CCL25 present in the media. Over a 24-hour incubation period PSCs secreted 67.4 pg/ml and PANC-1 secreted 77.6 pg/ml. Data shown represent the average of two independent experiments, error bars show standard deviation

melanoma cells to the small intestine [30]. The importance of CCL25-CCR9 signaling in cancer metastasis has been further tested in other cancers. These studies have found that CCL25-CCR9 interactions increase cancer cell survival, enhance migration and invasion, confer resistance to chemotherapy, and mediate anti-apoptotic signals [16–18, 20–22, 31]. Although CCR9 has been examined in several solid organ cancers, it has not been well studied in pancreatic or other gastrointestinal cancers. We have previously shown in vitro that exogenously added CCL25 increased pancreatic cancer cell proliferation [19]. In the present study we expand on our findings to show that CCL25 exposure increases pancreatic cancer cell invasion, although we did not observe clinical correlations with CCR9 expression. The CCL25-mediated invasion may result from both autocrine

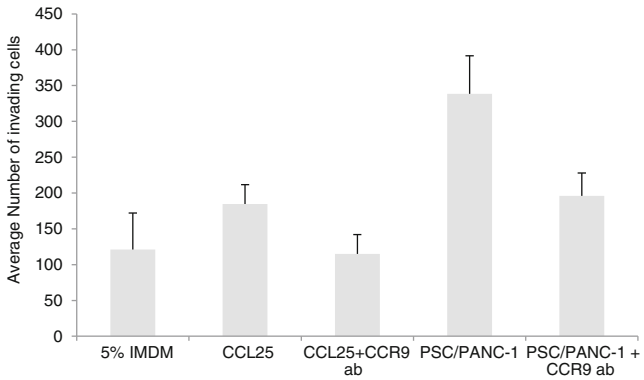

Fig. 3 PANC-1 cells show increased invasion towards CCL25, PSC and PANC-1 conditioned media. CCL25 or PSC and PANC-1 cells were placed in the bottom of a matrigel chamber. 24 h later, PANC-1 cells were seeded on top of the matrigel +/− a CCR9-neutralizing antibody and allowed to invade for 24 h prior to fixation and quantification. There was an average increase in invasion towards CCL25 and the PSC and PANC-1 cells from 121 cells in the control condition to 185 and 339, respectively. Data shown represent the average of two independent experiments, error bars show standard deviation

and paracrine signaling as evidenced by the activation of the CCL25-CCR9 axis by cancer cell and PSC secreted proteins.

In recent years, attention to the impact of the tumor microenvironment on cancer development and progression has increased. Therapies targeting tumors alone may prove insufficient due to growth factors and cytokines secreted by the surrounding stromal cells that continue to promote growth and invasion. In pancreatic cancer, a dense stroma forms around the tumor cells and collagen synthesis by PSCs increases after exposure to inflammatory cytokines such as TGF-β, TNF-α and IL-10 [31, 32]. In fact, prior studies have shown decreased response to gemcitabine and radiation when cancer cells were exposed to PSCs [7]. In addition, interactions between the tumor and surrounding stromal cells have been linked with tumorigenesis, metastasis, and an increased expression of cancer stem cell-related genes [6, 33]. Through IHC and ELISA experiments, we have shown that chemokine CCL25 is expressed by both PANC-1 pancreatic cancer cells and PSCs. Through autocrine signaling, CCL25 released by PANC-1 cancer cells binds to and activates CCR9. In addition, CCL25 secreted by PSCs activates CCR9 on PANC-1 pancreatic cancer cells through paracrine signaling. While we acknowledge that these experiments may be replicated in other pancreatic cancer cell lines, the PSCs used in this study were non-immortalized, grew very slowly, and were difficult to obtain from patients with pancreatic cancer. Furthermore, we observed in our previous studies, both published and unpublished, that other pancreatic cancer cells behaved similarly regarding CCR9 expression and signaling [19]. Taken together, we believed that PANC-1 cells alone were sufficient for the current investigations.

Targeting CCR9 may hold promise in diminishing the invasiveness of pancreatic cancer cells. Central to the use of CCR9 as a therapeutic target is its interaction with PSCs because of the secretion of CCL25, as demonstrated in our study. Future studies are warranted to expand our understanding of autocrine and paracrine CCR9-mediated signaling in pancreatic cancer and to develop novel therapeutic agents to target this pathway not only in cancer cells but also the cells of the cancer microenvironment.

Table 1 Average number of invading pancreatic cancer cells

Chemoattractant	Average number of invading cells	Average number of invading cells with CCR9 antibody
5% FBS	121	NA
CCL25	185	115
PSC/PANC-1	339	196

Acknowledgments Presented in part at the American College of Surgeons Surgical Forum Meeting on October 24, 2011 in San Francisco, CA.

Conflict of interest The authors declare they have no conflict of interest.

Financial support This work was supported in part by a Research Scholar Grant (120687-RSG-11-070-01-TBE) from the American Cancer Society. Additional financial support was provided by the City of Hope Comprehensive Cancer Center (P30CA33572-27), The National Institutes of Health (5K22CA134637-2), and the Leo and Anne Albert Charitable Trust.

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