Interaction between neoplastic cells and cancer-associated fibroblasts through the CXCL12/CXCR4 axis: Role in non–small cell lung cancer tumor proliferation

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Objectives: Carcinoma-associated fibroblasts are reported to communicate microenvironment-derived signals through chemokine/chemokine receptor interaction, influencing carcinogenesis. We sought to characterize roles of CXCL12/CXCR4 in crosstalk between non–small cell lung cancer epithelial cell and carcinoma-associated fibroblasts and in tumor growth.

Methods: Non–small cell lung cancer tumor samples obtained at surgery and from tumor arrays, as well as primary carcinoma-associated fibroblast and epithelial cell lines generated from fresh tumors, were assessed for CXCL12/CXCR4 expression, tissue localization, and production. Colony assays, extracellular signal-regulated kinase signaling, and chemokine production were measured to assess cancer cell responsiveness to CXCL12 stimulation with or without CXCR4 antagonists.

Results: CXCL12 and CXCR4 were detected in all major subtypes of non–small cell lung cancer. CXCL12-expressing carcinoma-associated fibroblasts were mostly located near CXCL12-negative tumor cells, whereas CXCL12-positive tumor cells were mostly surrounded by CXCL12-negative stroma. Intratumoral CXCL12 levels were significantly higher than serum levels. CXCL12 expression correlated with advanced disease stage. In vitro, tumor cell lines produced variable amounts of CXCL12 and expressed high levels of CXCR4. Carcinoma-associated fibroblasts cell lines produced high amounts of CXCL12 and expressed variable levels of CXCR4. Stimulation of non–small cell lung cancer neoplastic cells with CXCL12 increased colony-forming capacity, induced extracellular signal-regulated kinase phosphorylation, and production of the proinflammatory chemokine CCL20. CXCR4 antagonists attenuated these effects.

Conclusions: Interaction between carcinoma-associated fibroblasts and tumor epithelial cells through the CXCL12/CXCR4 axis plays a role in non–small cell lung cancer tumor proliferation, marking this axis as a target for immune intervention. (J Thorac Cardiovasc Surg 2011;141:1503-12)

Neoplastic cells, carcinoma-associated fibroblasts (CAFs), tumor-infiltrating immune cells, and vascular epithelial cells are key components of the non–small cell lung cancer (NSCLC) microenvironment. The interaction between tumor cells and stromal cells is believed to influence carcinogenesis. Previous studies have shown that NSCLC tumors contain macrophages, immature dendritic cells, and Foxp3 regulatory T cells infiltrate, suggesting that the crosstalk among these cells and the neoplastic cells may enhance local tumor progression and metastasis.¹⁻³

Chemokines, a family of chemotactic cytokines, are master regulators of immune cell trafficking in the body under physiologic and pathologic conditions.⁴ Chemokines interact with 7 transmembrane G-protein-coupled receptors to exert their effects.⁴ Distinct immune cell subtypes express specific repertoires of chemokine receptors, which guide their trafficking, retention, and function in target organs.⁵ A variety of tumor cells express chemokines and chemokine receptors.⁶ Activation of the chemokine/chemokine receptor axis within tumors induces autocrine and paracrine loops, promoting tumor growth and angiogenesis and subverting antitumor immune response.^{6,7} For example, the chemokine/chemokine receptor pair CXCL12/CXCR4 has been shown to be a key mediator of tumor spread, site-specific metastasis, and patient survival.^{6,8} Intense CXCR4 cytomembranous staining in NSCLC was associated with distant metastasis and decreased disease-free survival in 2 human studies.^{9,10} In experimental models, expression of CXCR4 by NSCLC cell lines was associated with metastatic spread to organs highly expressing CXCL12.¹¹

Multiple signaling pathway such as extracellular signal-regulated kinase (ERK, p44/42 mitogen-activated protein

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Disclosures: Authors have nothing to disclose with regard to commercial support.

Read at the 90th Annual Meeting of The American Association for Thoracic Surgery, Toronto, Ontario, Canada, May 1–5, 2010.

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Received for publication March 31, 2010; revisions received Sept 23, 2010; accepted for publication Nov 2, 2010; available ahead of print April 4, 2011.

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0022-5223/\$36.00

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doi:10.1016/j.jtcvs.2010.11.056

Abbreviations and Acronyms

CAF	= carcinoma-associated fibroblast
ELISA	= enzyme-linked immunosorbent assay
ERK	= extracellular signal-regulated kinase
NSCLC	= non-small cell lung cancer
PCR	= polymerase chain reaction
RPMI	= Roswell Park Memorial Institute medium

kinases), PI3 kinase, and nuclear factor κ B are activated through CXCL12/CXCR4 interaction. In nonmalignant cells, ERK signaling cascades are involved in regulating cell proliferation, survival, and differentiation.¹² Chemokine/chemokine receptor-mediated activation of ERK also plays a key role in cancer cell proliferation and invasiveness.¹³⁻¹⁵ CXCR4 signaling through ERK has recently been shown to promote pancreatic cancer cell proliferation and survival.¹⁶

In breast carcinoma, CAFs have been shown to promote growth of mammary carcinoma cells and to enhance tumor angiogenesis. This effect is in part mediated by CXCL12.^{17,18} CAFs comprise a significant component of NSCLC; however, their role in NSCLC pathogenesis has not been well defined. Limited data are available on the role of the CXCL12/CXCR4 axis and its signaling pathway in NSCLC. We sought to characterize the roles of the CXCL12/CXCR4 pair in the crosstalk between NSCLC epithelial cell and CAFs and in tumor growth.

MATERIALS AND METHODS

Tissue Collection and Patient-Specific Clinical Data

To exclude confounding effects, fresh tumor specimens, paraffin-embedded tissue sections, and fresh blood samples were obtained from patients ($n = 20$) undergoing complete resection of early-stage NSCLC (clinical stage IA–IIB) who had not undergone preoperative chemotherapy or radiotherapy. Histologic sections were prepared from formalin-fixed, paraffin-embedded tissues. Histopathologic diagnosis was confirmed by an experienced pathologist (G.A.). To enlarge our tissue sample pool and to allow clinical correlation, we also studied the expression of the CXCL12/CXCR4 axis in 2 NSCLC arrays containing 59 and 99 samples, respectively. Both arrays contained various subtypes of NSCLC.

The human component of the study was approved by the Hadassah Hospital ethics committee, and the animal component was approved by the animal experiment committee.

Immunohistochemistry of CXCL12 and CXCR4

NSCLC tissue samples were fixed with formalin and embedded in paraffin. Antigen retrieval was performed in ethylenediaminetetraacetic acid buffer for 20 minutes in a microwave, and sections were stained with the monoclonal antibody 79018 (R&D Systems, Inc, Minneapolis, Minn) for CXCL12 (1:100) and monoclonal antibody clone 12G5 (R&D Systems) for CXCR4 (1:100) with a standard antimouse horseradish peroxidase method according to the manufacturer's instructions. For color development, 3-amino-9-ethylcarbazole was used, and sections were counterstained with hematoxylin. Negative control sections were stained with

either no primary antibody (phosphate-buffered saline solution) or with isotype-matched control antibody.

Enzyme-Linked Immunosorbent Assays of CXCL12 and CCL20

Tumor tissue homogenates were prepared on ice from weighed tissue samples. Plasma and NSCLC tissue homogenate CXCL12 levels, as well as CCL20 levels in the supernatant collected from NSCLC cell lines, were measured with enzyme-linked immunosorbent assays (ELISAs) performed with a Quantikine kit (R&D Systems) according to the manufacturer's instructions. Levels of CCL20 in the supernatant of the L4 cell line in response to CXCL12 were measured in the presence or absence of anti-CXCL12 antibody (10 μ g/mL) and pertussis toxin (1 μ g/mL).

Isolation of NSCLC Tumor Cells and CAFs

Fresh NSCLC tumor tissue samples were washed twice in 10 mL of phosphate-buffered saline solution to remove remaining blood. Tissue was cut into approximately 1-mm pieces and plated in Roswell Park Memorial Institute medium (RPMI) 1640 plus 10% fetal calf serum (Gibco; Invitrogen Ltd, Paisley, UK). Tumor and fibroblast cells sprouting from the tumor explants were observed. We tried to generate primary culture tumor cells and CAFs from 20 samples and succeed in selectively transferring and expanding only 2 tumor cell and 4 fibroblast cell lines. To distinguish tumor-derived neoplastic cells from tumor-derived fibroblasts, cells were initially plated, stained with hematoxylin and eosin, and evaluated by an experienced histopathologist (G.A.). Cells were then immunofluorescently stained for cytokeratin and fibronectin. Tumor-derived neoplastic cells (L3 and L4) were defined as cytokeratin positive and fibronectin negative. The L3 line was generated from lung adenocarcinoma and the L4 line from large cell carcinoma. Tumor-derived fibroblasts (F1, F2, F3, and F4) were defined as fibronectin positive and cytokeratin negative and generated from adenocarcinoma (F1, F3, and F4) and squamous cell carcinoma (F2) tumors.

In Vivo Experiments

A sample of 1.5×10^6 NSCLC-derived neoplastic cells (L3 and L4) were injected into the tail veins of NOD-SCID mice. Two mice were injected with each cell line. Ten weeks later, the mice were killed. The lungs were fixed with formalin, embedded in paraffin, and assessed for tumor formation by histologic and immunohistochemical methods.

Flow Cytometric Analysis of Neoplastic Cells and CAFs

We performed flow cytometric analysis of 1.5×10^5 cells/mL of primary tumor-derived neoplastic cells (L3 and L4), CAFs (F1, F2, F3, and F4), and the NSCLC cell line A549 diluted in 0.1 mL phosphate-buffered saline solution containing 0.1% bovine serum albumin (Biological Industries Israel Beit-Haemek Ltd; Kibbutz Beit-Haemek, Israel) and 0.01% sodium azide (fluorescence-activated cell sorting buffer). Nonspecific staining was avoided by incubation with 1% human plasma for 15 minutes on ice. Blocked cells were then mixed with phycoerythrin-conjugated anti-CXCR4 monoclonal antibody (clone 12G5, 1:40; R&D Systems), with polyclonal anti-CXCR4-N-terminus antibody (Chemicon International, Temecula, Calif), or with isotype control for 20 minutes on ice and were washed with fluorescence-activated cell sorting buffer. Immunostained cells were analyzed by flow cytometry with the FACSCalibur Flow Cytometer (BD Biosciences, Mountain View, Calif). For all analyses, gating was performed using the forward scatter versus side scatter plot. Histogram overlays of control and stained cells were generated. Data were analyzed with the CellQuest software package (version 3.3; BD Biosciences).

Western Blot of ERK and Phosphorylated ERK

Western blot analysis for ERK and phosphorylated ERK was carried out as previously described in accordance with the manufacturer's

FIGURE 1. A, Expressions of CXCL12 and CXCR4 in non-small cell lung cancer tissue samples and plasma polymerase chain reaction assays for CXCL12, CXCR4, and β -actin in 3 non-small cell lung cancer tumors. B, Enzyme-linked immunosorbent assay for CXCL12 concentration in fresh tumor tissue and in the serum of patients operated on for non-small cell lung cancer; 4 cases are shown. C through H, Immunohistochemical staining for CXCL12. C, Tumor-adjacent normal lung tissue. D, CXCL12-positive fibroblasts surrounding CXCL12-negative adenocarcinoma cells. E, CXCL12-positive fibroblasts and vascular structures surrounding CXCL12-negative adenocarcinoma cells. F, Both adenocarcinoma cells and tumor stroma staining positively for CXCL12. G, CXCL12-positive fibroblasts and vascular structures surrounding CXCL12-negative squamous carcinoma cells. H, CXCL12-positive squamous cell carcinoma. I, CXCR4-positive adenocarcinoma.

recommendations.¹⁹ In brief, cells were seeded at 1×10^5 cells in RPMI and 10% fetal calf serum. After 24 hours, the medium was changed to RPMI without fetal calf serum for an additional 24 hours. We then added 500 ng/mL of CXCL12 (R&D Systems). Cells were then harvested at 0, 5, 15, 30, and 60 minutes, and 0.1 mL of lysis buffer was added to each culture and incubated on ice for 5 minutes. Cell lysates were boiled for 5 minutes, and samples consisting of 60 μ g protein were fractionated on 7% to 15% sodium dodecyl sulfate–polyacrylamide gel for 4 hours and transferred to nitrocellulose paper at room temperature overnight. The samples were then incubated with the appropriate antibody (rabbit polyclonal antibodies specific for ERK and phosphorylated ERK; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) according to the manufacturer's recommendations. Goat antirabbit horseradish peroxidase–conjugated antibody was added for 30 minutes of incubation at room temperature. The nitrocellulose blots were washed, enhanced chemiluminescence reagent was applied, and autoradiography was developed on x-ray films in cassettes. The developed bands were scanned and quantified with a densitometer ImageMaster VDS-CL machine and the BIS 303 PC bioimaging systems (Pharmacia Biotech, Piscataway, NJ).

Colony Assays

Agar base layer was prepared as follows: 45 mL of RPMI plus 12% fetal calf serum was mixed with 15 mL of RPMI X2 plus 12% fetal calf serum and with 15 mL of 2.5% agar in double distilled water. Tumor cells were suspended in RPMI plus 10% fetal calf serum. Cell suspension was mixed in a ratio of 1:3 with the agar base solution. This mixture was then plated on top of a preformed solid agar base. CXCL12 was added to the mixture at the concentrations of 100 ng/mL and 500 ng/mL. Fourteen days later, the numbers of colonies were counted in 10 different fields. These experiments were performed in the absence or presence of the CXCR4 inhibitor AMD 3100 at a concentration of 10 μ g/mL.

Polymerase Chain Reaction

The following primers were used for polymerase chain reaction (PCR): for CCL2, sense ATGAAAGTCTCTGCCGCCCT, antisense ACAG ATCTCCTTGCCACAA; for CXCR4, sense GCCTGAGTGCTCCAGT AGCC, antisense GGAGTCATAGTCCCCTGAGC; for CXCL12, sense ATGAACGCCAAGGTCGTGGTCG, antisense TGTTGTTGTTCTTCA

FIGURE 2. Production of non–small cell lung cancer–derived neoplastic and carcinoma-associated fibroblast cell lines. Hematoxylin and eosin staining of cancer epithelial cells (A) and cancer-derived fibroblasts (B). Cancer epithelial cells stained positively for cytokeratin (C) and negatively for fibronectin (E). Carcinoma-associated fibroblasts were cytokeratin negative (D) and fibronectin positive (F).

GCCG; for CXCL8, sense TGCCAAGGAGTGCTAAAG, antisense TCTCAGCCCTCTTCAAAA; and for vascular endothelial growth factor, sense ACATTGTTGGAAGAAGCTGC, antisense ATCCTGCCCTGTCTCTCTGT.

Statistical Analysis

Data are expressed as mean $\pm$ SE and as absolute values with percentages. Continuous data with normal distribution were analyzed with the *t* test. Categorical data were analyzed with the Mann–Whitney U test. Statistical analyses were performed with the SPSS statistical software package (SPSS, an IBM Company, Chicago, Ill).

RESULTS

To assess the potential role of the CXCL12/CXCR4 axis in NSCLC cancer, we examined fresh tumor tissues obtained from our patients during surgery, plasma obtained from these patients, and NSCLC tumor arrays, as well as cell lines we generated from NSCLC tumors.

Expression of CXCL12/CXCR4 in NSCLC Tissue Samples and Plasma

The expressions of CXCL12 and CXCR4 in fresh NSCLC samples were assessed by PCR. Figure 1, A, depicts

3 representative patients, documenting expressions of the ligand and its receptor.

In immunohistochemical analysis, we found distinct patterns of CXCL12 expression by NSCLC neoplastic cells and CAFs. Tumor-adjacent normal lung epithelial cells stained negative for CXCL12 (Figure 1, C). CXCL12-positive fibroblasts surrounded CXCL12-negative tumor tissue (Figure 1, D, adenocarcinoma, Figure 1, G, squamous cell carcinoma). CXCL12 was also expressed in small vessels and stroma adjacent to CXCL12-negative neoplastic cells (Figure 1, E, adenocarcinoma). In some cases, neoplastic cells expressed high levels of CXCL12 and stromal cells were negative (Figure 1, H, squamous cell carcinoma). Finally, we observed a pattern of CXCL12 expression by both neoplastic and stromal cells (Figure 1, F, adenocarcinoma).

Both neoplastic cells and CAFs produced CXCL12. CXCL12-expressing CAFs and stroma were mostly detected in proximity to CXCL12-negative tumor tissues. CXCL12-positive tumor cells were mostly surrounded by CXCL12-negative stroma.

FIGURE 3. Characterization of non-small cell lung cancer-derived neoplastic cell lines. A, Polymerase chain reaction results for CXCL12, CXCR4, and β -actin in the cancer epithelial cell lines L3, L4, and A549. B, ELISA assay for CXCL12 secretion to the medium at 24, 48, and 72 hours after plating of L3, L4, and A549 cells. C, Flow cytometric analysis of CXCR4 expressions by L3, L4, and A549. Representative forward and side scatter dot plot and cell gating of L3 is shown at *right*. Histogram of control antibody and overlay with anti CXCR4 antibody for L3, L4, and A549 are shown at *left*. Representative hematoxylin and eosin (D and E) and cytokeratin (F) staining of L4-derived tumors forming in the lungs of NOD-SCID mice after injection of the cells into the tail vein. SSC, Side scatter; FSC, forward scatter; PE, phycoerythrin.

In line with previous publications, we found high CXCR4 expression in NSCLC tumors (Figure 3, I, adenocarcinoma).

CXCL12 levels within NSCLC tumors were significantly higher than the serum levels of the protein (Figure 2, B).

CXCL12 Expression and NSCLC Pathologic Stage

Expression of CXCL12 was detected in all major subtypes of NSCLC: adenocarcinoma, squamous cell carcinoma, bronchoalveolar carcinoma, and large cell carcinoma. Among the 59 samples in the first array, 48 samples (81%) stained positively for CXCL12 and 11 (19%) stained negatively. The proportions of CXCL12-positive samples according to disease stage were 23 of 31 (74%) for stage I, 15 of 17 (88%) for stage II, 9 of 10 (90%) for stage III, and 1 of 1 (100%) for stage IV ($P = .145$). Nearly all samples in the second array stained positively for CXCL12 in variable intensity. We classified the tumor samples as having high or low CXCL12 expression. High expression of CXCL12 was noted if more than 50% of neoplastic cells stained positively for CXCL12. The

proportions of tumors with high CXCL12 expression according to disease stage were 17 of 44 (39%) for stage I, 11 of 23 (48%) for stage II, 19 of 31 (61%) for stage III, and 1 of 1 (100%) for stage IV ($P = .043$). Thus CXCL12 expression appears to correlate with a more advanced disease stage.

Production and Characterization of NSCLC-Derived Neoplastic and CAF cell lines

Four primary cell cultures of CAFs (F1, F2, F3, and F4) and 2 of NSCLC neoplastic cells (L3 and L4) were generated from fresh tissue samples of NSCLC tumors removed during surgery. Representative staining of these cells with hematoxylin and eosin is shown in Figure 2 (Figure 2, A and B). Cells were further characterized with immunohistochemistry. NSCLC neoplastic cells stained positively for cytokeratin and negatively for fibronectin (Figure 2, C and E). In contrast, CAFs stained negatively for cytokeratin and positively for fibronectin (Figure 2, D and F).

PCR analysis of NSCLC-derived neoplastic cell lines L3 and L4 and the established NSCLC cell line A549 showed

FIGURE 4. Characterization of non-small cell lung cancer-derived carcinoma-associated fibroblast cell lines. A, Polymerase chain reaction results for CXCL8, CXCL12, CCL2, and β -actin in 2 carcinoma-associated cell lines, F1 and F2. B, Enzyme-linked immunosorbent assay results for CXCL12 secretion to the medium at 24, 48, and 72 hours after plating of F1 and F2 cells. C, Immunohistochemical staining for CXCR4; black arrows point to CXCR4-positive fibroblast. D, Flow cytometric analysis of CXCR4 expression by carcinoma-associated fibroblast cell lines F1, F2, and F3. Histogram of control antibody and overlay with anti-CXCR4 antibody for F1, F2, and F3 are shown. PE, Phycoerythrin.

differential expression of CXCL12 (Figure 3, A). L3 cells showed a strong PCR signal for CXCL12. Production of CXCL12 by L3 cells was time dependent, reaching high levels of the chemokine after 72 hours (Figure 3, B). In contrast, L4 and A549 cell lines showed low PCR signal and reduced secretion of the chemokine (Figure 3, A and B). PCR signal for CXCR4 was detected in all neoplastic cells (Figure 3, A), and high membranous expression was detected by flow cytometry (Figure 3, C).

To demonstrate the tumorigenic potential of these cells, L3 and L4 neoplastic cells were injected into NOD-SCID mice, and the lungs were histologically assessed as described in the Methods section. All mice showed development of tumors. Figure 3 (D, magnification $\times 4$, and E, magnification $\times 20$) shows representative sections from these mice with tumor foci in the lungs, staining positive for cytokeratin (Figure 3, F).

By PCR analysis, we found all 4 CAF cell lines to express an array of tumor-promoting chemokines. Figure 4,

A, demonstrates the expressions of CXCL8, CXCL12, and CCL2 in F1 and F2 CAF lines. With ELISA, we showed that CAFs in culture produce CXCL12 in a time-dependent fashion similar to that of L3 neoplastic cells (Figure 4, B).

Staining of NSCLC tissue sections for CXCR4 indicated that some of the tumor fibroblasts expressed CXCR4 (Figure 4, C). Flow cytometry confirmed differential expression of CXCR4 by 3 CAF lines (Figure 4D).

CXCL12 Induces NSCLC Proliferation, ERK Phosphorylation, and CCL20 Production

The effects of CXCL12 stimulation on neoplastic cell proliferation were studied with colony assays. Stimulation of L3, L4, and A549 cells by increasing concentrations of CXCL12 promoted colony formation in a dose-dependent fashion (Figure 5, A, B, and C). CXCL12-induced colony formation could be blocked by the CXCR4-specific inhibitor AMD3100 (Figure 5, D, E, and F).

FIGURE 5. CXCL12-induced non-small cell lung cancer proliferation, extracellular signal-regulated kinase phosphorylation, and CCL20 production. A to C, Colony formation by L3, L4, and A549 cells in response to increasing concentration of CXCL12. D to F, Colony formation by L3, L4, and A549 cells in response to increasing concentration of CXCL12 with and without the addition of the CXCR4 antagonist AMD3100. G to I, Western blot analysis for phosphorylated extracellular signal-regulated kinase (*Perk*, upper panel) and extracellular signal-regulated kinase (*erk*, lower panel) after stimulation of L3, L4, and A549 cells with CXCL12. J to L, Enzyme-linked immunosorbent assay results for CCL20 secretion after stimulation with increasing concentrations of CXCL12 for L3, L4, and A549 cell lines. M, Enzyme-linked immunosorbent assay results for CCL20 secretion after CXCL12 stimulation with and without the addition of anti-CXCR4 antibodies or pertussis toxin for L4 cell line. Asterisk indicates $P < .05$; double asterisk indicates $P < .01$.

CXCL12 stimulation of L3, L4, and A549 cells induced ERK phosphorylation 15 and 30 minutes after exposure (Figure 5, G, H, and I, upper panel). There were no changes in total ERK levels (lower panel).

Finally, we found that stimulation of all 3 neoplastic cell lines by CXCL12 induced the secretion of the proinflammatory chemokine CCL20 (Figure 5, J, K, and L). The effects were more pronounced in the L4 and A549 lines. Pertussis toxin and anti-CXCR4 antibodies abrogated CXCL12-induced CCL20 secretion in L4 cells (Figure 5, M).

DISCUSSION

Previous studies have shown that all major subtypes of NSCLC tumors express CXCR4.^{9-11,20,21} CXCR4 expression has been detected in the nucleus, the cytomembranous compartment, or both.^{9-11, 20-22} Su and colleagues⁶ have reported that high cytomembranous CXCR4-expressing tumors were more prone to metastasis than were low CXCR4-expressing tumors. Spano and associates²¹ have reported that strong CXCR4-positive nuclear staining was associated with improved outcomes in early-stage NSCLC. Wagner and colleagues¹⁰ have confirmed these findings and suggested that cytomembranous expression of the CXCR4 in lung adenocarcinoma is an independent risk factor for decreased disease-free survival, whereas nuclear staining confers a survival benefit. These clinical studies indicate cytomembranous expression of CXCR4 in NSCLC as a prognostic marker.

The prognosis of patients with NSCLC is related to tumor metastasis. The primary sites of lung cancer metastasis, such as bone, liver, adrenal glands, and brain, express high levels of CXCL12.²³ Thus CXCR4 may play an important role in guiding NSCLC sites of metastasis. Phillips and coworkers¹¹ showed that administration of anti-CXCL12 neutralizing antibodies to immunodeficient mice with human NSCLC abrogated organ metastasis. Economidou and associates²⁰ suggest that high CXCL12 levels in pleural effusion of patients with NSCLC may be associated with tumor seeding of pleural space.

In this study, we showed that more than 80% of NSCLC expressed CXCL12. Intratumoral levels of the protein were much higher than in peripheral blood. Thus CXCR4-mediated metastatic spread of NSCLC may not be solely explained by migration of tumor cells into CXCL12-expressing tissues. The local concentration of CXCL12 and the expression of CXCR4 by neoplastic cells in the microenvironment seem to be of critical importance.²⁴ Taken together, our data suggest that CXCL12/CXCR4 interactions occur in the tumor microenvironment and may act locally to support tumor growth.

With immunohistochemical staining, we observed CXCL12 expression by both CAFs and neoplastic cells. All primary CAF cell lines produced CXCL12 in vitro. In contrast, the production of this chemokine by neoplastic cell lines

was variable. The neoplastic cell lines consistently expressed high levels of CXCR4. This led us to examine the effects of CXCL12 stimulation on the neoplastic cell lines. We found that CXCL12 promoted the colony-forming capacity of NSCLC cells, an effect that was inhibited by the CXCR4 antagonist AMD3100. Thus CXCL12 may act locally to support tumor cell growth in either an autocrine or paracrine manner. The detection of CXCL12-expressing CAFs in the tumor microenvironment is intriguing. It is possible that these cells participate in the process of neoangiogenesis. Orimo and colleagues¹⁷ described such a mechanism for tumor propagation in a model of breast carcinoma.

We have previously reported that the prognosis of patients with NSCLC might be related to tumor CXCL12 levels.³ Recurrence rates were higher (7/21) among patients with intense CXCL12 staining than among those who had weak staining (0/8).³ This study supports that observation by showing a correlation between the pathologic disease stage and CXCL12 levels. These findings are also in accordance with other studies showing a linkage of advanced T score, lymph node metastasis, and high tumoral CXCL12 levels. Lymph node metastasis has also been linked to strong nuclear CXCR4 staining.²² These data suggest that CXCR4 might not be the sole mediator guiding trafficking of tumor cells into lymph nodes. Other chemokines, such as CCL21 and CCL19, may participate in the process and have been shown to regulate CCR7-dependent T-cell and dendritic cell trafficking into lymph nodes.⁴ Takanami²⁵ showed that 57.8% of patients with positive tumoral CCR7 messenger RNA expression had lymph node metastasis, whereas only 11.5% of those without CCR7 messenger RNA expression had lymph involvement. Overall, these clinical and experimental data suggest that the CXCL12/CXCR4 signaling pathway promotes tumor cell proliferation. Trafficking of cancerous cells into draining lymph nodes may depend on other chemokines and receptors. Once in the lymph node, CXCL12/CXCR4 autotranssignaling may augment metastasis growth.

The mechanisms and signaling pathways involved in CXCL12/CXCR4 activation in NSCLC have not been fully identified. A recent report by Lee and coworkers²⁶ marks ERK activation as a key pathway in NSCLC development. In this study, we found that CXCL12 induced ERK phosphorylation in all neoplastic cell lines tested. ERK phosphorylation is a common pathway of extracellular-stimulated signaling cascades involved in the regulation of tumor cell proliferation and cytoskeletal rearrangement.^{12,27} CXCL12-induced ERK phosphorylation has been shown to induce matrix metalloproteinase 9 secretion and to augment the motility of NSCLC tumor cells.^{13,28} We found that CXCL12-expressing CAFs and stroma were mainly detected in proximity to CXCL12-negative tumor tissues. It is therefore possible that such sites represent tumor niches that facilitate tumor cell detachment and release

to the circulation. Furthermore, distant sites where CXCL12 is highly expressed may serve as favorable niches for metastasis to occur. At these locations, a paracrine CXCL12/CXCR4 loop may stimulate tumor cell proliferation and induce extracellular matrix rearrangement, necessary for metastasis formation.

Finally, we showed that CXCL12 stimulation resulted in production of the proinflammatory chemokine CCL20 by neoplastic cells. This may explain our previous observation that CXCL12 expression in lung adenocarcinoma was associated with increased tumor inflammatory load and the accumulation and retention of CD4⁺ T cells.³ In addition to CD4⁺ T cells, immature dendritic cells were also shown to accumulate in NSCLC.² The trafficking of such cells is governed in part by both CXCL12 and CCL20.^{2,5} Dumitriu and colleagues²⁹ recently described the potential of dendritic cells to induce differentiation of FoxP3 regulatory T cells on exposure to NSCLC cells.²⁹ Thus augmentation of tumor inflammation and recruitment of tumor promoting rather than suppression of immune cells may be an additional mechanism by which the CXCL12/CXCR4 axis promotes NSCLC growth and spread.

In conclusion, our findings suggest that the interaction of CAFs and tumor neoplastic cells and the CXCL12/CXCR4 axis play a role in tumor cell proliferation, thus marking CXCL12/CXCR4 axis as a target for immune intervention in the treatment of NSCLC.

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Discussion

Dr Steven J. Mentzer (Boston, Mass). Thank you, Dr Wald. That was a nice presentation, and your written article was equally thoughtfully put together.

For those of us who sometimes blur when we think about chemokines—and I think there are now roughly 44 or 45 chemokines known, so I think often people tune out—there are a few that are very important to thoracic surgeons. CCL2, or the old MCP-1, seems to be involved in a lot of different lung diseases, and CXCR4 is very important in terms of human cancer. I think it has been demonstrated in more than 20 human cancers. One of the things that I think is interesting about your study is the heterogeneity

in the samples that you showed, at least in the written article. Did you look at metastatic disease? If you imagine a subpopulation and if, for example, CXCR4 expression were important, you would perhaps expect that to be selected for in metastatic disease. Did you have an opportunity to look at that in any metastatic lesions?

Dr Wald. Well, we have conducted 2 studies focusing mainly on CXCL12, because some data regarding the expression of CXCR4 had already been published. In 2 different series, 1 that we reported in the past and 1 that we have now, we found some correlation between the level of CXCL12 expression and recurrence of disease. I assume that the balance of expression between the chemokine and the receptor will determine the outcome in many patterns. If you have expression of both the chemokine and the receptor in the tumor microenvironment, you might think that an autocrine loop or a paracrine loop is actually acting and may support tumor growth locally, whereas if you have, for example, expression of the chemokine on vascular structures and fibroblasts at the tumor edge or border with expression of the chemokine itself by the tumor cells, then this pattern might favor the possibility of guiding tumor metastatic spread. I think, however, that this is something that would be difficult to prove.

Dr Mentzer. One of the problems with a lot of chemokine research is that people kind of reach too far. They try to overinterpret their data. The CXCL12, it's actually stromal cell-derived factor 1 (SDF-1); it stops lymphocytes on a dime, it recruits endothelial progenitor cells, and it is expressed in a lot of tumors. It does a lot of different things. I don't think you have to try to explain it in in vitro work. And particularly the problem with in vitro work is that the cells are responding to polystyrene, not to the tumor microenvironment, which I think is a practical problem.

I think back to 8 or 10 years ago, when this was first discovered in breast cancer metastatic disease, Dynex and a variety of

other companies were developing small-molecule inhibitors. Can you envisage that kind of therapeutic potential in treating lung cancer?

Dr Wald. Well, today there is 1 molecule that is already approved for clinical use, I think it is AMD3100 (Plerixafor). I think that the method of delivery is highly important, because, as you mentioned, the CXCL12/CXCR4 axis is important in many homeostatic processes in the body. So if one wants to look at this as a therapeutic target, one must look for a way to deliver the drug specifically into the tumor, or at least use molecules that have a short half-life, maybe that predispose the cells to the action of other therapeutic agents, such as chemotherapy. I think this might be the path.

Dr Mentzer. Nice work. Thank you.

Dr Nasser K. Altorki (*New York, NY*). I want to congratulate you on pursuing this idea of crosstalk between the CAFs and the tumor. This will be an area of great interest going forward.

I understood you to say that these were primary-derived cultures from tumors, and I wondered whether you could share with us some of the tricks that you used to develop epithelial cell lines, because in our hands the success rate is less than 10%.

Dr Wald. I'm afraid, as we wrote in the article, that our percentage of success is around 10%. I think that it's very tough to isolate the primary tumor cells. I know that other people in our institution have tried to collect them from pleural effusions, and they have had a relatively high rate of success. What we have done, and sometimes it seems promising, is initially grow the cells on plates and then use like a colony assay and select specific colonies. There are also kits available for that, which seemed to be a bit more useful in some of the cases, but the success rate is still low.

Dr Altorki. Thank you, and congratulations.